UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Functional Neurorehabilitation and Stem Cell Therapy in Canine Degenerative Myelopathy

Dissertation of the Integrated Master in Veterinary Medicine

Inês da Silva Ferreira Dias

Supervisor: Professor Doutor Artur Severo Proença Varejão

Co-supervisor: Mestre Ângela Paula Neves Rocha Martins

Vila Real, 2020

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I declare that this master's dissertation is the result of my research and personal work and the guidance of my supervisors. Its content is original, and all sources consulted are duly mentioned in the text and in the references. I further declare that this work was not presented in any other institution to obtain any academic degree.

Vila Real, February 2020,

Inês da Silva Ferreira Dias

Dedication

To my parents…

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Abstract

Canine degenerative myelopathy is a progressive and fatal neurodegenerative disease with an adult-onset, is described in several breeds and currently doesn't have an effective treatment. Some forms of amyotrophic lateral sclerosis in humans have homology to degenerative myelopathy, sharing a mutation in the superoxide dismutase 1 (SOD1), which is considered a risk factor to the development of the disease.

Functional neurorehabilitation is a modality of restorative neurology and has a fundamental role in neurologic patients. Furthermore, mesenchymal stem cells, due to its differentiation, immunomodulation and regeneration capacity, are an interesting therapeutic option for diverse neurodegenerative conditions.

The present study was carried out in Hospital Veterinário da Arrábida, Centro de Reabilitação Animal da Arrábida and Veterinary Medicine School of Lisbon University. The main aim was to evaluate the therapeutic potential of adipose-derived mesenchymal stem cells (MSC) associated with an intensive protocol of functional neurorehabilitation (FNR) in degenerative myelopathy affected dogs. For such, a sample of ten dogs with a diagnosis of exclusion of degenerative myelopathy was selected, four constituted the control group and performed a physical therapy protocol and the other six comprised the study group and were submitted to a stem cell based protocol (MSC and FNR).

The results revealed an increase in survival time of the study group's animals comparing to the ones in the control group. The average survival time since the beginning of physical therapy protocol for the control group was 15 weeks, in contrast with the study group, in which a dog survived 36 weeks and another 88 weeks (death from clinical occurrence) since the initiation of stem cell based protocol. Additionally, two animals in the study group had follow-ups for 88 and 91 weeks, still with acquired motor functionality. It was also possible to verify that 67% of study group's animals improved according to a functional scoring system (FSS), after the stem cells transplantation.

In conclusion, the current study reveals a synergetic effect of the stem cell based protocol in canine degenerative myelopathy.

Keywords: Degenerative myelopathy, functional neurorehabilitation, mesenchymal stem cells, amyotrophic lateral sclerosis, dog.

Resumo

A mielopatia degenerativa é uma doença neurodegenerativa de caracter progressivo e fatal, que afeta cães adultos descrita em diversas raças, não tendo atualmente um tratamento eficaz. Algumas formas de esclerose lateral amiotrófica em humanos apresentam características homólogas com a mielopatia degenerativa, partilhando uma mutação no gene superóxido dismutase 1 (SOD1), sendo este um fator de risco para o desenvolvimento da doença.

A neurorreabilitação funcional é uma medida de neurologia restaurativa com um papel fundamental nos doentes neurológicos. Por outro lado, as células estaminais mesenquimatosas, devido às suas capacidades de diferenciação, de imunomodulação e regeneração, são consideradas uma fascinante opção terapêutica para diversas doenças neurodegenerativas.

O presente trabalho foi realizado no Hospital Veterinário da Arrábida, no Centro de Reabilitação Animal da Arrábida e na Faculdade de Medicina Veterinária da Universidade de Lisboa. Teve como objetivo avaliar o potencial terapêutico das células estaminais mesenquimatosas (MSC) derivadas de tecido adiposo juntamente com protocolos intensivos de neurorreabilitação funcional (PINRF) no tratamento de cães com mielopatia degenerativa. Foram incluídos dez cães com diagnóstico de exclusão de mielopatia degenerativa, quatro representaram o grupo de controlo e realizaram um protocolo de fisioterapia e os restantes seis constituíram o grupo de estudo, tendo estes sido sujeitos a um protocolo baseado em células estaminais (MSC e PINRF).

Os resultados obtidos revelaram um aumento do tempo de sobrevivência dos animais do grupo de estudo em relação aos do grupo de controlo. O tempo médio de vida desde o início do protocolo de fisioterapia no grupo de controlo foi de 15 semanas, contrastando com um animal do grupo de estudo que sobreviveu 36 semanas e outro que sobreviveu 88 semanas (morte por ocorrência clínica) após o início do protocolo baseado em células estaminais. Para além disso, dois animais do grupo de estudo têm um seguimento de 88 e 91 semanas, continuando com funcionalidade motora adquirida. Foi ainda possível verificar que 67% dos animais do grupo de estudo tiveram uma melhoria segundo uma escala de pontuação funcional (FSS) depois da transplantação das células estaminais.

Em conclusão, o presente estudo evidencia o papel sinergético do protocolo baseado em células estaminais na mielopatia degenerativa.

Palavras-chave: Mielopatia degenerativa, neurorreabilitação funcional, células estaminais mesenquimatosas, esclerose lateral amiotrófica, cão.

List of abbreviations and acronyms

DM – Degenerative Myelopathy

CSF – Cerebrospinal Fluid

ALS – Amyotrophic Lateral Sclerosis

PWC - Pembroke Welsh Corgi

LMN – Lower Motor Neuron

UMN – Upper Motor Neuron

PL – Pelvic Limbs

SOD1 – Superoxide Dismutase 1

MRI – Magnetic Resonance Imaging

EMG - Electromyography

CT – Computed Tomography

MSC – Mesenchymal stem cells

CNS - Central Nervous System

FNR – Functional Neurorehabilitation

CPG – Central Pattern Generator

SCI – Spinal Cord Injury

TENS - Transcutaneous Electrical Nerve Stimulation

NMES - Neuromuscular Electrical Stimulation

FES - Functional Electrical Stimulation

LT - Locomotor Training

SC – Stem Cell

iPSC - Induced Pluripotent Stem Cell

HVA/CRAA - Hospital Veterinário da Arrábida/Centro de Reabilitação Animal da Arrábida

PTP - Physical Therapy Protocol

SCBP – Stem Cell Based Protocol

CCRP - Certified Canine Rehabilitation Practitioner

MFS – Modified Frankel Scale

FSS – Functional Scoring System

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1. Introduction

Canine degenerative myelopathy (DM) is a progressive and fatal neurodegenerative disorder that affects the spinal cord of adult dogs (Yokota *et al*., 2018).

DM was first described by Averill in 1973, when it was recognized as a specific neurological disease (Nardone *et al*., 2016). Other terms used were chronic degenerative radiculomyelopathy, German Shepherd Dog myelopathy, and progressive myelopathy (Kathmann *et al*., 2006).

German Shepherd dog is the most commonly affected breed and the mainly represented in the initial reports of DM. Nowadays this disease is identified in numerous breeds and mixed breeds (Granger & Neeves, 2015; March *et al*., 2009; Shelton *et al*., 2012). Predominantly it occurs in large size dogs and the overall prevalence rate is 0,19% (Capucchio *et al*., 2014; Steven & Coates, 2018).

DM was histopathologically confirmed in the following breeds: German shepherd Dog, Siberian Husky, Miniature Poodle, Boxer, Pembroke Welsh Corgi, Chesapeake Bai Retriever, Rhodesian Ridgeback, Bernese Mountain Dog, Standarf Poodle, Kerry Blue Terrier, Cardigan Welsh Corgi, Golden Retriever, Wire Fox Terrier, American Eskimo Dog, Pug, Soft-coated Wheaten Terrier and mixed breeds. However, based on a presumptive diagnosis without histopathologic confirmation, many other dog breeds were previously reported to be affected with this disease (Coates & Wininger, 2010; Granger & Neeves, 2015).

There is no sex predilection in DM, although one study revealed a predominance of females in a clinical characterization in Pembroke Welsh Corgi dogs and older studies indicated a bigger incidence in males in samples of large size breeds (Averill, 1973; Griffiths & Duncan, 1975; Coates *et al*., 2007).

Even though DM is considered an adult onset disease, this condition has been described in dogs aged between 6 months and 15 years old (Cherubini *et al*., 2010; Longhofer, *et al.,* 1990*).*

Most dogs affected are at least 8 years old before the onset of neurologic signs (Coates & Wininger, 2010; Kobatake *et al*., 2016; Zeng *et al*., 2014) and the mean age of onset of clinical signs in large dog breeds is 9 years old, although study in Pembroke Welsh Corgis (PWC) refer a mean age of 11 years (Coates *et al*., 2007)

2. Spinal Cord Histopathology

The spinal cord histopathological changes of DM affected dogs are consistent with a noninflammatory axonal degeneration and demyelination. The lesions are identified in all funiculi and involves the somatic sensory, general proprioceptive sensory and motor tracts of the spinal cord (Coates & Wininger, 2010; Holder *et al*., 2014; Nakamae *et al*., 2015).

The degenerative changes in the white matter funiculi are characterized by dilation of the myelin sheath, axon cylinder vacuolization and concurrent fragmentation and phagocytosis of axonal and myelin debris. Regional axonal loss is severe in many dogs, as evidenced by complete loss of recognizable axonal and myelin profiles and replacement by large areas of astrogliosis, with proliferation of fibrous or reactive astrocytes. It is described the occasional presence of macrophages and a study demonstrated that there is an increase of CD18-positive macrophages in severe lesions (Crisp *et al*., 2013; March *et al*., 2009).

The most severely affected region of white matter is the dorsolateral portion of the lateral funiculus and the dorsal funiculi in some dogs (Averill, 1973; Braund & Vandevelde, 1978; Bichsel *et al*., 1983; Matthews & de Lahunta, 1985; Johnston *et al*., 2000; Coates *et al*., 2007; Nardone *et al*., 2016).

Location of lesions coincide with the anatomic distribution of descending and ascending tracts in the spinal cord, with no apparent predilection for a specific proprioceptive or upper motor neuron (UMN) system. The dorsal portion of the lateral funiculus comprises the spinocerebellar, reticulospinal, rubrospinal and corticospinal tracts. Within the dorsal funiculus, lesions tend to localize medially, corresponding to the location of the fasciculus gracilis (Coates & Wininger, 2010; Nardone *et al*., 2016).

Neuronal degeneration and loss were identified in the red nucleus, the lateral vestibular nucleus, the fastigeal nucleus, and the dentate nucleus of German Shepherd Dogs with DM (Johnston *et al*., 2000).

Neuropathologic lesions occur at all levels of the spinal cord, however are characteristically more severe and extensive in the mid to caudal thoracic segments, where degeneration and loss of large-diameter myelinated fibers are consistently

greater, with mild to moderate degeneration found in cervical and lumbar segments (Wininger *et al*., 2011; Bichsel *et al*., 1983; Matthews & de Lahunta, 1985).

It was proposed that predilection for lesion severity in the thoracic spinal cord may be a result of decreased perfusion from smaller diameter radicular artery and paucity of vessels when compared with other spinal cord regions. This fact may predispose neural tissue to ischemic processes associated with oxidative stress and excitotoxicity (Matthews & de Lahunta, 1985; Coates *et al*., 2007; Caulkins *et al*., 1989).

Descriptions of histopathological changes in DM vary within and across different breeds. Yet with different degrees of lesion severity and funicular distribution, they present similar patterns of axonal and myelin degeneration (March *et al*., 2009). In affected German Shepherd dogs, degenerative lesions are described as discontinuous, multifocal, bilateral, and asymmetric. Pathologic studies in this breed rarely report extensive areas of complete fiber loss in large regions of spinal cord white matter. In PWC and in an adult Miniature Poodle with degenerative myelopathy, severe and continuous regions of axonal and myelin degeneration were described (Coates *et al*., 2007; Miller *et al*., 2009; de Lahunta A., 1985).

Severity of lesions in individual dogs positively correlated with the duration of clinical signs. In longer clinical course cases it is possible to observe increased neural degeneration within funicular tracts across a longitudinally greater number of spinal cord segments (Johnston *et al*., 2001; March *et al*., 2009).

Lesions in the ascending and descending white matter pathways can explain the proprioception loss and paraparesis. The extension of lesions to the UMN pathways in the cranial thoracic and cervical spinal cord explain the clinical progression from paraparesis to tetraparesis. Moreover, fecal and urinary incontinence present in some dogs may be associated with histopathologic changes in the sensory pathways of the dorsal funiculus of the spinal cord, which signaling colorectal and urinary bladder distension. Involvement of these pathways may also contribute to a lack of visceral sensory feedback to the brain centers, altering recognition of visceral distention and involuntary evacuation. Nevertheless, urinary incontinence may also be caused by lower motor neuron (LMN) abnormality in the micturition pathway (Coates & Wininger, 2010; March *et al*., 2009).

2.1 Brain Pathology

The major hallmark histopathology of DM is the degenerative lesions in the white matter of the spinal cord, as described before. There is very limited information about the brain pathology in this condition, and some studies who examined brains from DM-affected dogs by light microscopy did not find lesions (March *et al*., 2009). However, one study revealed that the main differences between controls and affected German Shepherd dogs were the more marked gliosis and neuronal loss in the red nucleous, lateral vestibular nucleous and, occasionally, in the dentate nucleous and fastigial nucleous of the cerebellum, and the presence of Wallerian degeneration in the ventral tegmental decussation of the dogs with DM (Johnston *et al*., 2000).

2.2 Nerve and Muscle Pathology

Initial studies in DM described pathologic changes in the lumbar dorsal nerve roots, including severe degeneration. It has been reported chromatolysis in the intermediate gray matter, however motor neuron loss has not been recognized as a pathologic feature of this disease (Griffiths & Duncan, 1975; Shelton *et al*., 2012).

Spinal nerve root axons have been characterized as normal or exhibiting axonal loss comparing with age matched controls (Katz *et al*., 2017; Morgan *et al*., 2014; Zeng *et al*., 2014).

Concerning skeletal muscles, histopathologic findings includes substantial change in the ratio of the two main muscle fiber types and in late stage disease can involve denervation atrophy and excessive variability in myofiber size (Coates & Wininger, 2010; Zeng *et al*., 2014). Regarding intercostal muscle atrophy describe DM-affected dogs, it is not preceded by physical loss of the motor neurons innervating these muscles, nor of their axons wich indicate that intercostal muscle atrophy is not secondary to loss of physical contact with motor neurons (Morgan *et al*., 2014). Similar to what is observed in the thoracic intercostal muscles, extensor carpi radialis muscle analysis shows that its acetylcholine recept complexes retained contact with motor nerve terminals, demonstrating that impairment of forelimb motor function is not secondary to denervation by lower motor neurons (Katz *et al*., 2017).

Thus, some findings suggest that changes in muscle fiber type and size in DM appear to be due to primary alterations in the muscles (Katz *et al*., 2017).

Other histopathologic lesions in dogs with DM can include nerve fiber loss in peripheral nerves resulting from axonal degeneration, endoneural fibrosis, numerous inappropriately thinly myelinated fibers, and secondary demyelination (Awano *et al*., 2009; Coates & Wininger, 2010; Zeng *et al*., 2014; Matthews & de Lahunta A., 1985). Studies of the common fibular (peroneal) nerve reveal that these changes are more severe as the disease progresses. (Awano *et al*., 2009; Coates & Wininger, 2010)

There is evidence that Pembroke Welsh Corgis and Boxers with chronic DM develop peripheral nerve pathology consistent with an axonopathy, and to a lesser degree demyelination. Reported pathologic changes within common peroneal nerve are consistent with axonal degeneration and to a lesser extent demyelination, which results in a neurogenic pattern of muscle fiber atrophy in longstanding DM. These dogs developed peripheral nerve pathology as the disease progresses, supported by the worsening of LMN weakness (Shelton *et al*., 2012).

In contrast, mild histologic changes in peripheral nerves reported in other studies appeared to be compatible with age-related changes and did not support an obvious peripheral nervous system component of this disorder (March *et al*., 2009).

3. Clinical Spectrum

The clinical presentation of DM comprises an insidious, progressive UMN spastic paraparesis and a general proprioceptive ataxia of the pelvic limbs (PL), usually asymmetric, which progresses to LMN paraparesis and then spreads to involve the thoracic limbs and brainstem (Coates & Wininger, 2010; March *et al*., 2009; Steven & Coates, 2018).

One of the key features of DM is the lack of paraspinal hyperesthesia, in any of the disease's phases (Coates *et al*., 2007).

Early disease

Initially, affected dog owners may report abnormal gait and falling. In the physical examination it is usually observable asymmetric lameness of the pelvic limbs, knuckling over of the toes, wearing of the nails, and stumbling. Thereby, in most dogs, clinical signs are consistent with a lesion in the UMN system that is characterized by proprioceptive deficits, mild paresis and increased muscle tone in the pelvic limbs (Braund & Valdevelde, 1978; Griffiths & Duncan, 1975; Kathmann *et al*., 2006; Okada *et al*., 2009). Spinal reflex abnormalities are consistent with a lesion between the 3rd thoracic and 3rd lumbar spinal cord segments. Patellar reflexes may be normal or exaggerated to clonus and flexor (withdrawal) reflexes may also be normal or show crossed extension (suggestive of chronic UMN dysfunction) (Coates *et al*., 2007; Averill, 1973).

However, at the disease onset LMN signs can be observed in 10-15% of affected dogs, involving decreased patellar reflex, which can be attributed to normal age-related changes. Other possible explanations are the involvement of dorsal spinal nerve roots or peripheral nerves (Coates *et al*., 2007; Okada *et al*., 2009; Griffiths & Duncan, 1975).

As the disease evolves, affected dogs lose the ability to ambulance in the pelvic limbs, often within 12 months from the time of detection of first clinical signs (Coates & Wininger, 2010; Nardone *et al*., 2016; Kanazono *et al*., 2013). At this point, large dogs are usually elected for euthanasia. Owners can care for smaller dog breeds over a longer time (Coates *et al*., 2007; Matthwes & de Lahunta, 1985). The median disease duration in the Pembroke Welsh Corgi until euthanasia is 19 months (Coates *et al*., 2007).

