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SPECIFIC IN-DEPTH INSIGHTS ON FEATURES OF IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE IN DORSAL ROOT GANGLIA AND CLINICAL SIGNS IN NEUROPATHIC AND NON-NEUROPATHIC DOGS

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Abstract

Disorders of the somatosensory system causing neuropathic pain (NP) are common in people. In small animals medicine, this condition remains underestimated probably due to the lack of standardized assessment methods and studies focused on the description of structures playing a key role in developing and maintaining neuropathic pain, such as dorsal root ganglia (DRG).

Hence, the aims of the present research project, structured in three different studies, were:

a) investigate the possible effects of the reduction in radicular blood flow on the vascular density, ganglionic and endoneurial ischemia and shift in neuronal metabolism, in fifteen L7-DRG from a total of eight dogs suffering from painful compressed spinal L7 nerve roots (NRs) and DRG;
b) perform a preliminary characterization of the expression of substance P, calcitonin gene-related peptide, calbindin D-28k, neuronal nitric oxide synthase and the colocalization of those molecules in lumbosacral DRG neurons of three neurologically normal dogs;
c) Investigate the prevalence, risk factors and owners' perception of their pets quality of life of the Phantom Complex (PC) in a client-owned population of 107 dogs with limb amputation through an online survey.

Main results were:

a) Our findings highlight a significant neuronal distress in compressed DRG. Despite the similarity of vascular density between controls and compressed L7-DRG, we found a significant increased in neuronal immunoreactivity for hypoxia-related markers and for markers related to glycolytic cellular metabolism.
b) The wide variability of CGRP-, SP-, CALB-, and nNOS-immunoreactivity among different species was confirmed. Further studies are required in order to better define the characterization of those markers in canine DRG.
c) Our study demonstrates for the first time the presence of PC.Significant risk factors associated with the frequency of post-amputation pain episodes are duration of pain before amputation and time between diagnosis and amputation.

Introduction

Neuropathic pain (NP) is defined as a type of chronic and maladaptive pain arising as direct consequence of a lesion (or a disease) affecting the nervous system. Independently from the origin of the lesion, pathologic neuropathic mechanisms may expand to involve both peripheral and central nervous structures, including the dorsal root ganglia, which play a key role in triggering and maintaining the "spinal central sensitization".

Despite different animal models have been intensively used to investigate the mechanisms involved in the development of neuropathic pain, currently there is a lack of studies focused on the expression, distribution, and immunocytochemical characterization of pain-related molecules in dorsal root ganglia of healthy dogs.

In human medicine, a recent study on neuropathic pain, found a prevalence ranging from 7% to 8%. [1, 2] Different standardised screening tools, such as PainDETECT, ID-Pain and DN4, have been developed to identify and classify neuropathic pain on the basis of patient-reported verbal descriptors of pain modalities.[3-7] Nevertheless, the diagnosis of neuropathic pain remains difficult and its real prevalence may still be considered underestimated. The clinical value of the screening tools consists in the possibility to better identify patients with neuropathic pain, in order to permit an appropriate pharmacological approach. However, despite many pharmacological treatments available, the unsuccessful management of neuropathic pain still represents the majority of cases.[8]

In veterinary medicine, during the past 10 years, pain has been a common topic for many studies.[9-14] Most of these papers were focused on pain management and,

actually, there is still a relevant lack of studies centered primarily on neuropathic pain.

Different conditions may cause neuropathic pain in dogs as in humans. One of the best known in the dog is represented by the Degenerative Lumbosacral Stenosis (DLSS), named “Lumbar Canal Stenosis” (LCS) in human medicine.

DLSS is caused by a complex of causes, including possible instability of the area, protrusion of the degenerated L7-S1 intervertebral disc, proliferation of the soft tissue surrounding the cauda equina, sacral osteochondrosis and vascular compromise of the spinal nerves, leading to the stenosis of the vertebral canal and compression of the cauda equina, nerve roots and dorsal root ganglia (this latter called “Neuro-Foraminal Stenosis” [NFS)]), in a relevant percentage of affected dogs.[15, 16]

In human beings, the most common consequence is the development of neuropathic pain, reported in 37% to 64.7% of patients affected by LCS.[17]

Although the direct contribution of vascular dysfunction to the development of neuropathic pain has not been fully clarified, evidence of microvascular disturbances has been reported in human and animal models of nerve compression, suggesting a correlation between the mechanical compression of the nerve roots and dorsal root ganglia and the haemodynamic factors.[18-21] However, the evidence of ischemia and changes of the vascular density in the affected nerve roots and dorsal root ganglia of patients with natural nerve root compression remains to be proven.