Late disease

In late disease, clinical signs progress to LMN paraplegia and ascend to affect the thoracic limbs within 18–24 months (Awano *et al*., 2009). The paresis becomes more symmetrical and progresses to flaccid tetraplegia, and severe muscle atrophy occurs in the axial and appendicular musculature (Awano *et al*., 2009; Matthwes & de Lahunta, 1985; Averill, 1973). Hereupon, neurologic examination includes hyporeflexia of the patellar and withdrawal reflexes. Although it is not an usual clinical sign on this condition, it has been described urinary and fecal incontinence that appears near the development of nonambulatory paraparesis (Steven & Coates, 2018).

If the disease is allowed to progress, DM affected dog can exhibit cranial nerve signs such as dysphagia and dysphonia (Steven & Coates, 2018).

Scientific evidence demonstrates that, in the later stages, DM leads to a decline in respiratory function that correlates with an alteration in respiratory movements and atrophic changes in the intercostal muscles. This hypoventilation results in hypoxemia and respiratory movement is consequently changed to the abdominal breathing pattern (Oyake *et al*., 2016). Hence many affected dogs are able to live with extensive care until respiratory failure develops, which is commonly approximately 3 years or later after the disease onset (Nakamae *et al*., 2015).

Table 1 - Classification scheme for clinical presentation of DM categorized into four stages. Adapted from (Coates & Wininger, 2010; Toedebusch *et al*., 2017)

-	Disease severity		+
Stage 1	Stage 2	Stage 3	Stage 4
6 – 12 months	9 – 18 months	14 – 24 months	>36 months
- Asymmetric and spastic paresis in PL - General proprioceptive ataxia in PL - Normal spinal reflexes	- Nonambulatory paraparesis to paraplegia in PL - Mild to moderate PL muscle atrophy - Decreased to absent PL spinal reflexes - Urinary/fecal incontinence (+/-)	- Flaccid paraplegia in PL - TL paresis - Severe PL muscle atrophy - Absent spinal reflexes - Urinary/fecal incontinence	- Flaccid tetraplegia - Severe widespread muscle atrophy - Absent spinal reflexes - Urinary/fecal incontinence - Dysphagia, dysphonia - Respiratory distress

4. Etiopathogenesis

Several studies have been considering possible causes underlying DM, however, conclusive evidence for a specific pathogenic mechanism is lacking (Cherubini *et al*., 2010; March *et al*., 2009). Genetic, metabolic, nutritional, vascular, and immune-mediated etiologies have been proposed (March *et al*., 2009).

In 2009, a study identified a mutation in superoxide dismutase 1 (SOD1) gene, which encodes Cu/Zn superoxide dismutase. A G to A transition in the *SOD1* gene predicted a missense underlying DM, which is a genetic change that results in the substitution of one amino acid for another, and there was a highly significant association between homozygosity for the mutant allele and the DM phenotype in Pembroke Welsh Corgis, Boxer, Chesapeake Bay Retriever, German Shepherd Dog, and Rhodesian Ridgeback. In humans, mutations in the *SOD1* gene are known to cause some forms of familial amyotrophic lateral sclerosis (ALS) (Rothstein, 2009; Steven & Coates, 2018).

The SOD1 protein is involved in antioxidant mechanisms to protect the cell from reactive oxygen species toxicity and his mutations do not lead to disease by loss of enzyme function. Instead, the mutation results in a misfolded protein, which accumulates in spinal neurons and astrocytes (Nakamae *et al*., 2015; Yokota *et al*., 2018).

To date, two SOD1 mutations were described in affected dogs, E40K and T18S. These two enzymatically active dimers possess an increased aggregation propensity in cell culture and support the proposed toxic role of SOD1 in DM (Crisp *et al*., 2013; Shelton *et al*., 2012).

Through medical genomics it is possible to use individual's genomic information to assist in diagnosis and to help assess the risk of developing a disease, in other words, to show the susceptibility to suffer from a determined illness.

Despite the connection between the SOD1 mutation and the development of the disease, mechanisms underlying his effect in neurodegeneration are still uncertain (Awano *et al*., 2009).

It appears to be probable that mutations in SOD1 determine MN death due to multifactorial and complex pathogenesis which are still not fully understood, as it happens in ALS. These mechanisms include a possible dysregulation of energy supply

to neurons by oligodendrocytes, supporting the thesis that glial components are affected (Golubczyk *et al*., 2019; Nardone *et al*., 2016). Moreover, selective vulnerability of MN with SOD1 mutation can be related to mitochondrial dysfunction, since motor neurons are cells that require for a high level of mitochondrial activity and axons with the mutation are less resistant to activity-induced changes in ion concentrations (Alvarez *et al*., 2013; Shaw & Eggett, 2002).

In another study, it was identified a modifier gene, SP110 nuclear body protein, involved in the regulation of gene transcription, which variations may affect overall disease risk and age of onset in Pembroke Welsh Corgis at risk for DM (Ivansson *et al*., 2016).

Based on what happens in SOD1-associated familial ALS, reports also suggest an association between endoplasmic reticulum stress and DM pathogenesis, demonstrated by the up-regulated expression of an endoplasmic reticulum stress marker in the spinal cords of affected dogs (Yokota *et al*., 2018).

Vitamin E deficiency was suggested to be involved in DM pathogenesis, although recent data indicate that it is unlikely (Johnston *et al*., 2001; Fechner *et al*., 2003).

Pro-inflammatory mediators are known to play a role in the pathogenesis of ALS and it is possible to occurs a pro-inflammatory state in DM, with future studies needed to clarify its significance (Lovett *et al*., 2014).

Other potential etiologies indicated are abnormalities of autophagy, that have recently been reported to occur in various neurodegenerative diseases (Nardone *et al*., 2016; Ogawa *et al*., 2015); disturbed immune response, with immunohistochemical evidence for immunoglobulin and complement deposition in spinal cord lesions of German Shepherd dogs suffering from DM (Barclay & Haines, 1994); excitotoxicity via deficiency of the glutamine/glutamate cycle, that may contribute to the striking neuron loss in the ventral horn of DM dogs during disease progression (Ogawa *et al*., 2014); and lastly, oxidative stress and accumulation of denatured ubiquitinated proteins in spinal cord lesions, revealed in a study of immunohistochemical features of DM in two PWC dogs with a homozygous mutation in SOD1 (Ogawa *et al*., 2011).

4.1 Mutation

Mutations in the SOD1 gene are known to cause some forms of familial ALS in humans. That fact plus the clinical similarities between DM and ALS, made this a viable candidate gene to investigate and subsequently a mutation in affected dogs was identified. In a study realized by Awano and colleagues, the sequencing of SOD1 gene revealed a G to A transition in exon 2 (SOD1:c.118A), which corresponds to nucleotide 118, resulting in a glutamic acid to lysine missense mutation at amino acid 40 (E40K) (Awano *et al*., 2009; Coates & Wininger, 2010).

Significant associations between the DM phenotype and homozygosity for the A allele were observed in 5 dog breeds: Pembroke Welsh corgi, Boxer, Rhodesian ridgeback, German Shepherd dog, and Chesapeake Bay retriever (Awano *et al*., 2009).

SOD1 is one of the most abundant proteins in the Central Nervous System (CNS), possesses 153 amino acids and functions as a homodimer, which converts superoxide radicals to hydrogen peroxide and molecular oxygen.

A second SOD1 mutation was detected in a Bernese Mountain dog suffering from DM. It predicts a T18S missense mutation that was homozygous (Wininger *et al*., 2011).

Later, another report associated the first discovered mutant SOD1 allele with DM in cross-bred dogs and 124 different canine breeds (Zeng *et al*., 2014). The great majority of histopathologic confirmed affected dogs are SOD1:c.118A homozygous but a small quantity are heterozygous, being homozygosity a major risk for this condition (Kobatake *et al*., 2017; Pfahler *et al*., 2014).

Still, dogs that only carry a single copy of the SOD1 mutation rarely develop the clinical signs of DM. It is possible that asymptomatic heterozygotes represent the subclinical state of this disease and therefore they can constitute one line of research regarding early pathologic events (Kobatake *et al*., 2017).

There has been no conclusive evidence, however, as a considerable number of homozygous dogs for the mutant allele didn’t develop clinical signs, it was suggested that DM is an autosomal recessive disease with incomplete penetrance (Awano *et al*., 2009; Steven & Coates, 2018).

Through immunohistochemical analysis, studies oftentimes detected accumulation and aggregate formation of SOD1 in the spinal neurons of affected dogs (Crisp *et al*., 2013; Kohyama *et al*., 2016; Nakamae *et al*., 2015).

5. Diagnosis

Diagnosis of DM can be challenging because its early signs can mimic several spinal cord diseases that also compromises UMN pathways. As this condition affects mainly older dogs, it is not uncommon for co-morbidities to exist and complicate the interpretation of neurologic examination (Crisp *et al*., 2013; Steven & Coates, 2018).

A complete history must be taken to establish the onset and recognize the insidious progression pattern of clinical signs (Cherubini *et al*., 2010). It is imperative to perform a thorough general clinical examination to rule out the presence of concomitant diseases, such as metabolic and systemic disorders and cardiovascular conditions (Cherubini *et al*., 2010). An orthopedic examination is particularly important in older dogs and degenerative joint disease, bilateral cranial cruciate disease and hip dysplasia must be considered in the list of differential diagnosis (Cherubini *et al*., 2010).

Regardless of age, assessment of any animal with suspected neurologic disease involves a complete and accurate neurologic examination (Bagley, 1997). Common differentials include intervertebral disk disease, spinal cord trauma, lumbosacral stenosis, inflammatory disease and spinal cord neoplasia (Coates *et al*., 2007; Wahl *et al*., 2008). Lack of paraspinal hyperesthesia is a key clinical feature of DM that differentiates it from compressive myelopathies (Steven & Coates, 2018). Furthermore, in contradiction to DM, orthopedic conditions are not associated with proprioceptive deficits, and lumbosacral disease is associated with LMN rather than UMN signs (Bagley, 1997).

Presently, there is no specific antemortem diagnostic test available and because SOD1 mutations are incompletely penetrant, genetic screening is insufficient for diagnosis (Bagley, 1997; Toedebusch *et al*., 2017). A definitive diagnosis of DM can only be made postmortem through histologic examination of the spinal cord and detection of axonal degeneration, demyelination along with astrogliosis (Nardone *et al*., 2016).

As such, having ruled out non-neurological causes for the clinical presentation and after a neurological examination is performed, tentative antemortem diagnosis is based on the anamnesis, the clinical signs and its progression pattern, SOD1 mutation and a process of elimination of other causes supported by complementary tests (Cherubini *et al*., 2010; Coates & Wininger, 2010).

Neurodiagnostic techniques for evaluation of spinal cord disease include cerebrospinal fluid (CSF) analysis, electrodiagnostic testing and spinal cord imaging procedures as myelography, computed tomography (CT), and magnetic resonance (Coates *et al*., 2007; Coates & Wininger, 2010).

A presumptive diagnosis of DM is, thereby, based on ruling out spinal cord compressive diseases (Steven & Coates, 2018).

If there are no other concurrent spinal diseases, radiographic and myelographic studies are normal and CT and magnetic resonance imaging (MRI) don't reveal significant abnormalities (Bagley, 1997; Okada *et al*., 2009). However, if another spinal disease is found with diagnostic testing, it is not possible to exclude degenerative myelopathy as a contributive factor to the clinical signs (Bagley, 1997).

Survey spinal radiographs can help rule out bone diseases such as diskospondylitis, bone neoplasia and spinal fractures. A lumbar myelography can be performed to detect spinal cord compression, nevertheless CT myelography is more sensitive for characterizing morphology of the spine (Cherubini *et al*., 2010; Jones *et al*., 2005). MRI is the modality of choice to investigate spinal cord diseases and is especially helpful for identifying early spinal cord neoplasia and evidence of extradural compressive myelopathy (Coates & Wininger, 2010).

Frequently, imaging reveals disc protrusions that can confound a diagnosis of DM. Ultimately, the clinician must be guided by clinical experience and evaluate the disease progression, paraspinal hyperesthesia, and amount of spinal cord compression to estimate the severity of the compressive myelopathy (Coates & Wininger, 2010; Steven & Coates, 2018).

In DM-affected dogs, results of CSF analysis are normal or, more commonly, indicate elevated total protein without a concurrent pleocytosis (Bagley, 1997; Cherubini *et al*., 2010). CSF analysis provides information to the existence of inflammation or infection of CNS and therefore can help rule out meningitis. It may also be a promising source for diagnostic biomarker of DM, and a possible help in establishing prognosis and mechanism of disease (Coates & Wininger, 2010).

Myelin basic protein is a protein restricted to the nervous system and in humans, its concentration in CSF is used as a biochemical marker to evaluate active demyelinating

disorders. One study reported an increased concentration of myelin basic protein in CSF collected from the lumbar cistern of dogs with DM, but more investigation is needed to have valid conclusions (Oji *et al*., 2007). CSF of affected dogs has also been evaluated for markers of immune responses with intrathecal formation of immunoglobulins by detecting the presence of oligoclonal banding, but results were not significantly different from control dogs and large-scale study including cases with other spinal cord disorders is warranted (Kamishina *et al*., 2008). Another study verified that chaperon protein clusterin, that is protective against oxidative stress, is elevated in the CSF of chronic spinal cord disorders compared to meningitis and idiopathic epilepsy, indicating that additional markers are required to differentiate DM from a concurrent condition (Shafie *et al*., 2014). More recently, CSF levels of phosphorylated neurofilament heavy (pNF-H) were investigated in all stages of DM. pNF-H is an abundant structural protein of myelinated motor axons and a promising biomarker for nervous system diseases and its concentrations in blood and CSF have shown high association with disease progression in humans diagnosed with ALS. The results from this report indicate that pNF-H is increased in the CSF but not in serum of affected dogs relative to control groups and that quantification of this protein can be used as an antemortem diagnostic tool for DM, however further studies are necessary (Toedebusch *et al*., 2017).

Electrodiagnostic testing is helpful for detecting evidence of neuromuscular disease, but the results vary in the various disease stages. Early in DM, electromyography (EMG) and nerve conduction velocity studies don't detect any change from normal limits, suggesting that peripheral nerves and motor fibers are not affected in this stage. Later in the disease course, EMG reveals multifocal spontaneous activity, fibrillation potentials, and positive sharp waves in the appendicular musculature. The proximal and distal motor neuron conduction velocities in stimulated tibial and ulnar nerves were decreased than normal, providing evidence of the presence of axonopathy and demyelination in disease's late stages (Awano *et al*., 2009; Steven & Coates, 2018).

5.1 Genetic testing

DM is associated until now with two single nucleotide variations in exon 1 (specific to Bernese Mountain dog) and exon 2 (not specific to the breed) of the SOD1 gene (Awano *et al*., 2009; Wininger *et al*., 2011). A genetic test based on the SOD1 mutation is commercially available and can assist in the diagnosis (Steven & Coates, 2018).

Due to the late onset and insidious nature of the disease process and because dogs homozygous for the mutation are likely as fertile as other genotypes, the natural selection is insignificant and they will contribute one chromosome with the mutant allele to all of their offspring. The heterozygotes are DM carriers and are less likely to develop clinical signs but could pass on a chromosome with the mutant allele to half of their offspring (Capucchio *et al*., 2014; Steven & Coates, 2018). Thus, the mutant allele may reach high frequencies in dog populations and an easy to implement diagnostic test may contribute to control the gene diffusion (Capucchio *et al*., 2014).

The *SOD1* DNA test for breeding strategies is strongly recommended in an effort to reduce the incidence of DM and ultimately leading to an eventual eradication of the disease in some breeds (Kohyama *et al*., 2016; Turba *et al*., 2013). Working dogs can also be submitted to genetic testing to identify at-risk genotypes prior to buying or training (Shaffer *et al*., 2018).

However, an overly aggressive breeding program to eliminate the mutant allele may create a "bottleneck" effect and possibly select for other diseases or alter desirable qualities of the breed. (Coates & Wininger, 2010)

6. Treatment

Currently, there is no prophylactic or curative treatment for DM. Therefore, management strategies have been empiric and aim to relieve clinical signs and maintain the quality of life of the affected dogs. However, long-term prognosis of DM is poor (Coates & Wininger, 2010; Oyake *et al*., 2016; Steven & Coates, 2018).

In 2008, a study evaluated a therapeutic protocol including the administration of ε-aminocaproic acid (a fibrinolysin inhibitor) and N-acetylcysteine (a glutathione precursor and free radical scavenger) along with supplementation of vitamins B, C and E and controlled daily exercise. The progression of the disease was not affected in any animal and the rate of neurological worsening did not change (Clemmons, 1992; Polizopoulou *et al*., 2008). Therapy with corticosteroids didn't delay the progression of DM, as well. Another report studied the long-term parenteral cobalamin or oral tocopherol administration and found no beneficial effects. Furthermore, there was no significant differences between serum concentrations of α-tocopherol in affected German Sheppard dogs and controls (Johnston *et al*., 2001; Polizopoulou *et al*., 2008).

Physiotherapy and principles of physical rehabilitation have shown to be crucial in DM management, because it may improve the quality of life and prolong the survival time. When affected animals are left untreated, non-ambulatory paraplegia is usually established within 6 months after the initial diagnosis (Clemmons, 1992; Polizopoulou *et al*., 2008).

Kathmann and colleagues reported survival data from 22 dogs with DM that received varying degrees of physiotherapy. Dogs that received intensive physiotherapy had significantly longer survival times (mean 255 days) compared with that for animals with moderate (mean 130 days) or no (mean 55 days) physiotherapy. The physiotherapy routine consisted of active and passive exercises regardless the disease stage (Kathmann *et al*., 2006).

As already mentioned, some of the earliest pathological changes in DM occur in the muscle fibers and upper motor and sensory neuron tracts in the spinal cord. A more thorough characterization of this cascade of pathological events will provide targeting therapeutic interventions most likely to be effective in slowing disease progression (Katz *et al*., 2017).

Recent studies examined therapeutic potential of the endocannabinoid system for the treatment of chronic neurodegenerative diseases of the CNS, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS that has many similar features with DM. As it is known, the endocannabinoid system exerts a modulatory effect of important functions such as neurotransmission, glial activation, oxidative stress, or protein homeostasis and dysregulation of this processes is a common neuropathological hallmark in neurodegenerative diseases (Aymerich *et al*., 2018; Fernández-Trapero *et al*., 2017).

Reports observed that CB2 receptors become strongly up-regulated in activated astrocytes recruited at the damaged spinal cord in mutant SOD1 mice, an experimental model of ALS. These results were also found in the spinal cord of DM affected dogs, a natural occurring model of some forms of ALS. These findings support that the elevation of CB2 receptors in activated glial elements is an endogenous response of the endocannabinoid to neuronal damage. Such up-regulation occurred in absence of changes in other endocannabinoid elements. Targeting the CB2 receptor afforded neuroprotection in transgenic rodent ALS models. Thereby, more research in cannabinoid-based therapies must be conducted and selectively targeting this receptor can be used to enhance the protective effects exerted by these glial cells to improve neuronal homeostasis and integrity and slow disease progression (Aymerich *et al*., 2018; Fernández-Trapero *et al*., 2017).

Perspectives for the future include targeted stem cell (SC) therapy and delivery of small molecule therapies to the spinal cord with viral vectors (Coatti *et al*., 2015; Nayak *et al*., 2006).

A promising adjunctive therapy for a variety of surgical and medical disorders is hyperbaric oxygen therapy and its use is increasing in both human and veterinary medicine (Birnie *et al*., 2018; Edwards, 2010). It is based on the therapeutic use of intermittent inhalation of 100% oxygen inside a chamber pressurized above atmospheric pressure, which increases the dissolved oxygen concentration in blood and can alter tissue responses to disease and injury (Birnie *et al*., 2018; Edwards, 2010). It has already been demonstrated that this treatment is well tolerated and safe in dogs and cats and that hyperoxia has many potential benefits (Birnie *et al*., 2018). Physiologic effects of hyperbaric oxygen may include a relief of oxidative stress, antimicrobial effects, inflammation decrease, enhanced neuroprotective mechanisms and even

immunomodulation, making this a promising adjuvant therapy for neurologic patients (Patel & Huang, 2017; Zhou *et al*., 2019).

6.1 Functional Neurorehabilitation

Physical Rehabilitation of companion animals has become increasingly common and it proves to be very important to attain recovery in many orthopedic and neurologic conditions (Drum, 2010; Riegger-Krugh *et al*., 2014).

Functional neurorehabilitation (FNR) is a practice dedicated to the restauration of function to a body impaired by injury or disease, in order to improve the quality of life of the animal (Millis & Ciuperca, 2015; Weigel *et al*., 2005).

After a neurologic injury, FNR intend to recover animal's postural control, balance, locomotion capacity and ultimately functional independence (Harkema *et al*., 2012).

A rehabilitation plan of care, created to each individual case, should be intense and applied as soon as possible, within the limitation of the disease, in order to achieve success (Martins, 2015; Norrie, 2005; Goncalves *et al*., 2016).

Rehabilitation of dogs with neurologic disease can involve a combination of many therapeutic strategies as passive and reflexive exercises, active exercises and therapeutic modalities such as electrical stimulation, ultrasound, cryotherapy and heat therapy (Olby *et al*., 2005). An important attention must be given to the possible consequences secondary to the condition in order to prevent and treat them like pain, decubital ulcers and muscle contractures (Riegger-Krugh *et al*., 2014).

FNR is based on neuroanatomical key principles and CNS properties as neuroplasticity and neuromodulation, presents in both human being and domestic animals (Martins, 2015; Goncalves *et al*., 2016; Rossignol & Frigon, 2011).

One of the goals of this growing branch of veterinary medicine is the translation of these principles into rational strategies to promote recovery of function (Celnik & Cohen, 2004).

6.1.1 Properties of Central Nervous System

6.1.1.1 Central Pattern Generators

Neuronal circuits within the spinal cord interacts with specific sensory information and are capable of generate self-sustained patterns of locomotor-like neural activity, independently of supraspinal and afferent input (Dietz & Harkema, 2004). These networks of interneurons are defined as Central Pattern Generators (CPG) and are located in the lumbar segments of the spinal cord (Dietz & Harkema, 2004; Guertin, 2014) After a spinal cord injury (SCI), different changes in cellular and circuit properties occur spontaneously and can be promoted by rehabilitation approaches based on these functional networks (Rossignol *et al*., 2006; Rossignol & Frigon, 2011).

6.1.1.2 Neuroplasticity

Neuroscience has demonstrated that plasticity occurs throughout the CNS and throughout life (Thompson & Wolpaw, 2014; Yue *et al*., 2017). In physiologic conditions the spinal cord is continuously interacting with brain and peripheral sensory input during postural and locomotor activities and the activity-dependent plasticity plays an important role in the acquisition and maintenance of motor skills and in the effects of SCI or a progressive disease of the spinal cord (Edgerton & Roy, 2009; Wolpaw & Tennissen, 2006). Thus, CNS has the potential to reorganize connections through synaptic plasticity of pre-existing circuits and anatomical plasticity whereupon new circuits might develop through anatomical reorganization (Edgerton *et al*. 2006; Grasso *et al*., 2004).

After a SCI, motor training can provide sufficient stimulation of specific neural pathways to facilitate functional reorganization within the spinal cord and improve motor output. The most successful type of training includes repetitive pattern and variability in the performed task (Dietz & Harkema, 2004; Edgerton & Roy, 2009). Thus, training-induced plasticity might be responsible for changes in the locomotor network, that are probably adaptive and learnt (being specific to the trained task) (Dietz, 2012; Grasso *et al*., 2004). Furthermore, appropriate sensory input during training is of high importance to achieve an optimal motor output of the spinal neuronal circuitry (Dietz & Harkema, 2004).

The use of robotic devices for training specific motor tasks has become more prevalent recently and can provide a high probability of successful rehabilitation (Edgerton & Roy, 2009).

Further work should aim to identify the timing, type, and quantity of exercise and pharmacological interventions that can be used in new therapeutic approaches to maximizing function after spinal cord injury or restoring function to a newly regenerated spinal cord (Jakeman *et al*., 2011; Wolpaw & Tennissen, 2006).

6.1.1.3 Neuromodulation

Neuroplasticity that occurs after a SCI doesn't always have beneficial effects, that is why FNR must modulate the spinal cord excitability and define specific protocols with multimodal approach (Edgerton *et al*., 2006).

Interventions that may contribute to improve sensorimotor function include practice of the specific motor task that needs to be improved; combining with modulation of the excitability of the spinal circuitry through pharmacological modulation and/or via epidural stimulation (Celnik & Cohen, 2004; Edgerton *et al*., 2006; Zbogar *et al*., 2017). Moreover, peripherally or centrally applied electrical stimulation is a valuable tool to promote functional improvement related to modulation of specific areas of CNS (Yue *et al*., 2017).

Several reports have shown the involvement of neurotrophic factors in mechanisms related to neuroplasticity, as their actions are seen in a wide range of neuronal events and are also factors for axonal growth and synaptic plasticity (Côté *et al*., 2010; Yue *et al*., 2017). Also, molecules involved in axon guidance during CNS development may have an important role in the process of neurorehabilitation, as well as some neurotransmitters that have a modulator function in locomotor patterns (Edgerton *et al*., 2004; Yue *et al*., 2017).

6.1.2 Locomotor Training

Locomotor training (LT) is a rehabilitation strategy and an effective method for improving the recovery of postural control, balance, postural standing, gait, independence of function and quality of life after a neurological injury (Dietz & Harkema, 2004; Martins, 2015). Intensive activity-based rehabilitation interventions

such as step-training on a treadmill can result in significant functional improvements in individuals with chronic incomplete SCI (Harkema *et al*., 2012). It was also shown to improve gait kinematics, recover of phase-dependent modulation of spinal reflexes, and prevent loss of muscle mass (Côté *et al*., 2010).

LT can activate and modulate spinal locomotor centers, the already mentioned central pattern generators, and so by exploiting plasticity of CNS, it is possible to improve recovery of hindlimb function (Dietz & Harkema, 2004). In the spinal cord, exercise can also improve microenvironment and regulate physiological and metabolic function of motor neurons (Fu *et al*., 2016). However, besides the improvement of spinal cord function, LT can improve the function in different levels from end-effector organ to cerebral cortex through reshaping skeletal muscle structure and muscle fiber type and remodeling function of the cerebral cortex, thereby promoting functional recovery by improving neural and muscular function (Fu *et al*., 2016).

In humans affected by multiple sclerosis, rehabilitation interventions including LT can reduce sequels of the disease and should be considered early for maintaining functional capacity and reducing risk for losing important abilities, to achieve the highest possible quality of life (Beer *et al*., 2012). Furthermore, regular exercise may promote anti-oxidant defenses and neurotrophic support that could reduce CNS vulnerability to neuronal degeneration (White & Castellano, 2008). In poststroke subjects, short-term intensive rehabilitation using body weight support treadmill training has also shown to be useful for improving gait capacity (Takao *et al*., 2015).

A study concerning individuals with ALS determined that repetitive rhythmic exercise of supported treadmill ambulation training is feasible, tolerated and safe and it is consistent with improved gait function (Sanjak *et al*., 2010).

Chronic spinal cord conditions, such as Hansen type II intervertebral disc disease and degenerative myelopathy, are typically associated with progressive neurologic deficits and warrant a therapeutic approach with less focus on aggressive management and a greater emphasis on frequent repetition of low-intensity activities. Thus, preserving neuromuscular and musculoskeletal function, and manage the pain in some animals (Sims *et al*., 2015).

Hydrotherapy is another rehabilitation strategy that results in improvement in motor functions. There is evidence that an aquatic training program is appropriate and beneficial for individuals with multiple sclerosis and SCI and can be an effective method in reducing spasticity severity (Kesiktas *et al*., 2004; Salem *et al*., 2011).

6.1.3 Electrical Stimulation

Therapeutic exercise in rehabilitation is often complemented by physical agent modalities that can assist to limit impairments and disability and to maximize function. Electrical stimulation, ultrasound, cryotherapy, and heat therapy are the most commonly used modalities in neurologic rehabilitation and they have been used to reduce swelling, relieve pain, enhance healing, increase muscle strength, improve muscle tone, affect the elasticity of connective tissue and promote of soft-tissue and fracture healing (Drum, 2010; Hanks *et al*., 2015).

Electrical stimulation (ES) is most commonly used to reduce acute and chronic pain and spasticity and increase muscle strength and it is broadly categorized transcutaneous electrical nerve stimulation (TENS) or neuromuscular electrical stimulation (NMES) (Drum, 2010; Hanks *et al*., 2015; Peckham & Knutson, 2005). Generally, the intensity must always be adapted to the needs, comfort, and response of the animal and varies with the objective of treatment and toleration (Hanks *et al*., 2015).

TENS is used primarily for pain, however fine muscle fasciculations can be attained. It can also be used in the acute phase around incision lines to reduce pain and edema (Drum, 2010). In combination with active therapy, TENS can be an adjunct therapy for management of limb spasticity and improve voluntary motor control (Hofstoetter *et al*., 2014; Mills & Dossa, 2016). Moreover, a study tested the effects of TENS on spasticity in rats with SCI and suggested that high frequency TENS alleviates spasticity by inhibiting activated microglia (Hahm *et al*., 2015).

NMES is used to stimulate a muscle contraction, slowing muscle atrophy but there is also a component of pain control. It can help simulate walking motion if applied in an alternating contracting fashion and can be combined with active exercise to maximize muscle contraction (Drum, 2010; Hanks *et al*., 2015).

Therefore, through electrical activation of intact lower motor neurons using electrodes placed on or near the innervating nerve fibers, paralyzed or paretic muscles can be made to contract in a coordinated manner. Functional electrical stimulation (FES) is the application of electrical current to excitable tissue to provide functional restoration in neurologically impaired individuals (Peckham & Knutson, 2005). By means of FES, it is possible to increase strength (muscle force) and endurance (fatigue resistance) and improve upper or lower limb mobility (Gordon & Mao, 1994; Hamid & Hayek, 2008). In addition to motor system, FES can also be applied to restoring function in bladder and bowel, and respiratory system (Hamid & Hayek, 2008; Peckham & Knutson, 2005).

6.1.4 Laser therapy

Among the various modalities of FNR, laser therapy is applied to several conditions in dogs, including neurologic disorders. Therapeutic lasers have become increasingly popular for rehabilitation purposes and although its mechanism of action has yet to be fully characterized, it may have direct beneficial effects on nerve cells and their supporting structures. Most studies of laser use in rehabilitation are focused on wound healing and pain management, however, it may help to modulate cellular functions, reduce degenerative changes in neurons, induce proliferation of astrocytes and oligodendrocytes, and may also have anti-inflammatory effects (Riegger-Krugh *et al*., 2014; Sims *et al*., 2015).

6.2 Stem Cell Therapy

Regenerative medicine is an emerging field of research in tissue engineering, biomaterials and cellular therapies that aims to restore structure and function of diseased, nonfunctional or malfunctional tissues or organs (Gugjoo *et al*., 2019). Cell therapy had his first steps in the 1950s with the first bone marrow transplant involving identical twins. For several decades now, in human medicine, the application of stem cells represents already a well-established clinical procedure (Ciervo *et al*., 2017).

Thus, stem cell sciences are a promising biologic tool for a better understanding of several diseases as well as a potential therapy in many conditions (Glicksman, 2018).

Stem cell term refers to an unspecialized and precursor cell characterized by the properties of self-renewal, multiplication and multi lineage differentiation potential (Bonafede & Mariotti, 2017; Haidet-Phillip *et al*., 2015; Quimby, 2019). Self-renewal reflect their capacity to undergo multiple/limitless divisions (Los *et al*.,2019).

According to their regenerative potential, SC can be classified as totipotent, pluripotent, multipotent, oligopotent and unipotent (Los *et al*., 2019). Totipotency is defined as the ability to differentiate into cell lineages from all three germ layers: mesoderm, endoderm, and ectoderm including placental cell. In a human organism, only zygote and first blastomere have this capability. Pluripotency is the ability to differentiate into several but not all cell types, which is a characteristic of cells composing blastocyst's inner cell mass, embryonic stem cells. Multipotent cells differentiate into many types of cells that originate from one germ layer. Oligopotent cells are capable of differentiate into a few types of cells and, finally, unipotency is the ability to produce cells of their own type (Los *et al*., 2019; Nayak *et al*., 2006).

Stem cells can also be classified according to their origin and divided into two major subtypes: embryonic stem cells that are able to differentiate to the more than 200 cell types present in the body, if provided the right conditions for differentiation, and adult stem cells, which are located in organs of mature body and can usually only form the cell types present in the organ from which they were derived, being multipotent (Gugjoo *et al*., 2019; Mummery *et al*., 2010).

The use of embryonic stem cells raises ethical concerns, as most methods of their isolation involve the destruction of embryo. Also, its use carries a risk of teratoma formation and immune rejection (Cashman *et al*., 2008; Los *et al*., 2019).

Adult stem cells are found in niches which are microenvironments in specific anatomic locations that influence and regulate stem cell fate (Los *et al*., 2019; Rossi & Salvetti, 2019). Examples are mesenchymal stem cells (MSC), hematopoietic SC, skeletal SC, pancreatic SC and neural SC. Neural stem cell are found in the brain of both growing and mature mammals and are defined based on their ability to differentiate into three major CNS cell types: neurons, astrocytes and oligodendrocytes. They may enable a better understanding of diseases pathology and in the future may become a treatment

option not only in neurodegenerative diseases (Andreotti *et al*., 2019; Grochowski *et al*., 2018; Meamar *et al*., 2013).

Through transgenic techniques, recent technological advancements have made it possible to reprogram adult somatic cells to pluripotency. Defined as induced pluripotent stem cells (iPSCs), they are probably among the most promising sources of stem cells (Higuchi et al., 2019; Mummery et al., 2010). iPSCs have the properties similar to embryonic stem cells as they have the ability to differentiate into cells from all three germ layers, with the exception of extra fetal tissues (i.e., placenta). Since these SC can serve as an unlimited source and have been shown to be expandable and stable over many cell passages, iPSCs are excellent candidates for regenerating and repairing damaged organs and tissues (Chow *et al*., 2017; Glicksman, 2018; Gugjoo *et al*., 2019).

There are numerous stem cell sources, including bone marrow and adipose derived mesenchymal stem cells, hematopoietic stem cells, neural precursor cells, olfactory mucosa, and embryonic cord blood (Gabel *et al*., 2017; Nayak *et al*., 2006; Quimby, 2019).

Autogenous SC are harvested directly from the patient and the advantage in their use is that they are not immunogenic. Contrariwise, allogenic SC are harvested from a donor and carry the risk of rejection and the need for long term immunosuppression (Gabel *et al*., 2017). The advantages of using this type of SC include sparing the patient from undergoing the harvest procedure and also the possibility of use cells from young healthy donor animals (Quimby, 2019).

Regarding the delivery routes, there are two main strategies for stem cell transplantation: systemic and local. Intravenous delivery is feasible and a minimal invasive approach, but it is not clear whether the stem cells can successfully cross the blood–brain barrier. Intrathecal injection can be very effective and there is evidence that transplantation of stem cells into the CNS does not require immunosuppression (Meamar *et al*., 2013).