In human beings, a well known syndrome related to neuropathic pain is the Phantom Complex (PC).[22-24] The PC includes three different elements: a) Phantom Limb Pain (PLP) which is defined as painful sensations referred to the absent limb; b) Phantom Limb Sensation (PLS) as any sensation in the absent limb, except pain; c) Stump Pain (SP) defined as pain localized in the stump.[22-24]

These elements often coexist in each patient and may be difficult to separate.

PLP is reported to occur in about 60 to 80% of patients within the first 2 years after amputation and persists during the whole life in up to 10% of the affected population.[25-27]

Despite PC may occur in veterinary patients, there are no focused studies in dogs and cats. The few reports published on amputated animals focused on the biomechanical adaptation of dogs and owners' satisfaction, without investigating the presence and characteristics of PC.

Hence, the present research project was focused on three different aspects of neuropathic pain in dogs:

1) The preliminary description and characterization of the expression of substance P (SP), calcitonin gene-related peptide (CGRP), calbindin D-28k (CB), neuronal nitric oxide synthase (nNOS) and the colocalization of those molecules in lumbosacral spinal ganglia (SG) neurons of dogs without pathologies related to the nervous system;

2) The investigation of the possible effects of the reduction in radicular blood flow on the vascular density, ganglionic and endoneurial ischemia and shift in neuronal metabolism, leading to neuronal dysfunction, in naturally compressed spinal L7 nerve roots and dorsal root ganglia (DRG) in a canine model of DLSS. To challenge this hypotesis we evaluated the ganglionic vascular density by antifactor VIII-related antibodies (factor VIII) and the presence of hypoxia through the expression of hypoxia-related markes as the Neuroglobin (NGB), the Carbonic Anhydrase-IX (CA-IX), and the Vascular Endothelial Growth Factor (VEGF). Furthermore, we investigated the lactic

stress by assessing the expression pattern of Monocarboxylate Transporter-1 (MCT-1) and -4 (MCT-4).

3) The assessment of the prevalence, risk factors and owners' perception of their pets quality of life associated to Phantom Complex in a client-owned population of dogs with limb amputation through an online survey.

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Marker expression suggests complex metabolic stress in compressed canine L7 root ganglia

Introduction

The term Lumbar Canal Stenosis (LCS) refers to any type of narrowing of the spinal canal, nerve root canals or intervertebral foramina by surrounding bone and soft tissues.[1-3] In human medicine, the LCS is a common disease, affecting from 21% to nearly 100% of persons over age 60.[4] LCS may involve central and/or lateral structures, compressing the conus medullaris and/or spinal nerve roots (NRs) and dorsal root ganglia (DRG), eventually resulting in a Neuro-Foraminal Stenosis (NFS).[1,2] The number of patients with LCS and NFS complaining of low back pain, lower extremity pain and/or numbness and neurogenic intermittent claudication has increased yearly, with the prevalence in humans ranging from 18.9% to 29.7%.[2,5]

In dogs, Degenerative Lumbosacral Stenosis (DLSS) largely resembles human LCS. DLSS refers to a degenerative disorder that is multifactorial origin. In DLSS, the intervertebral disc (IVD) degeneration and bony and soft tissue proliferations contribute to spinal stenosis, cauda equina compression and NFS in 68% of dogs.[6,7] The clinical signs of DLSS in dogs have been well documented, with a high resemblance with those reported in humans. The most commons comprise pelvic limb lameness, hyperesthesia of the lumbosacral area or pelvic limbs, sensory and motor disturbances and urinary or fecal incontinence.[6-9]

In humans, as in dogs, the most common consequence of LCS/DLSS is the development of a chronic neuropathic pain (NP), which affects 37% to 64.7% of human patients suffering from low back pain.[10] Whereas, dogs affected by DLSS revealed signs of caudal lumbar pain from 68.6% to 97.7%.[11,12]

NP is defined as a type of chronic and maladaptive pain arising as direct consequence of a lesion or a disease of the nervous system. Independently of the origin of the lesion, neuropathic mechanisms may involve peripheral and central nervous structures, such as the DRG.

Several experimental studies *in vivo* and *in vitro* have focused on NRs and DRG compression, so far. The results showed nerve fiber deformation, intraganglionic edema with increase endoneurial pressure, changes in nerve root microcirculation, break down of blood-nerve barrier, demyelination, myelinated axon loss, Wallerian degeneration, inflammatory reactions and cell death.[13-17] These type of alterations appear not to be confined to the site of compression only, but also extend to extrafocal areas and in particular the DRG.[18-20] One of the explanations for the extension of changes would be a circulatory compromise with impact on nerve root physiology and metabolism.