In human medicine, cell transplantation has been proposed as a treatment for several neurological disorders, including ALS, Parkinson disease, Huntington disease, stroke, epilepsy, multiple sclerosis and SCI (Boulis *et al*., 2012). However, there are still many undetermined factors related with their use as a therapy.

The therapeutic potential of SC in neurological diseases is due to their ability to regenerate cells of the CNS such as neurons, astrocytes, and oligodendrocytes (Nayak *et al*., 2006). Furthermore, SC can be used as a biological tool to understand a disease as well as the potential of endogenous stem cells present in the mammalian nervous system can be exploited and expanded to repair damaged tissue. Finally, stem cells can be transplanted into the injured nervous system as they may provide trophic support to host cells, slow a degenerative process, facilitate axonal growth or glial function and differentiate into CNS cells (Nayak *et al*., 2006).

Neural stem cell sciences have a promising future, considering that induced pluripotent stem cells may allow the creation of defined neuron populations, particularly for neural studies of neurodegenerative diseases as well as ischemic events (Grochowski *et al*., 2018). iPSCs derived from cancer cells could be also a novel strategy for studying some of the molecular mechanisms associated with cancer progression (Chao & Chern, 2018).

In veterinary medicine, SC therapy is a rapidly growing field for clinical application, especially in horses. Recently, this treatment option has also been commonly applied in dogs, mainly in diseases of the musculoskeletal system (Quimby, 2019).

As companion animals naturally develop many diseases analogous to human conditions, they can act as preclinical models. Also, spontaneously affected animals can be recruited into clinical trials and provide realistic insight into feasibility, safety, and biological mechanisms of SC therapies (Hoffman & Dow, 2016).

One of the biggest challenges is the determination of which cells have the best potential for which applications. Amongst the various types of SC used in the treatment of neurodegenerative diseases, MSC seem to be the most promising candidates (Bonafede & Mariotti, 2017).

6.2.1 Mesenchymal Stem Cells

MSCs are multipotent stem cells that possesses self-renewal ability and can differentiate into several types of mesenchymal tissues. Defined as adult stem cells of mesodermal origin, MSC represent an ideal source for cell therapy given their immunosuppressive nature, low potential for immunogenicity, trans-differentiation capacity and ability to secrete regenerative bioactive molecules (Ciervo *et al*., 2017; Marx *et al*., 2015).

In human medicine, for over a decade, MSCs have been used in the clinic and have been shown to be effective in several neurological conditions (Srivastava *et al*., 2014). According to the International Society for Cellular Therapy, Human MSCs must have the following characteristics: plastic adherence when maintained in standard culture conditions, expression of a specific cell surface antigen marker panel (CD73, CD90 and CD105) and absence of other cell surface markers (CD11b, CD14, CD19, CD29a, CD34, CD45 and HLA-DE) to differentiate MSCs from endothelial, epithelial and muscle cells and leastwise tri-lineage (osteogenic, chondrogenic and adipogenic) differentiation potential (Bakker *et al*., 2013).

MSCs have a significant supportive role in regenerative therapies, particularly in the CNS, supporting axonal growth and maintenance of synaptic connections and preventing neuronal death by reducing apoptosis and free radical generation (Boido *et al*., 2014; Srivastava *et al*., 2014). Moreover, MSC carry little tendency to form teratoma and, as the other stem cells, after transplantation, have the ability to migrate to the damaged tissues (Bonafede & Mariotti, 2017; Gugjoo *et al*., 2019). In fact, a study from 2012 reports that MSCs survive and migrate toward neural cells in SCI and help preserve axon and neural cells, resulting in improved hind-limb function (Ryu *et al*., 2012).

Their therapeutic potential is currently explained by the production of bioactive molecules, such us growth factors, cytokines, anti-inflammatory mediators, anti-apoptotic and neurotrophic factors. Therefore, many effects produced by the MSC result from their paracrine activity, including decreased inflammation, neuronal protection, angiogenesis stimulation, and antioxidant effect (Lewis *et al*., 2019; Marx *et al*., 2015; Penha *et al*., 2014). Also in spinal cord injured dogs, umbilical cord blood-MSC transplantations appeared to improve the functional outcome by new neuronal formation (Lim *et al*., 2007).

In addition, MSCs have a further interesting characteristic, related to the immunomodulatory activity on cells of adaptive and innate immunity, such as T and B lymphocytes, dendritic cells, natural killer cells and monocytes (Chow *et al*., 2017; Marx *et al*., 2015; Quimby, 2019).

As they reside in a perivascular niche, MSCs are virtually present in all tissues and organs (Marx *et al*., 2015). That is why MSCs have been harvested from a wide variety of fetal and adult tissues: adipose tissue, amniotic fluid, amniotic membrane, bone marrow, dental pulp, endometrium, limbal epithelium, liver, skeletal muscle, olfactory epithelium, omentum, ovary, periodontal ligament, periosteum, peripheral blood, placenta, synovium, umbilical cord blood, umbilical cord tissue, umbilical cord vein, and Wharton's jelly (Bonafede *et al*., 2017; Gugjoo *et al*., 2019; Srivastava *et al*., 2014). Among all these, adipose tissue and bone marrow are the most extensively studied and investigated for clinical applications (Gugjoo et al., 2019; Marx et al., 2015). Depending on the tissue of origin, the number of MSCs recovered varies and adipose tissue shown to be more abundant in MSC than the bone marrow (Enciso *et al*., 2018; Marx *et al*., 2015).

SCs derived from the adipose tissue is gaining increasing interest in cell therapy in humans and animals, because it is available in large amount, both as freshly isolated, stromal vascular fraction cells, or as cultivated adipose-derived mesenchymal stem cells (Bonafede & Mariotti, 2017; Marx *et al*., 2015).

Moreover, subcutaneous (buttocks and abdomen) and visceral (omentum) white adipose tissue can be obtained by minimally invasive, simple procedures such as liposuction or lipectomy (Ciervo *et al*., 2017).

There are several technical advantages in the application of MSC. First of all, their isolation is safe and easy. Secondly, MSCs can be easily cultured to a large scale in vitro, under appropriate conditions, to obtain sufficient numbers for either therapy or research purposes (Bonafede & Mariotti, 2017; Lewis *et al*., 2014).

Culture-expanded MSCs are often preserved at ultralow temperatures during manufacturing process, storage and transportation (Devireddy *et al*., 2017; Yousefi *et al*., 2019). Although cryopreservation effects on all properties of MSCs has not been fully investigated, fresh cells are better than cryopreserved (Quimby, 2019).

Additionally, recent studies in humans and rodents suggest that MSC obtained from young healthy individuals have superior proliferation and therapeutic potential compared to those collected from elderly diseased individuals (Quimby, 2019).

Regarding the route of administration, the main advantage in using intrathecal delivery in spinal cord conditions is that the trophic factors produced by MSC remaining in CSF can bypass the blood brain barrier to reach the spinal cord parenchyma, contrary to systemic route (Mazzini *et al*., 2012; Meamar *et al*., 2013).

Scientific interest on MSC as potential therapeutic approach in neurodegenerative diseases is increasing, due to its unique properties that provide an approach to achieving neural repair and protection (Lewis *et al*., 2014; Mazzini *et al*., 2012; Ryu *et al*., 2009). Thus, they have become attractive therapeutic cells that can potentially modify the ALS-specific inflammatory and excitotoxic microenvironment in the spinal cord (Srivastava *et al*., 2014).

Currently, MSCs are widely studied in veterinary regenerative medicine and mainly contribute to the stem cell therapy in dogs (Devireddy *et al*., 2017; Enciso *et al*., 2018). There are numerous canine studies that have shown safety and efficacy for stem cell therapies, however, continued efforts to increase understanding of canine SCs is critical to progressive treatment advances and high-impact study outcomes (Duan & Lopez, 2018; Gabel *et al*., 2017).

Canine patients constitute a major component of veterinary practice, and the dog is an established preclinical animal model for diverse human disease conditions. Thus, dog studies may provide an important insight into the MSCs applications in novel treatments for humans (Bakker *et al*., 2013; Duan & Lopez, 2018; Gugjoo *et al*., 2019).

Guidelines and criteria established for human MSC-based products were adopted by veterinary medicine, as the information for characterizing and identifying MSCs from veterinary species have lagged behind the information available for humans (Devireddy *et al*., 2017). In fact, canine MSC properties are overall similar to those described for human cells (Marx *et al*., 2015). For example, a recent study identified the dynamic release of various bioactive molecules, from canine bone marrow MSC, revealing the paracrine component already described (Humenik *et al*., 2019).

MSC can now be isolated from a wide spectrum of adult and fetal tissues, however, the tissue source with the highest MSC proliferation potential appears to vary from species to species (Quimby, 2019). Veterinary MSCs are commonly sourced from naive bone marrow, bone marrow concentrate, peripheral blood, umbilical cord blood, stromal

vascular fraction of adipose tissue, and umbilical cord tissue (Devireddy *et al*., 2017). A recent study concluded that canine amniotic membrane is a good and accessible source for obtaining MSCs of low immunogenic and tumorigenic potential and may represent a good candidate to be used therapeutically in veterinary regenerative medicine studies (Borghesi *et al*., 2019). Another one referred that ovary or uterus removed during castration may be used to harvest MSCs in the future, since such tissues carry good number of the SC (Sultana *et al*., 2018).

A study from 2011 in SCI dogs reported that transplantation of umbilical cord-blood-derived MSC one week after the lesion was more effective compared to other transplantation times evaluated, thus it may contribute to the optimization of timing for the use of this therapeutic modality (Park *et al*., 2011).

Clinical application has preferred bone marrow and adipose tissue because of their relative ease obtaining and the minimal donor-site morbidity (Bakker *et al*., 2013).

The donor is another source of variability in veterinary MSCs, due to different breed, age, gender, and health status that significantly contribute to quantitative and qualitative differences in MSCs. Preferentially, young donor MSCs should be utilized in the therapeutics for the better results, because they appear to carry higher expression profile of surface and pluripotency markers and a higher proliferative potential, when compared with cells harvested from aged dogs (Gugjoo *et al*., 2019). Also, to keep this variation to a minimum in sourcing allogenic veterinary MSCs-based products, would be very helpful to develop standardized diagnostic tests validated for determining absence of disease agents in an otherwise healthy appearing donor (Devireddy *et al*., 2017).

In a study comparing autologous and allogenic bone marrow-derived MSC in canine SCI reported that the first ones showed more beneficial effects, however both transplantations improved functional recovery following SCI (Jung *et al*., 2009).

There is little information about alterations of biologic characteristics in preservation of veterinary MSC, however, their cryopreservation has been shown not to affect their morphology, immunophenotype and differentiation potential, albeit inducing a minor decrease in the proliferation ratio and telomerase activity (Devireddy *et al*., 2017; Marx *et al*., 2015).

MSCs are being evaluated for their application in a variety of conditions, including musculoskeletal and neurological disease in dogs, cats, and horses (Devireddy *et al*., 2017). In vivo potential therapeutic applications include: osteoarthritis, osteogenic defects, periodontal defects, ligaments/tendon injuries, muscle tears, myocardial diseases, wounds, vocal fold injury, stomatitis, anal fistula, inflammatory bowel disease, Keratoconjunctivitis sicca, acute kidney injury, chronic renal disease, SCI, intervertebral disc degeneration and acute nuclear herniation (Gugjoo *et al*., 2019; Quimby, 2019). Predominantly, MSCs-based therapies in dogs have been focusing on bone and cartilage repair and inflammatory diseases such as osteoarthritis (Bakker *et al*., 2013).

In the future, the use of MSC derived from canine induced pluripotent stem cells may represent an important new source of cells for therapeutic modulation of inflammatory disorders, as they have almost unlimited proliferative potential and exhibit in vitro phenotypic stability (Chow *et al*., 2017).

6.2.2 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis is an adult-onset neurodegenerative disorder characterized by degeneration and death of upper and lower motor neurons, leading to progressive motor weakness and ultimately death within a few years of diagnosis. The cause and pathogenesis of the motor neuron degeneration in ALS appears to be a complex and multifactorial process. ALS is mainly sporadic in origin (without a family history), however approximately 10% of ALS cases are familial (hereditary forms of the disease). About 20% of familial ALS are caused by mutations in the gene encoding copper-zinc superoxide dismutase (SOD1) (Golubczyk *et al*., 2019; Papadeas & Maragakis, 2009).

DM is a neurodegenerative disease with clinical, histopathologic, and genetic parallels to human ALS, including presence of mutated SOD1. The similarities between the canine and human nervous systems, exposition to similar environmental factors, the analogy in the onset and clinical progression of both diseases, and the ability to study naturally affected dogs with DM represent a uniquely valuable large animal disease model for therapeutic development. And also advances in familial ALS studies can

contribute to develop new therapeutic approaches to DM research (Golubczyk *et al*., 2019; Papadeas & Maragakis, 2009).

The development of relevant therapies for ALS is challenging particularly because of the insidious, neurodegenerative course of the disease however stem cells can provide promising potential for understanding and treating the disease (Srivastava & Morgan, 2014). SC therapy could potentially target several mechanisms responsible for the etiology of ALS, through cellular replacement and neural protection (Meamar *et al*., 2013). The first would be achieved by the ability of SCs to differentiate into specific cell subtypes involved in the disease (Bonafede & Mariotti, 2017). The second involves the use of SCs based on their capacity to release trophic factors and to scavenging neurotoxic molecules, providing a local support in the microenvironment of the damaged area, acting as neural protectors (Bonafede & Mariotti, 2017; Srivastava *et al*., 2014).

Among different types of stem cells, MSCs represent a strong candidate for ALS treatment, due to their neuroprotective and immunomodulatory potential that match very well with the multifactorial nature of this disease (Ciervo *et al*., 2017; Lewis *et al*., 2014).

Easy to harvest and expand, MSCs are recognized for their neuronal and non-neuronal cell replacement capacity, trophic factor delivery and modulation of the immune response. Moreover, MSCs are also known for playing a crucial role in nourishing neurons, reducing neuronal sensitivity to glutamate receptor ligands, altering gene expression, and thus reactivating cell plasticity in the CNS (Forostyak *et al*., 2014; Lewis *et al*., 2014; Srivastava *et al*., 2014).

In recent years, stem cell transplantation as a new therapy for ALS patients has been extensively investigated. In several preclinical studies using the transgenic mouse model of familial ALS, that expresses a mutated form of the human SOD1, MSCs were demonstrated to be neuroprotective, effectively delay disease onset, improve motor performance, prolong survival time, and decrease local inflammatory response in treated animals (Bonafede & Mariotti, 2017; Ciervo *et al*., 2017).

Interestingly, one of the studies described that, after intravenous injection, human MSC migrated into the parenchyma of brain and spinal cord and showed neuroglia

differentiation and that transplanted mice showed significantly delayed disease onset (14 days), increased lifespan (18 days) and delayed disease progression compared with untreated mice (Zhao *et al*., 2007).

Moreover, intrathecally transplanted human MSCs inhibited inflammatory response in SOD1 transgenic mice, which was evidenced by the decreases in microglial activation and the secretion of inflammatory factors (Zhou *et al*., 2013).

Another study added that the intrathecal administration of human MSCs is a safe procedure that is able to remodel the recipient's gene expression profile, thus reactivating CNS plasticity. It also provided evidences that extracellular matrix (involved in the formation of synaptic connections and in CNS plasticity) is affected in SOD1 rats (Forostyak *et al*., 2014).

Furthermore, it has been demonstrated that even though overall astrogliosis was not modified, MSCs differentiated massively into astrocytes at the site of degeneration. The intrathecal delivery of MSCs and the subsequent generation of healthy astrocytes decreased motor neuron loss in the lumbar spinal cord, preserving motor functions and extending the survival of rats. Along with that, the neuroprotection was correlated with decreased inflammation, as shown by the lower proliferation of microglial cells and the reduced expression of COX-2 and NOX-2 (Boucherie et al., 2009).

Intravenous, intrathecal, intracerebral, and intraspinal delivery of autologous MSCs in SOD1 mice confers a range of beneficial effects on the disease course, including improved motor function, attenuated motor neuron loss, and prolonged survival (Lunn et al., 2015). In addition, multiple intrathecal transplantation of MSC have those beneficial effect in transgenic mice, contrary to one single administration that didn't have a therapeutic effect (Zhang *et al*., 2009). However, if systemically transplanted near the onset ages, a single treatment with neural inducted MSCs was sufficient to enhance motor functions during the symptomatic period of the condition, whereas unprocessed MSCs required repeated transplantation to achieve similar levels of improvement (Choi *et al*., 2013).

One study provided evidence that MSC, when intra cisternally administered (cisterna lumbaris) in SOD1 mouse, can exert strongly positive effects such as delay of

motoneuron death and motor decay, reduction of astrogliosis and modulation of microglial activation (Boido *et al*., 2014).

Dose-dependent effects were also investigated in SOD1 mice transplanted via intrathecal with human MSCs obtained from an ALS patient. Cell dose of 1×10^6 cells significantly prolonged life span and delayed the decline of motor performance, when compared to lower quantity cell treated groups, suggesting that intrathecal injection with an optimized cell number could be a potential route for stem cell therapy in ALS patients (Kim *et al*., 2010).

Fundamental to the initial development of stem cell transplantation into patients with ALS is the demonstration that transplanted cells lack tumorigenicity and have the appropriate biodistribution to ensure the safety of treated ALS patient (Haidet-Phillips *et al*., 2015).

Studies revealed that transplantation of autologous MSCs in patients with ALS is safe and clinically feasible, through intrathecal, intravenous and intraspinal administration (Karussis *et al*., 2010; L. Mazzini *et al*., 2010; Mazzini *et al*., 2008; Prabhakar *et al*., 2012).

Long-term monitoring of 19 ALS patients during 9 years also showed the safety of autologous MSC transplantation (Mazzini *et al*., 2012).

The results of a 2016 study using a culture-based method for inducing MSC to secrete neurotrophic factors (NTF) suggest that intrathecal and intramuscular administration of MSC-NTF cells in patients with ALS is safe and provide indications of possible clinical benefits, to be confirmed in upcoming clinical trials (Petrou *et al*., 2016).

Finally, in a phase I clinical trial, two repeated intrathecal injections of autologous MSCs were found to be safe and feasible throughout the duration of the 12-month follow-up period (Oh *et al*., 2015).

6.2.3 Advancements for the future

One of the future directions may be the identification of MSC biological markers predictive of a positive/negative therapeutic response. A study concluded that perineuronal nets and cytokine homeostasis are altered in the SOD1 rat model of ALS

and that these changes could potentially serve as biological markers for the diagnosis, assessment of treatment efficacy, and prognosis of ALS (Forostyak *et al*., 2014).

Another possible advancement for the future of SC therapies are the use of biomaterials for cell transplantation, because cell culture materials could sustain favorable conditions for stem cell survival, growth, migration and maturation. Transplantation of stem cells using biomaterials (scaffolds or hydrogels) shows promising results in experimental models of SCI, traumatic brain injury and nerve regeneration, and must be considered in ALS models where the presence of a hostile microenvironment is one of the main factors that negatively affects stem cell engraftment (Ciervo *et al*., 2017; Higuchi *et al*., 2019).

It is also important to consider a combination of SC and alternative therapies, such as gene therapy, that may in the future offer a better solution. Harnessing the power of the genes that code for trophic factors will likely improve tissue integration and the possibility of transplantation of stem cells that overexpress trophic factors and neuroprotectors would be beneficial in several neurodegenerative diseases (Gabel *et al*., 2017; Meamar *et al*., 2013; Mummery *et al*., 2010).

Finally, the success of any cell therapy depends on several critical issues and further investigation must determine scientific basis for each technical factor and establish standardized protocols (Devireddy *et al*., 2017). Those factors include optimal cell dose, ideal MSC source, isolation methods, culture conditions, cryopreservation methods, route of administration, the timing and number of applications and impact of tissue donor status on MSC function (Ciervo *et al*., 2017; Quimby, 2019).

7. Objectives

The evolution of neuroscience in human neurodegenerative diseases allowed a transposition of knowledge for the same conditions that affect dogs. Nowadays science considers that both human and veterinary medicine constitutes a single medicine. Reinforcing this statement, ALS and DM are examples of that "symbiosis".

The development of veterinary medicine and, more specifically the advance in animal neurorehabilitation, made it possible for DM-affected dogs to have quality of life. Functional neurorehabilitation protocols allow the appearance of locomotor pattern, which is paramount to gait achievement. Nevertheless, it was verified that memorization of flexion/extension locomotor pattern is limited in time (Marques, 2018).

Thus, as evidenced by Ciervo *et al*. (2017), the best therapeutic approach in a near future for these neurodegenerative patients may be the SC therapy. MSC have the ability to modulate synaptic and anatomic neuroplasticity, regenerate neural tissue, and possibly help to promote activation of spinal neural circuitry.

Therefore, the present prospective study with a control group intends to assess the synergetic capacity of intensive locomotor training based on movement repetition associated with stem cells administrated via intrathecal, aiming to achieve motor functionality, in order to evaluate an integrative multidisciplinary program between FNR and regenerative medicine.

Furthermore, the second objective is to perform a clinical follow-up in order to detect the mean number of days with quality of life and autonomy for patients with DM.

8. Materials and Methods

8.1 Participants

The prospective study comprises ten dogs diagnosed by exclusion with degenerative myelopathy. Four of them constitutes a control group and were submitted to a physical therapy protocol and a study group that is composed by six of these animals that were subjected to a stem cell based protocol (SCBP) that includes an FNR protocol along with stem cell therapy. A more detailed population characterization will be described in the results. This study was performed at Hospital Veterinário da Arrábida/Centro de Reabilitação Animal da Arrábida (HVA/CRAA), during a 2-year and 2 months period, from 30 July of 2017 till 30 September of 2019, and after approval by the Lisbon Veterinary School ethics committee. Written informed consent was taken from the owners of all animals, in respect to the SCBP.

In order to minimize data variability and avoid less valid results, all patients were submitted to an NRF examination performed by the same veterinary, Dr Ângela Martins (AM), a Certified Canine Rehabilitation Practitioner (CCRP) instructor/examiner certified by the University of Tennessee. All patients were recorded and regularly evaluated during the hospitalization.

8.2 Study Design

Regarding the study group, the study design performed in the six dogs is depicted in the following diagram (figure 1).

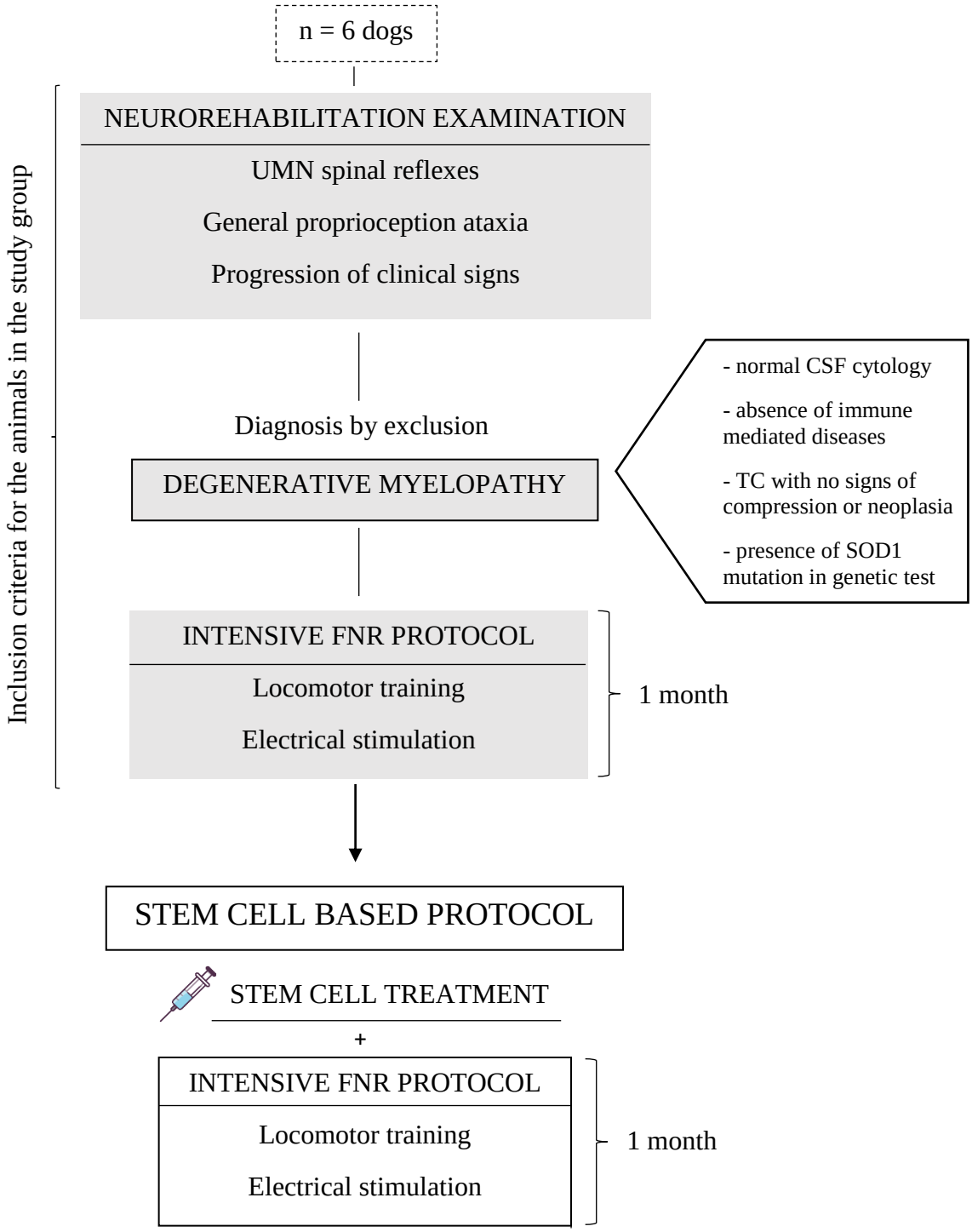


Figure 1 - Study design performed in the study group's animals

In the present study, dogs were selected to the study group if they presented classic motor and sensory deficits of UMN lesion. According to the Modified Frankel Scale (MFS), at admission two dogs were in score 3 (non-ambulatory) and in stage 3(8) on the Functional Scoring System (FSS). Three dogs were in score 4 (ambulatory paraparesis or general proprioception ataxia) on MFS and on the FSS 2 of those dogs were in stage 5(13) and one of them were in stage 5(12). One dog had score 2 on MSS and stage 2(5) on FSS (Levine *et al*., 2009). Previously they were all subjected to one month of intensive neurorehabilitation, after a presumptive diagnosis of degenerative myelopathy.

The diagnosis by exclusion comprised a serologic analysis of samples of blood and CSF for the study of immune mediated diseases, such as: tick-borne diseases (Ehrlichia, Rickettsia, Babesia), Leishmaniosis; parasitic diseases (neosporosis and toxoplasmosis) and viral diseases such as canine distemper. Furthermore, CSF was also analyzed for pleomorphism and protein presence.

To exclude the differential diagnosis of spinal cord compression, it was required a CT performed in the Neurology department of Veterinary Medicine School of Lisbon University by Professor Doctor António Ferreira (AF).

To reinforce the diagnosis of DM, it was performed a genetic test to detect a SOD1 mutation, including both homozygous and heterozygous animals, in Genevet® laboratory, Portugal.

All animals in the study group were evaluated in a functional neurorehabilitation examination, after one-month application of FNR protocol. In that appointment, they were prescribed an intrathecal administration of mesenchymal stem cells, which arrived from the Vetherapy® laboratory, Belgium, on the day before its application.

An evaluation was performed immediately before the stem cell treatment and, on the day after administration, patients begin another month of intensive FNR protocol. During this protocol the six dogs were hospitalized. Outcomes of neurologic examinations were performed one week after the SC therapy (week 2) and then two weeks after (week 4). Each of these patients have medical release at week 5, when the FNR protocol ends, and follow-ups are performed on week 6, 8 and 10, in all animals. From week 10, according to the examination, they had follow-ups with appropriate time intervals for each case, depending on the evolution of individual functionality, during the maximum of 91 weeks (namely animal 2).

The control group is composed by four dogs that presented the same inclusion criteria than the described for the study group. However, the therapeutic program comprises a physical therapy protocol (PTP), similar to the one defined by Kathman *et al.* (2006), performed at HVA/CRAA, during a 2-month period.

This group of animals was evaluated in a neurologic examination by the same doctor that evaluated the study group and the outcomes were registered according to the MFS and the FSS, in the moment of admission and at medical release, and according to the FSS in the moment before euthanasia, however there were no follow-ups in between.

At admission, three dogs in the control group presented score 3 (non-ambulatory paraparesis) on the MFS and stage 3 (6) on the FSS and one was scored 4 (ambulatory paraparesis or general proprioception ataxia) on the MFS and 4 (10) on the FSS.

To conclude, and as was already referred, the present prospective study is divided in two groups, a control group (n=4) and a study group (n=6). Classifications of neurologic status, according to MFS and FSS were registered at admission and medical release of Physical therapy protocol (2 months) for the control group and equally at admission and medical release of the first of intensive FNR protocol (1 month) for the study group' animals, that later initiate the SCBP. These two neurologic evaluations are described in table 2.

Table 2 - Classification of patients according to MFS and FSS, at admission and medical release of PTP (control group) or intensive FNR protocol (study group)

	Animal	MFS Admission	MFS Medical release	FSS Admission	FSS Medical release
Control group	1	3	1	3 (6)	1 (1)
	2	4	3	4 (10)	2 (3)
	3	3	2	3 (6)	1 (1)
	4	3	2	3 (6)	2 (3)
Study Group	1	4	4	5 (12)	5 (13)
	2	3	3	2 (5)	3 (8)
	3	3	3	2 (5)	3 (8)
	4	3	4	5 (12)	5 (13)
	5	4	4	5 (12)	5 (12)
	6	2	2	2 (5)	2 (5)

8.3 Functional Neurorehabilitation Examination

An FNR examination comprised the observation of the patient's gait, and the motor and sensory deficits were detected according to the MFS (Levine *et al*., 2009) and the FSS (Olby *et al*., 2001). Description of both scales are present in the table 4 and 5 in appendix I. This dynamic examination was recorded in all patients by a rehabilitation technician from HVA/CRAA, with the help of a Canon EOS Rebel T6 (EOS 1300D).

Afterwards, the static examination included the evaluation of posture to verify symmetries, postural reactions such us paw replacement and observation of support bases, and spinal reflexes (patellar reflex, withdrawal reflex, crossed extensor reflex, Babinski reflex and cutaneous trunci reflex), using a 12 cm Halstead mosquito curved forceps and a 18 cm Taylor reflex hammer.

Muscle tone was also assessed by palpation of both flexor and extensor muscles of the limbs. Finally, a simultaneous reflex movement of pelvic limb joints was performed, in order to search for signs of spasticity, muscle stiffness and articular pain.

8.4 Functional Neurorehabilitation Protocol

All the study group patients were prescribed the same intensive FNR protocol, starting daily at 9 am until 7 pm, six days a week. This protocol aims to stimulate the regenerative capacity of neural tissue.

As evidenced in neuroscience reports, locomotor training has a neuromodulatory role and allows for neural reorganization, contributing to regeneration (Côté *et al*., 2010). Therefore, treadmill (*Superior Fit Fur Life*) locomotor training was performed during 30 to 60 minutes, one or two times a day, five to six days a week, at 2 to 3,5 km/h, according to the cardiorespiratory capacity of each patient. All animals were already adapted to the locomotor training, because this was the second month of intensive protocol application. When exercise was well tolerated and the dog were able to attain 40 minutes minimum of locomotor training, treadmill slope was increased 5 to 10%.

Figure 2 – Animal 1 from study group performing locomotor training

(photograph kindly provided by HVA/CRAA)

The protocol also included a water treadmill training (*Hidro Psysio EUA*) during one hour in the morning period, 5 days a week, at 2-3,5 km/h, with the water level line at femur lateral epicondyle level and slope was increased to 5%.

In association with locomotor training, a segmental technique of electrical stimulation of the sciatic nerve was initiated (*BTL-4000 Premium*). The positive electrode was placed near the L7-S1 nerve root anatomic region and the negative electrode over the neuromuscular junction as near as possible to the motor point, in order to stimulate the intrinsic circuit of motor neurons and CPG. This stimulation was alternated with another one where the electrodes were inversely positioned, aiming to achieve a better

contraction of the flexor muscles group. Trichotomy was previously performed at these described locations and an ultrasound examination gel was applied before the electrodes placement. The parameters for electrical stimulation were 60 Hz and 24-36 mA, contraction/relaxation cycle of 1:4, 4 seconds ascending contraction ramp, 8 seconds plateau and 2 seconds descending contraction ramp. This therapeutic modality was prescribed three times a day, during six days a week.

Furthermore, kinesiotherapy exercises were prescribed and included 5 circuits of cavaletti rail training, 5 circuits of walking in different ground types and walking on ramps, two times a day, 5 days a week.

8.5 Physical Therapy Protocol

A physical therapy protocol was applied to the control group's animals for two months, whose owners didn't consent with SCBP, and was similar to the one described in Kathman and colleagues report from 2006.

Thus, these animals performed active exercise for 20 minutes, four times a day, five days a week, including gait stimulation on different type of ground and stair climbing and walking uphill. Equally 5 days a week, they completed a 20 minutes locomotor training on the water treadmill, at 2 km/h.

Due to delayed protrusion phase of movement in some patients, a paw protection was needed to prevent wounds on the digits' dorsal surface, whenever they were not performing the locomotor training.

8.6 Stem Cell Protocol

The Stem cell administration procedure took place in Veterinary Medicine School of University of Lisbon and was executed by the head of neurologic department Professor Doctor António Ferreira (AF), after a complete neurologic examination of the patient.

At this point, a peripheral vascular access was performed in all the study group's animals and dose-effect propofol (starting at 2 mg/kg) was administrated to induce hypnosis required to perform endotracheal intubation. Dogs were connected to an anesthesia machine, with an isoflurane maintenance with minimum alveolar concentration of 1,5, and received physiological saline infusion according to their maintenance rate.

Then, the hair of the dorsal cervical area was clipped and the skin was prepared for an aseptic procedure. The anatomical landmarks for the midline are the external occipital protuberance and the spinous process of the axis (C2). A 22-gauge 1,5-inch disposable spinal needle with a stylet was inserted in the middle near the cranial border of the wings of the atlas. A slight loss of resistance was felt as the needle enters the subarachnoid space, the stylet was removed and 1 ml of CSF was allowed to flow through the needle into a sterile tube, and SC were administrated, restoring the volume of fluid.

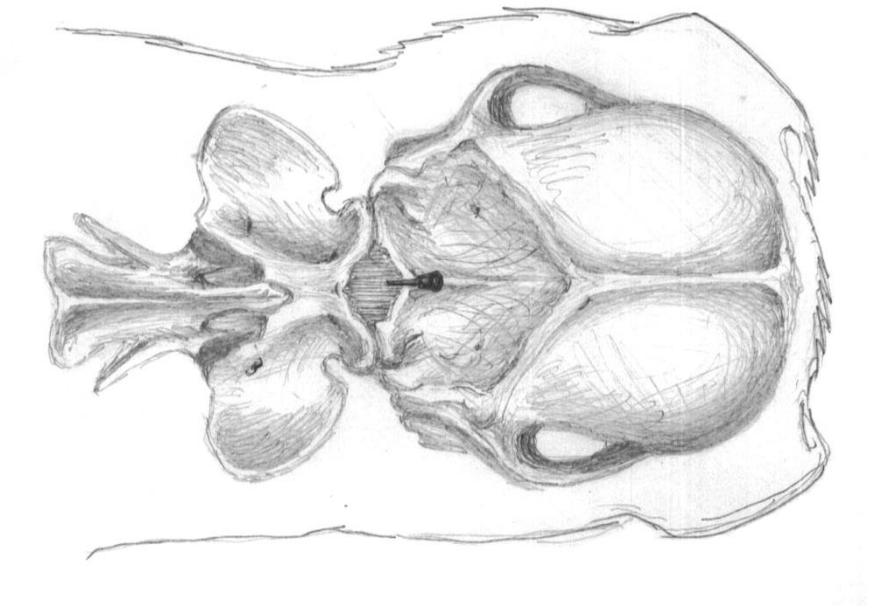


Figure 3 – Anatomical landmarks for cerebellomedullary cisternal stem cell transplantation in the dog, dorsoventral view of the cerebellomedullary cistern region (Author's illustration)

After the administration, the dog was placed in a more vertical position during 10 minutes, to allow CSF to flow down, according to myelography guidelines.