In previous studies, it was shown that dogs under iatrogenic spinal NRs compression developed a reduction of 50-60% of the radicular blood flow. The blood flow remained diminished for a critical period even after the compression had been removed.[2] In an experimental rat study, authors correlated the vascular supply to an increased expression of Vascular Endothelial Growth Factor (VEGF) after application of mechanical compression to the DRG.[21] VEGF seems to be an important trigger for angiogenesis and vascular permeability as consequence to hypoxia.[21] In a preceding investigation we were able to demonstrate structural vascular abnormalities in dogs with NRs compression.[22] Furthermore, a recent study in mice showed that a traumatic nerve injury resulting in painful peripheral

neuropathy can lead to a vascular abnormalities and subsequent endoneurial hypoxia.[18]

All these studies suggest a correlation between the mechanical compression of NRs, DRG changes and the haemodynamic factors. However, the evidence of ischemia and changes to the vascular density in affected nerve roots of patients with natural nerve root compression remains to be proven.

On top of numerous valuable experimental models of nerve root compression, the disease development in dogs suffering from spinal NRs entrapment may more closely resemble human cases of rhizopathic sciatica, suggesting the possibility of employing canine DLSS as translational platform.

Hence, the aim of this study was to elucidate the contribution of vascular compromise and its metabolic consequences to neuropathic pain in canine nerve root compression. A better understanding of the pathophysiology of compressed NRs and DRG in the dog allows for development of better taylored causal treatments of NP in these animals and their human counterpart.

Materials and Methods

Ethics Statement

This study was performed on archived nerve root biopsies and autoptic samples submitted for histological evaluation of compressive radiculopathy and possible differentials.

Stereological Assesment

Image analysis and quantification of vascular density were evaluated by a single rater blinded for the origin of the sample using new-Computed Assisted Stereology Toolbox software (new-CAST™, Visopharm, Horsholm, Denmark) connected to a light microscope equipped with a motorized XY stage, after immunohistochemical labelling for von Willebrand factor (factor VIII).

Using new-CAST, series of DRG sections were outlined at low magnification (4x objective). Subsequently, the outlined DRG section was divided in fields and, using an higher magnification (100x objective), the total area fraction of every field was assessed using a double crosses grid that overlapped every field.

The double crosses grid was composed by a first order of 10x10 crosses (100 crosses/field) and by a second order of 25x25 crosses (2.500 crosses/field) with a 1:25 ratio between first and second order crosses.

For the first order crosses were counted those hitting neither the DRG nor the perineurium (a) and those hitting only the perineurium (b), obtaining the number of

first order crosses hitting the DRG and perineurium (c = 100-b). For the second order crosses were counted those hitting the blood vessels in the DRG and perineurium (d) and those hitting only the blood vessels in the perineurium (e), obtaining the number of second order crosses hitting the blood vessels in DRG without perineurium (f = d-e), the number of second order crosses hitting the DRG and perineurium (g = c*25), the number of second order crosses hitting the perineurium (h = b*25) and the number of second order crosses hitting the DRG (i = g-h) .

Finally, the calculated parameters were: the volume fraction of the blood vessels in the DRG and perineurium ($Vv_{(vessels/DRG\ with\ perineurium)}$) (d/g), the volume fraction of the blood vessels in the DRG without counting the blood vessels in the perineurium ($Vv_{(vessels/DRG\ without\ perineurium)}$) (f/i), and the volume fraction of the blood vessels in the perineurium ($Vv_{(vessels/perineurium)}$) (e/h).

Tissue Sampling

L7 DRG were sampled from dogs with history of lumbosacral pain due to NFS confirmed by magnetic resonance imaging as described elsewhere.[6] Clinical assessment and MRI reading were carried out by veterinary neurology and imaging specialists. Further DRG, routinely harvested during postmortem examination (PME) of age- and breed-matched dogs without clinical evidence of back and limb pain or neurological deficits and with autoptic exclusion of lumbosacral pathologies and hind limb orthopaedic disorders were chosen as controls. Autopsy in these dogs was requested for purposes unrelated to the aims of this study.

Wedge shaped nerve roots/DRG biopsies were collected from NFS patients during foraminotomy surgery.

In dogs subjected to PME, the spinal cord and associated nerve roots, including the DRG, were removed in toto after dissection of the epaxial and paraxial musculature, extensive laminectomy, removal of transverse processes and the crista iliaca and desmotomy of ligamentous nerve root attachments.[23]

The tissues were immediately transferred in 10% neutral-buffered formalin for 24 hours. Fixed DRG were trimmed transversely with a razor blade into slices of 1 mm thickness. This study in particular concentrated on the aequatorial level of postmortem DRG and on serial transverse sections of wedge biopsies. These were postfixed in formalin for another 12 h and underwent subsequent processing by an automatic histoprocessor (TP 1020 Leica Instruments, Nussloch, Germany) running on a specific program for nervous tissue as described elsewhere.[24] After completing the cycle the tissues were embedded in paraffin (Paraplast Plus®, Leica Biosystems, St. Louis, Mo., USA), sectioned at 5 µm slice thickness and mounted on positively charged amino-propyl-ethoxy-silane-coated slides (Star Frost Adhesive, Engelbrecht Medizin- und Labortechnik, Edermunde, Germany).