The SC administered intrathecally were allogenic and derived from adipose tissue. The source of adipose tissue was about 4-5 cm3 and it is used for isolation of mesenchymal stem cells as a primary cell system, that are further expanded in vitro. The product contains $>15x10^6$ cells provided in 1 ml certified tube, by FAT-Stem Laboratories®, Belgium.

Vetherapy® was responsible for SC product transportation, that arrived the day before their administration, respecting the optimal conditions of storage.

8.7 Outcomes and Follow-ups

The animals in the study group were evaluated immediately before they start the SCBP by a CCRP instructor/examiner certified by the University of Tennessee, AM. Exactly one week later another outcome is registered, and then neurologic examinations are performed each 15 days for all animals until the week 10 (week 1, 2, 4, 6, 8, 10). According to the neurologic examinations of each animal, follow-ups assessments were reprogrammed. After the first 10 weeks, in the dogs that presented stable neurologic status, follow-ups evaluations were performed every month during 3 months and then, every two months (week 14, 18, 22, 30, 38) until, in some cases, reach a total period of 91 weeks. (Figure 4)

The control group was evaluated at admission, at medical release of PTP and in the moment before euthanasia.

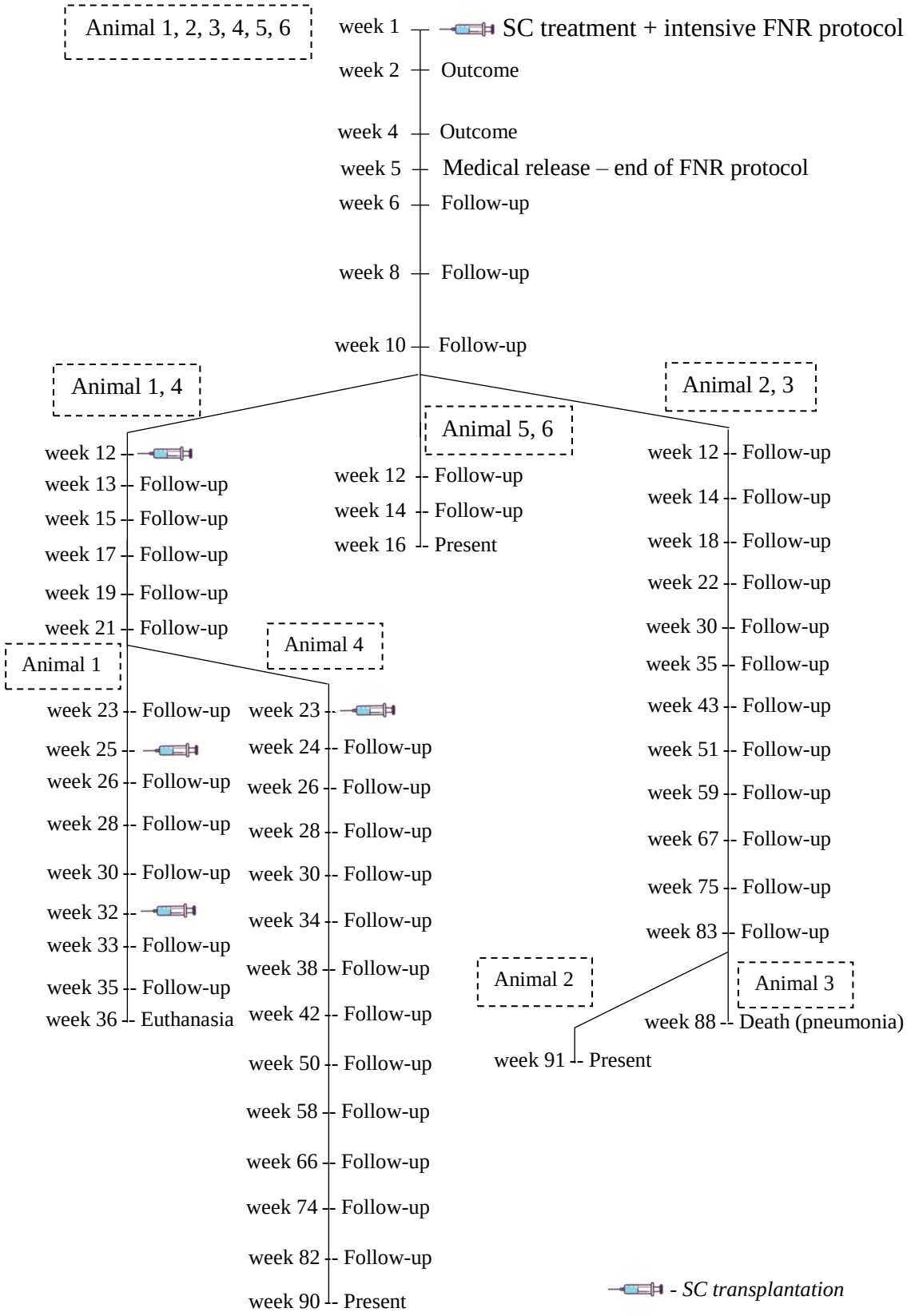


Figure 4 - Representation of outcomes and follow-ups performed in the study group's animals

8.8 Home Rehabilitation Program

All owners of the dogs in the study group received careful instruction on how to perform adequate rehabilitation exercises at home. Dogs should be stimulated to do locomotor training during 1 to 2 hours a day, 5 days a week. Whenever was possible, this exercise should consist in stair climbing, walking forming an eight shape on the floor, and changing of ground (grass, asphalt, wet sand). The sequence of these kinesiotherapy exercises should change each 5 days, in order to stimulate the maximum capacity of proprioception pathways regeneration. Furthermore, to avoid overtraining, owners were taught to control mucous membrane color and capillary refill time.

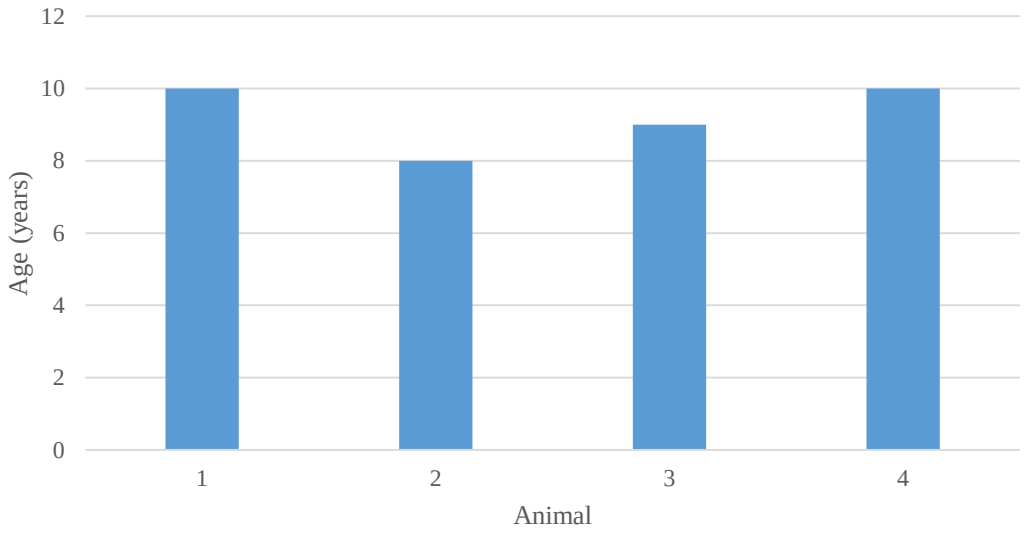
8.9 Statistical analysis

The spreadsheet program Microsoft Office Excel ® was used for statistical analysis. The sample characterization included the following topics: age, weight, gender, breed, genetic test result, survival time, information from neurologic examination, such as patellar reflex, withdrawal reflex, cutaneous trunci reflex, crossed extensor reflex and Babinski reflex and clinical condition evolution over time.

9. Results

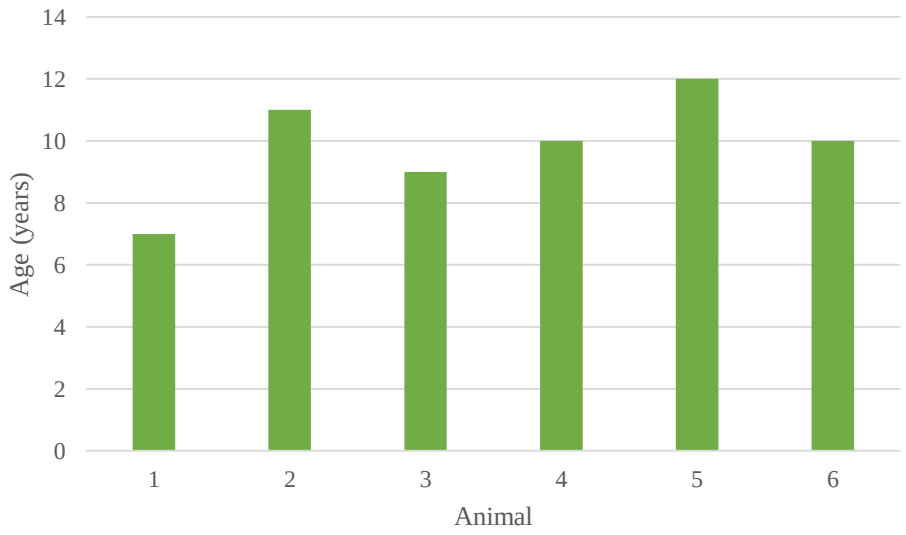
Population Characterization

The age distribution of the control group was composed by 4 dogs aged between 8 and 10 years old, with an average age of 9,3 years old (graph 1).



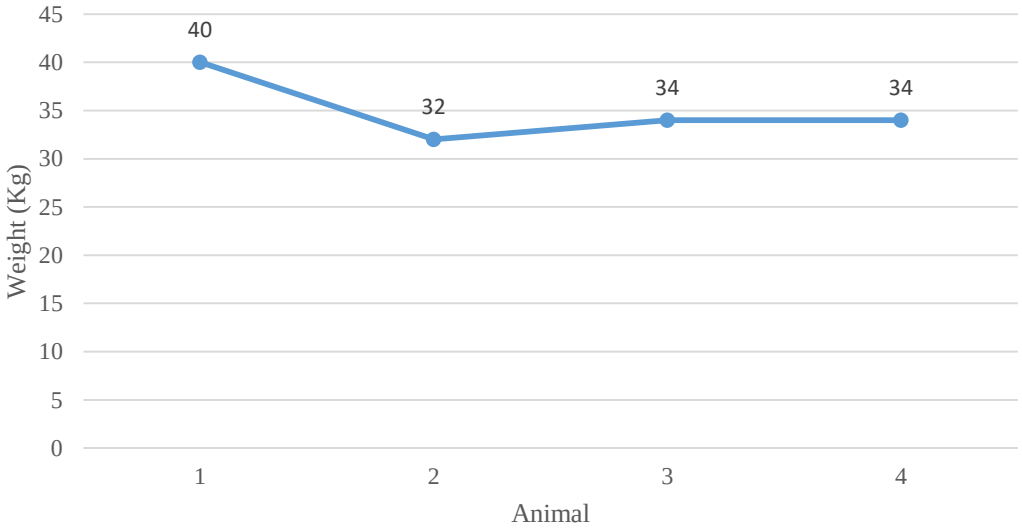
Graph 1 - Distribution of the control group by age

According to the study group, the six dogs were aged among 7 and 12 years old and the average age was 9,8 years old (graph 2)



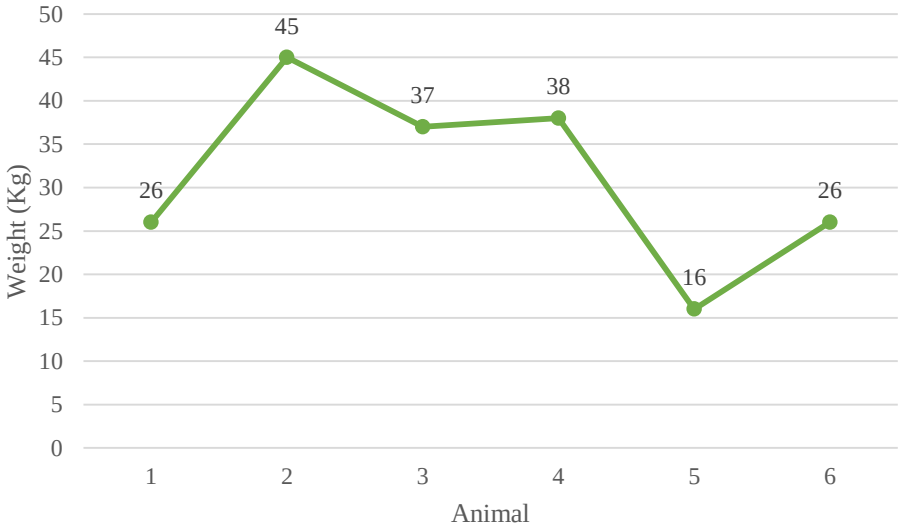
Graph 2 - Distribution of the study group by age

Relatively to the weight of the animals in the control group, values ranged from 32 kg to 40 kg, being the average weight 35kg (graph 3)



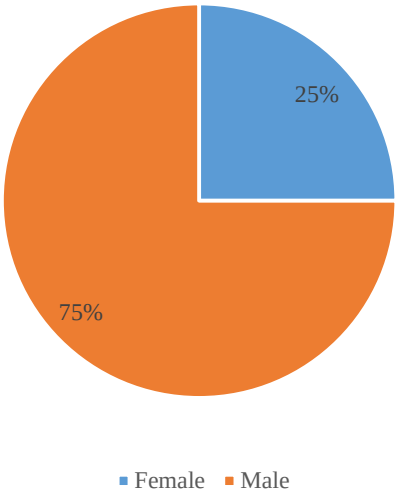
Graph 3 - Distribution of the control group by weight

In the study population, animals weighed between 16 kg and 45 kg. The average weight was 31,3 kg (graph 4)



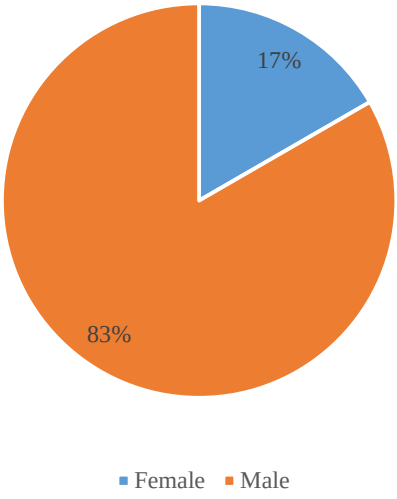
Graph 4 - Distribution of the study group by weight

The gender of the dogs in the control group is represented by 25% of females and a majority of 75% males (graph 5).



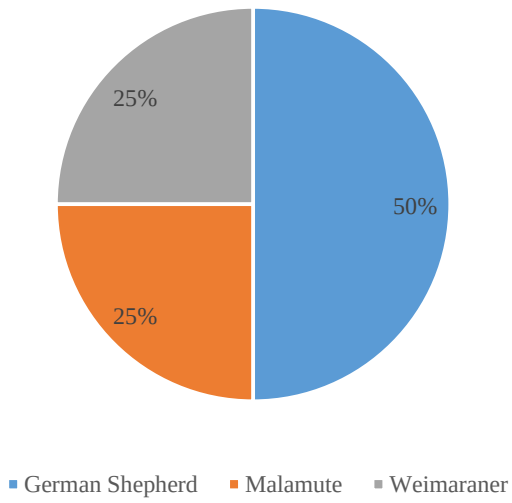
Graph 5 - Distribution of the control group by gender

The study group population is characterized by 17% of females and 83% of males (graph 6).



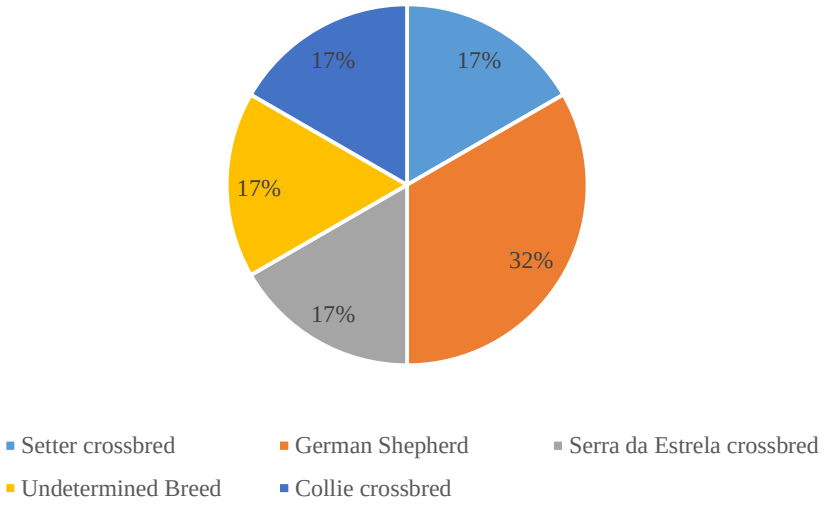
Graph 6 - Distribution of the study group by gender

Regarding the distribution of the control group by breed, German Shepherd dogs represents 50% of the population and Malamute and Weimaraner each represents 25% (graph 7).



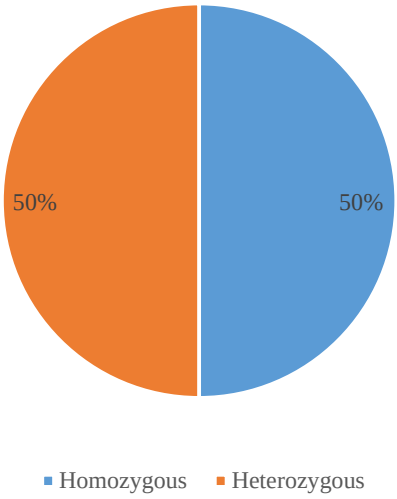
Graph 7 - Distribution of the control group by breed

In the study group, 2 dogs are German Shepherd (32%) and there is one dog (17%) representing each of the other breeds: Setter crossbred, Serra da Estrela crossbred, Collie crossbred and undetermined breed (graph 8).



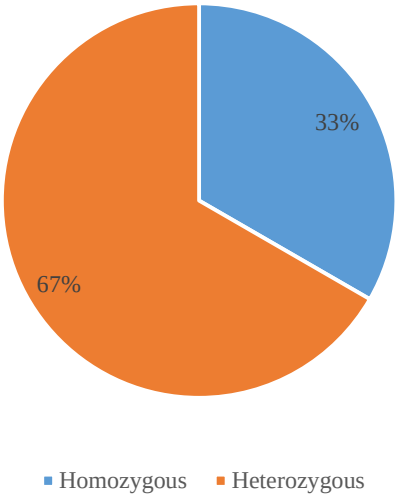
Graph 8 - Distribution of the study group by breed

In regard to the genetic test results of the control group, 2 dogs are homozygous (A/A) for SOD1 mutation (50%) and 2 dogs are heterozygous (A/G) (50%) (graph 9).



Graph 9 - Distribution of the control group by the genetic test result

In the study group, 2 dogs are homozygous (A/A) (33%) and 4 dogs are heterozygous (A/G) (67%) (graph 10)



Graph 10 - Distribution of the study group by the genetic test result

According to the survival time of the animals in the control group, the average is 15 weeks after PTP initiation. Two patients died within 12 weeks, in stage 1(1) and 1(2). One animal died in 16 weeks, in stage 1(1) and lastly one animal died 20 weeks after PTP initiation in stage 1 (2). All of them were euthanized.

In the study group, one animal was euthanized 36 weeks after SCBP initiation and another one died 88 weeks after the beginning of SCBP. The other 4 dogs in this group are still alive.

The next table represents the evolution of classifications according to the FSS of the animals in the study group, and we can observe that the average time interval between stem cell treatments is 11 weeks.

Table 3 - Evolution of the study group patients' classifications according to the FSS (outcomes and follow-ups)

Animal											
1		2		3		4		5		6	
week	FSS	week	FSS	week	FSS	week	FSS	week	FSS	week	FSS
1	5 (13)	1	3 (8)	1	3 (8)	1	5 (13)	1	5 (12)	1	2 (5)
2	5 (13)	2	4 (11)	2	4 (10)	2	5 (13)	2	5 (13)	2	3 (6)
4	5 (14)	4	5 (13)	4	5 (12)	4	5 (14)	4	5 (12)	4	3 (7)
6	5 (12)	6	5 (12)	6	5 (13)	6	5 (14)	6	4 (11)	6	3 (7)
8	4 (10)	8	4 (11)	8	5 (12)	8	5 (12)	8	4 (11)	8	3 (7)
10	4 (10)	10	4 (11)	10	5 (12)	10	4 (11)	10	4 (10)	10	2 (5)
12	4 (10)	12	5 (12)	12	5 (12)	12	4 (11)	12	4 (10)	12	2 (5)
13	4 (10)	14	5 (12)	14	5 (12)	13	4 (11)	14	4 (11)	14	2 (5)
15	4 (10)	18	5 (12)	18	5 (13)	15	5 (12)	16	4 (11)	16	2 (5)
17	4 (10)	22	5 (13)	22	5 (13)	17	5 (12)				
19	4 (11)	30	5 (13)	30	5 (13)	19	5 (12)				
21	4 (9)	35	5 (13)	35	5 (13)	21	4 (11)				
23	4 (9)	43	5 (13)	43	5 (13)	23	4 (11)				
25	4 (9)	51	5 (13)	51	5 (13)	24	4 (11)				
26	4 (10)	59	5 (13)	59	5 (13)	26	5 (12)				
28	4 (11)	67	5 (13)	67	5 (13)	28	5 (12)				
30	4 (9)	75	5 (13)	75	5 (13)	30	5 (12)				
32	4 (9)	83	5 (13)	83	5 (13)	34	5 (12)				
33	4 (10)	91	5 (13)	88	5 (13)	38	5 (12)				
35	3 (8)					42	5 (12)				
36	3 (8)					50	5 (12)				
						58	5 (12)				
						66	5 (13)				
						74	5 (13)				
						82	5 (13)				

- *SC transplantation*
- *Outcome/Follow-up*

In the present study, along with the FSS classifications, the following spinal reflexes were also evaluated: patellar reflex, withdrawal reflex, cutaneous trunci reflex, crossed extensor reflex and Babinski reflex.

According to the patellar reflex, after SC administration, four patients (66,7%) turned from clonic to normal and another patient (16,7%), who demonstrated absence of patellar reflex, after SC administration this became normal. (Figure 5)

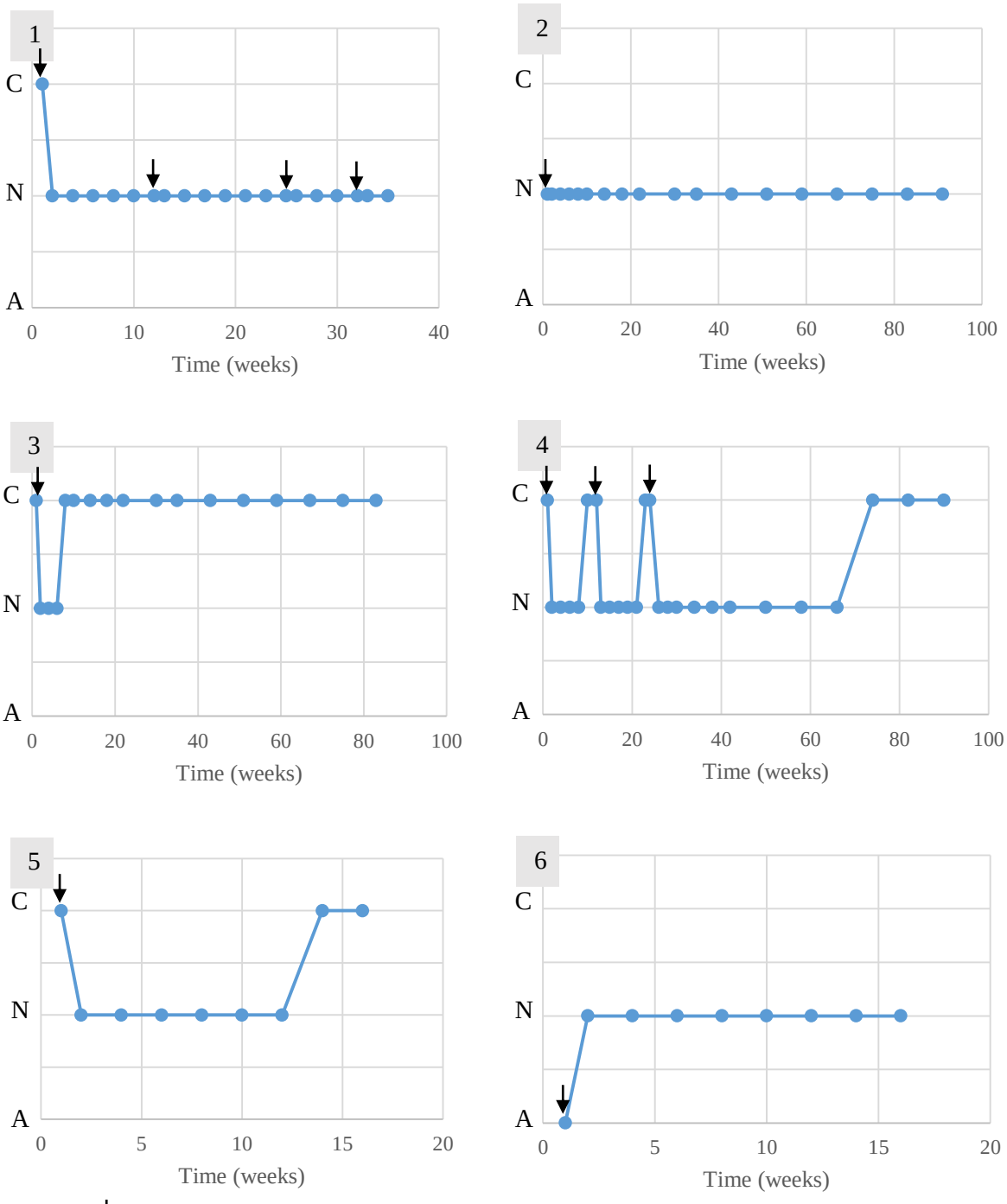
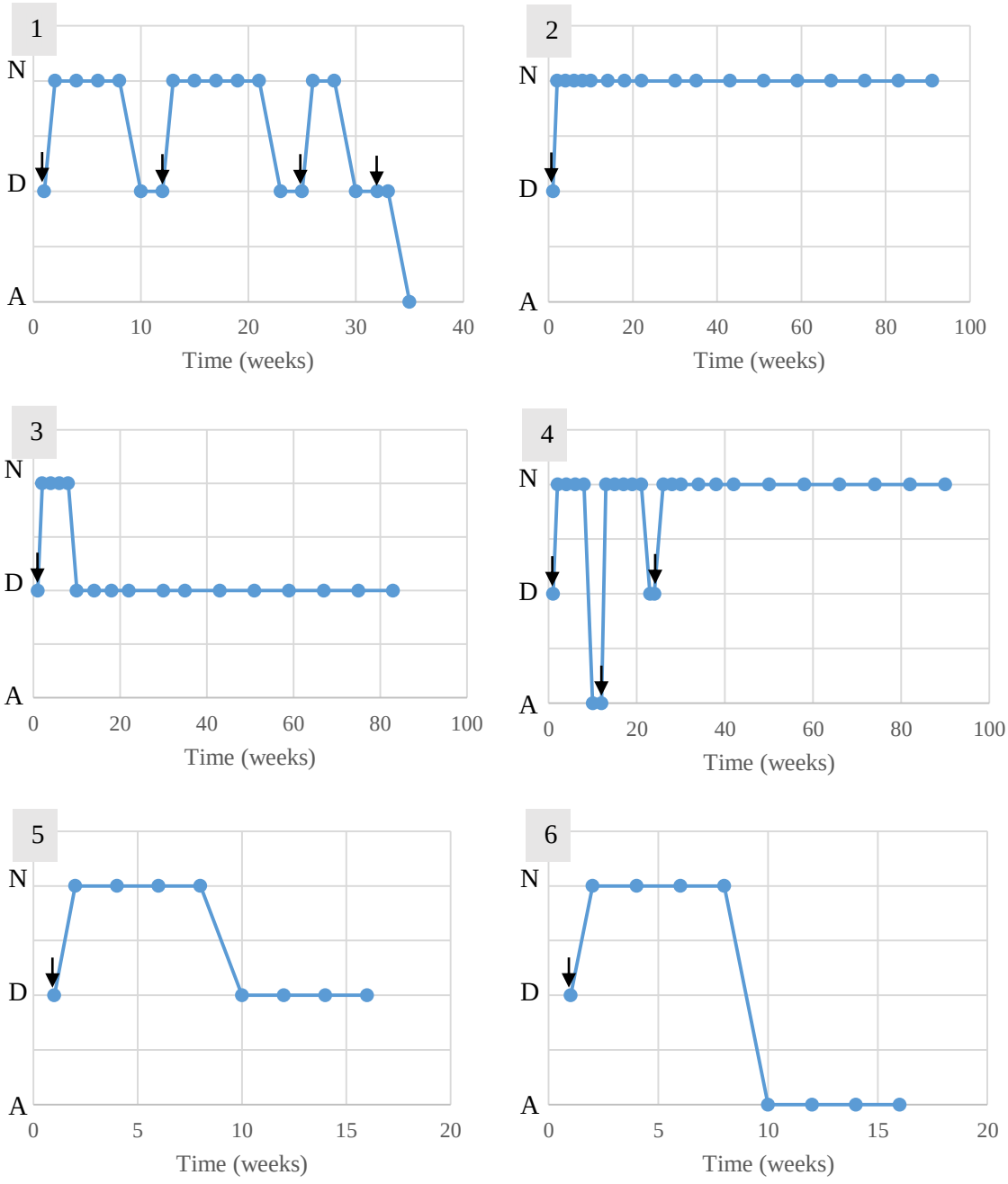


Figure 5 - Evolution of the patellar reflex in the study group's animals

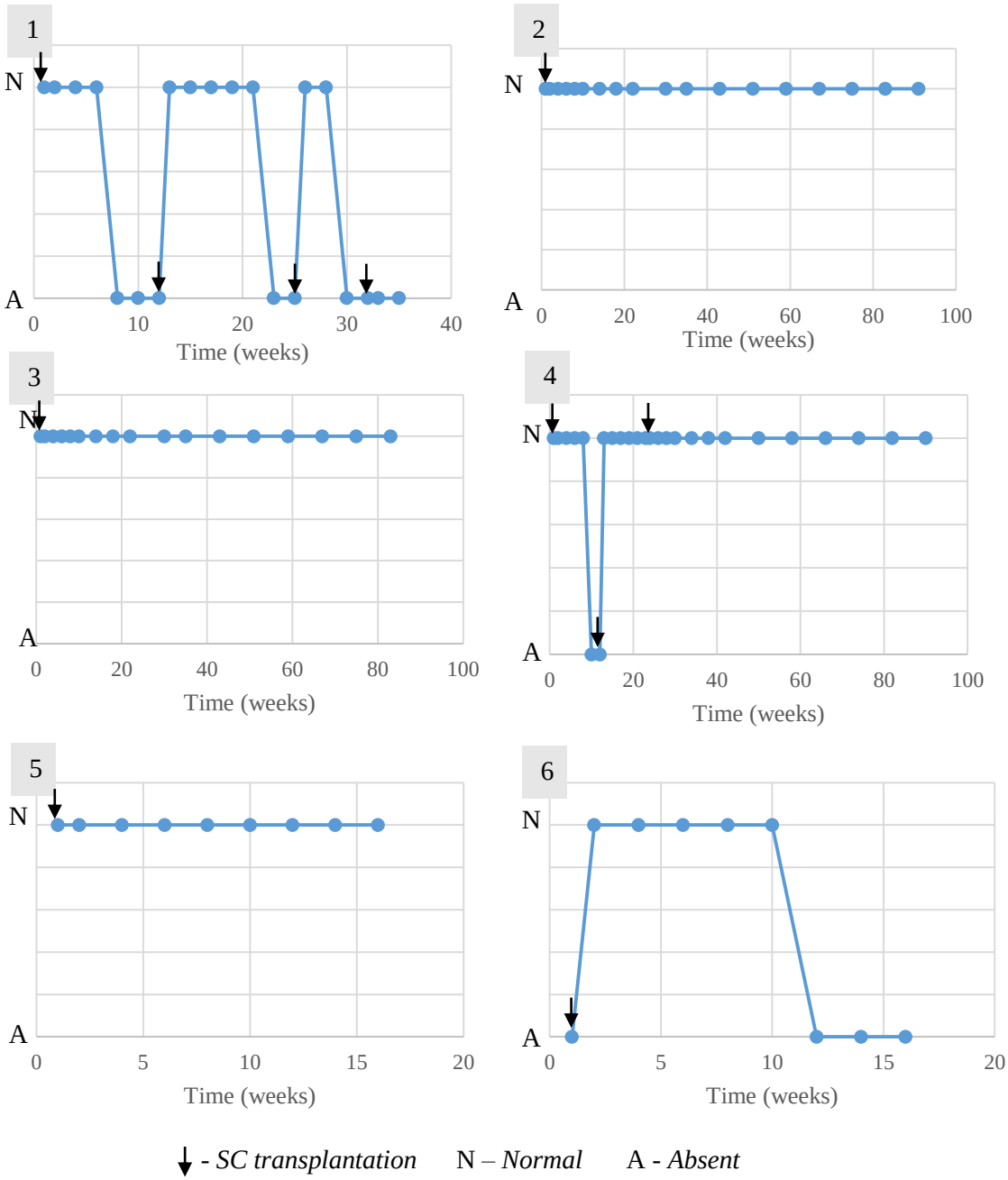
In reference to the withdrawal reflex, after one month of intensive FNR the six patients (100%) had a decreased withdrawal reflex and, in the observation following the SC injection, this became normal. (Figure 6)



↓ - *SC transplantation* N – *Normal* D – *Decreased* A - *Absent*

Figure 6 - Evolution of the withdrawal reflex in the study group's animals

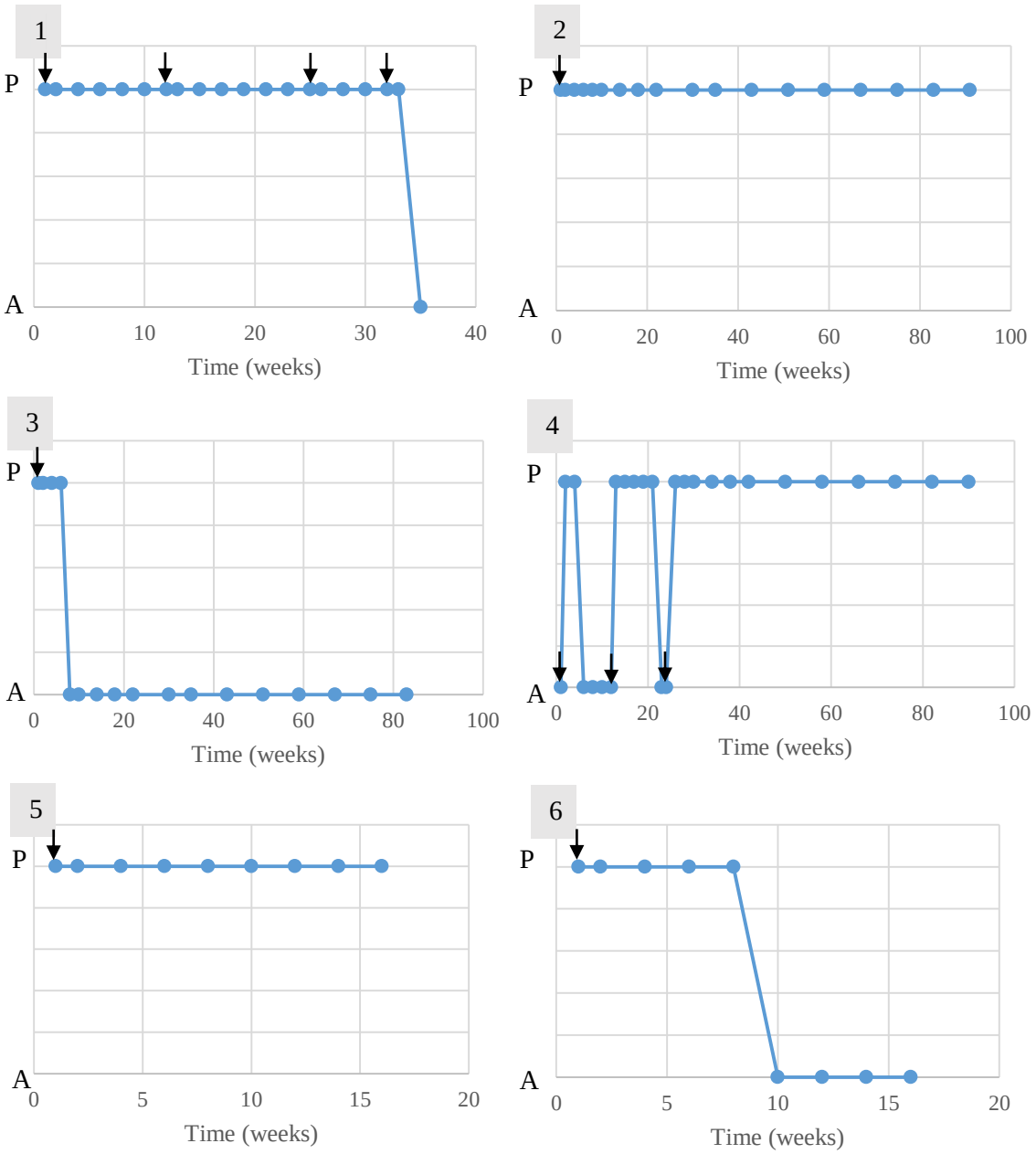
Regarding the cutaneous trunci reflex, five patients (83,3%) had normal reflex at study admission (cutaneous trunci reflex between the T2 region to the edge of the ilium wings). One animal (16,7%) demonstrated absence of this reflex (ascending reflex cranially from cutaneous region of the ilium wings). Throughout the study, patients who had no reflex, after SC administration and home rehabilitation program, recovered the reflex, as shown in figure 7.



↓ - *SC transplantation* N – *Normal* A - *Absent*

Figure 7 - Evolution of the cutaneous trunci reflex in the study group's animals

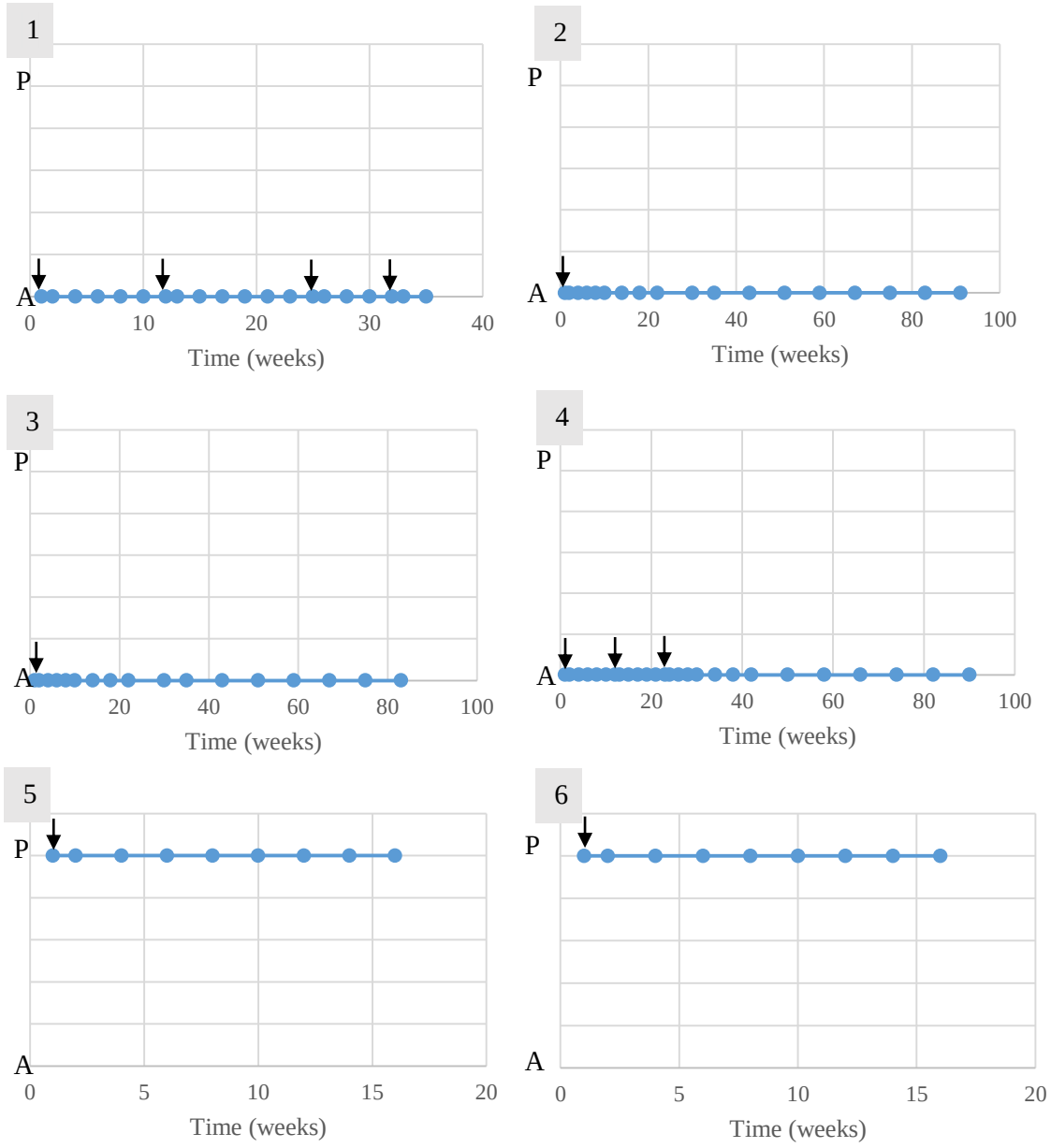
In regard to the crossed extensor reflex, in animal 4 (16,7%), who exhibit no reflex, the several SC administrations performed over time allowed it to reappear. (Figure 8)



↓ - *SC transplantation* P – *Present* A - *Absent*

Figure 8 - Evolution of the crossed extensor reflex in the study group's animals

As for the Babinski reflex, four patients (66,7%) exhibit no response (normal) and the other two (33,3%) exhibit the abnormal reflex. (Figure 9)



↓ - *SC transplantation* P – *Present* A - *Absent*

Figure 9 - Evolution of the Babinski reflex in the study group's animals

10. Discussion

The present study is balanced according to the average age, between the control group and the study group, which is about 9 years old, in accordance with an article from 2010 of Coates and Wininger.

The same is noticed for the registered weight of the animals in both groups, because the averages were between 30 and 35 kg, which is also in conformity with some studies (Capucchio *et al*., 2014; Steven *et al*., 2018), that refers that large size dogs are predominant for this condition.

In the gender category, once again there is a balance between the two groups, and it is observed that more than 70% of the animals are males. This is in line with old reports (Griffiths & Duncan, 1975; Averill, 1973).

In both groups, German shepherd is the main breed, 50% in the control group and 32% in the study group, although there is a breed diversity in the last one, it is in conformity with Steven & Coates, 2018.

Regarding the genetic test result, the groups are not balanced, because there are more heterozygous animals in the study group. The presence of heterozygous affected animals is normal in DM studies (Ogawa *et al*., 2014), however homozygous are in prevalence in most of the reports (Kobatake *et al*., 2017; Pfahler *et al*., 2014).

Although this is a controlled study, in which patients are followed until the present, the number of animals in both groups is small, but the follow-ups were performed over time (as referred in figure 4), and patients were evaluated according the FSS (table 5, appendix I).

Several studies report the SC properties and effects, but for Park *et al.* (2011), MSC transplantation allows a reduction of neural tissue atrophy after a week as well as an upregulation of inflammatory cytokines associated with intracellular signs modulation that decreases astrogliosis. These effects are demonstrated in 67% of patients (n=4/6), who improved according with FSS classification, in three, two and one level.

The other patients (animal 1 and 4) stayed on the same FSS classification after SC transplantation, however patellar reflex turned from clonic to normal (figure 5) and withdrawal reflex also turned from decreased to normal (figure 6). Regarding the animal 4, the crossed extensor reflex reappeared after the treatment (figure 8).

Reflexes reappearance related to monosynaptic reflex neuromodulation suggests some neural regeneration seven days after MSC transplantation, as Penha *et al*. (2014) reports, although in that study the progressive recovery evaluation had occurred in the tenth day.

In the same study, 24 weeks after SC transplantation, it was not observed clinical gains. Nonetheless, in the present study, 33,3% (n=2) of the animals kept the FSS classification of 5(13) in weeks 22 to 30, which corresponds to a normal locomotion with sensorial deficits. It should also be noted that mentioned patients (animals 2 and 3) kept a proprioceptive ataxia locomotion for 91 weeks and 88 weeks, respectively. This is possibly due to anatomic and synaptic neuroplasticity (Srivastava *et al*.,2014; Ducati *et al*., 2014), inherent to depolarizations of neural connections synapses through the amplitudes necessary to motor neurons intrinsic circuits and CPG activation, allowing an improvement in functional outcomes (Côté *et al*., 2010).

In this study, SC transplantation and the intensive FNR protocols had a therapeutic synergetic effect, although the applied cells were allogenic. According to Jung *et al.* (2009), this type of SC is less effective than autologous ones, however in 67% of animals in the present study have better follow-ups evaluations comparing to the mentioned study.

In a study from 2009, Jung and his colleagues report a significant decrease of functionality around the fourth week after transplantation, and also mention that there is a reduction in growth factors in this period.

This growth factor reduction tendency can be a possible explanation for the decreased withdrawal reflex in the present study, observed in the follow-up FNR examination between the sixth and eighth week. In addition, it is possible to notice in table 1 that patient 1 and 4 had a SC transplantation reinforcement after 10 or 11 weeks, thus contradicting Lim *et al*. (2007), that reports a functionality stability for only 4 to 8 weeks after transplantation.

The therapeutic synergetic effect can possibly be the reason for the temporal extension of animal's functionality and consequently postponement of SC therapy reinforcement. This synergetic result can be partly due to the release of growth factor such as neurotrophic growth factors, whose production is stimulated by locomotor training and stem cells (Petrou *et al*., 2016). The SC neuroregenerative influence is thus mediated by

growth factors such as the nerve growth factor and fibroblastic growth factor, BNDF, glial cell derived neurotrophic factor (Yousefi *et al*., 2019).

For Ryu *et al*. (2012) and Sykiva *et al*. (2005) the functional improvement resultant from SC transplantations occurs after the first 2 or 3 weeks, whereas in this study 55% of the SC transplantations (6/11) had evident effect in the first follow-up (one week after). The possible cause for this result is the increase in neural tissue activation enhanced by the intensive FNR protocol (Edgerton & Roy, 2009).

Therefore, this study is in conformity with Ryu *et al*. (2009) that also use allogenic adipose-derived SC, and observe an improvement in neurologic functions related to the release of growth factors, intercellular signals, angiogenic and anti-apoptotic cytokines and chemotactic factors. These factors support endogenous spinal cord-derived neural progenitor cells differentiation, possibly located in neural degeneration regions and allowing new neural pathways to be created (Lim *et al*., 2007).

Ryu and colleagues (2009) describe that survival SC can produce large amounts of basic fibroblast growth factor and vascular endothelial growth factor, as reported by Yang *et al*. (2008) in human medicine. Furthermore, for Ryu *et al*. (2012), the SC neuroinflammatory modulation ability can help prevent astrocytosis.

As already mentioned, DM is a neurodegenerative disease with clinical, histopathologic and genetic parallels with human ALS (Golubczyk *et al*., 2019). In that condition, MSC are also used because they are less immunogenic, have immunomodulator capacity, neuroprotection and tissue repair ability (Forostyak *et al*., 2014) and allow motor function improvement and prolonged motor neuron survival in affected patients (Ciervo *et al*., 2017).

Zhou and colleagues (2013) suggest once again the importance of MSC neuroprotective effect and relate it to a decrease in microglia activation. They also report that the inhibitory effect on microglial activation of MSC can be more effective in early stages of ALS. However, it is important to highlight that in the present study the six patients were non-ambulatory paraparesic to paraplegic when admitted to the first FNR examination. They all reacted to the first intensive FNR protocol, before the clinical therapeutic program, but didn’t reach motor functionality, hence the SC therapy approach.

In the current study, the animal 4 was submitted to three SC transplantations and after the last one manifested clinical signs of rejection, probably because the SC used were allogenic.

The average survival time of the control group's animals was 15 weeks after PTP initiation. In contrast to the study group, where one patient (animal 1) survived 36 weeks since the beginning of SCBP and another (animal 3) survived 88 weeks, in which the cause of death was a clinical occurrence (pneumonia). The other 4 animals in the study group are alive and in process of FNR evaluations. In conclusion, the SCBP may be a possible and potential therapeutic option in DM-affected animals, as a multidisciplinary protocol.

A study limitation is the small size of the sample, both the study group and the control group. Regarding the last mentioned, the number of follow-ups is also limitative, contrasting with the study group, where are animals with a minimum follow-up of 16 weeks and other with a maximum of follow-ups during 88 or 91 weeks. Another evident limitation is the fact that no histopathologic examination was performed in the control group's animals and neither in animal 1 and 3 in the study group after their death. In order to use a more effectual scientific evidence of neural regeneration, it would be interesting to use inflammation biomarkers, to demonstrate the anti-inflammatory and immunomodulatory role of stem cells. Finally, in a future prospect it would be important to continue this study, prolonging follow-ups and increasing the number of animals.

11. Conclusion

As a progressive neurodegenerative disease, DM changes the clinical condition of affected dogs, and becomes a limiting cause of functionality, especially with regard to their family integration. In close parallel to ALS in humans, a protocol based on regenerative medicine is a possible potential therapeutic approach that, in the current study, reaffirms its synergetic power in association with intensive functional neurorehabilitation.

In conclusion, the suggested stem cell based protocol is a multidisciplinary strategy that has been shown to increase the number of days with quality of life and autonomy in patients with degenerative myelopathy.

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Appendix I

Table 4 - Adapted Modified Frankel Spinal Cord Injury Scale for Dogs

(Levine *et al*., 2009)

Score	Description of neurologic condition
0	Para/tetraplegia without deep nociception
1	Para/tetraplegia without superficial nociception
2	Para/tetraplegia with intact nociception
3	Non-ambulatory para/tetraparesis
4	Ambulatory para/tetraparesis or ataxia
5	Normal gait and nociception

Table 5 - Functional Scoring System in Dogs with Acute Spinal Cord Injuries (Olby *et al*., 2001)

Stage	Level	Description of neurologic condition
1	0	No pelvic limb movement and no deep pain sensation
	1	No pelvic limb movement with deep pain sensation
	2	No pelvic limb movement but voluntary tail movement
2	3	Minimal non–weight-bearing protraction of the pelvic limb (movement of 1 joint)
	4	Non–weight-bearing protraction of the pelvic limb with 1 joint involved 50% of the time
	5	Non–weight-bearing protraction of the pelvic limb with 1 joint involved 50% of the time
3	6	Weight-bearing protraction of pelvic limb 10% of the time
	7	Weight-bearing protraction of pelvic limb 10 to 50% of the time
	8	Weight-bearing protraction of pelvic limb 50% of the time
4	9	Weight-bearing protraction 100% of the time with reduced strength of pelvic limb. Mistakes 90% of the time (eg, crossing of pelvic limbs, scuffing foot on protraction, standing on dorsum of foot, falling)
	10	Weight-bearing protraction of pelvic limb 100% of the time with reduced strength. Mistakes 50 to 90% of the time
	11	Weight-bearing protraction of pelvic limb 100% of the time with reduced strength. Mistakes 50% of the time
5	12	Ataxic pelvic limb gait with normal strength, but mistakes 50% of the time (eg, lack of coordination with thoracic limb, crossing of pelvic limbs, skipping steps, bunny-hopping, scuffing foot on protraction)
	13	Ataxic pelvic limb gait with normal strength, but mistakes made 50% of the time
	14	Normal pelvic limb gait