Prior to histological and immunohistochemical staining, the sections underwent a standard dewaxing protocol employing xylene treatment followed by a decreasing alcohol series and washes with distilled water and phosphate-buffered saline (PBS; pH 7.4) (Factor VIII, VEGF, NGB, CA-IX) or 0.1% Triton X-100 (MCT-1, MCT-4).

Histological Investigations

All L7-DRG underwent a routine histopathological examination with regards to ganglionic pathology, employing sections stained with haematoxylin and eosin (HE) and Picrosirius Red-Alcian Blue staining. In addition to general algorithms for prevertebral ganglia pathology (Fix 2000) there was a special focus on vascular

density, vessel wall morphology, lumen-wall ratio and course and orientation of the vessels. All investigations were conducted using Axioplan light microscope (Zeiss, Germany).

Immunohistochemistry

Optimal antibody concentrations and pretreatments were established for the respective tissue in a pilot investigation (data not shown). To enhance immunoreactivity, dedicated sections were pretreated as illustrated in the Table 1. Endogenous peroxidase activity was blocked using 3 ml of 30% hydrogen peroxide in 100 ml methanol for Factor VIII, VEGF, NGB and CA-IX and using 10 ml of 30% hydrogen peroxide in 90 ml methanol for MCT-1 and MCT-4. The incubation was carried out for 30 minutes, with subsequent washing steps in PBS (Factor VIII, VEGF, NGB, CA-IX) or in PBS + 0.1% Triton X-100 (PBS-T) (MCT-1, MCT-4) for 15 minutes each. After blocking with 2.5% normal horse serum (ImmPRESS™ HRP anti-rabbit IgG Polymer Detection Kit, Vector Laboratories, Burlingame, California, USA) applied for 20 minutes at room temperature, the slides were incubated with the primary antibodies (Table 1) in a humidified chamber at 4°C for 18 hours. Incubation was followed by repeated rinses in PBS (Factor VIII, VEGF, NGB, CA-IX) or PBS-T (MCT-1, MCT-4) with a final immersion for 15 minutes followed by incubation with the polymer (ImmPRESS™ HRP anti-rabbit IgG Polymer Detection Kit, Vector Laboratories, Burlingame, California, USA) in a humidified chamber at room temperature for 30 minutes. After repeated washes by PBS or PBS-T to remove the unbound secondary antibodies, sections were covered with 3,3'-diaminobenzidine (DAB) chromagen according to the manifacturer's protocol (Vector Laboratories

Burlingame, California, USA). The sections were counterstained with hematoxylin and coverslipped routinely using a xylene-based mounting medium.

Data collection

Sections were evaluated using Axioplan light microscope (Zeiss, Germany) at either x100 or x200 optical resolution. DRG cells (neurons and satellite cells) and blood vessels were scored according to the intensity of their cytoplasmic staining, using a 5-tiered scale (0 to 4). Scores were given as follows: 0 = immunonegative, 1 = very mild staining, 2 = mild staining, 3 = moderate staining, 4 = extensive staining. Furthermore, a value was given to the satellite cells stain following the overall percentage of immunopositivity (0: <5%; 1: 5-25%; 2: 25-50%; 3: 50-75%; 4: >75%).
Medial, adventitial or endothelial immunopositivity and location of stained blood vessels (endoneurium, perineurium or epineurium) were evaluated.
All data were fed into Microsoft Excel® and PAST® software for statistical analysis. As for the stereological analysis, the investigator was blinded for the origin of the tissues.

Statistical analysis

The distribution characteristics of the values were checked for each linear parameter by Shapiro-Wilk test and normal probability plotting. Association between categorical variables was assessed with chi-squared test or Fisher's exact test. Normal data were compared by Student's t test and Z test. Non normal data were compared by Mann-Whitney's test. P values ≤ 0.05 were considered significant.

Results

Tissue collection and histopathology

Fifteen L7-DRG were harvested from a total of eight dogs suffering from painful NFS (NFS-L7-DRG) and compared to L7-DRG from five non-affected dogs (NA-L7-DRG).
Histopathology showed a panel of vascular abnormalities comprising disproportionately thick arterial walls in 7/15 L7-DRG (46.6%), microvascular endothelial prominence in 4/15 L7-DRG (26.6%) and phlebectasia of capsular veins in 2/15 L7-DRG (13.3%). In 11/15 L7-DRG (73.3%) the perineurium was fibrotically enlarged.

Stereological findings

A mean of 26 ±13.2 fields for each slide in controls and 24±14 fields for each slide in NFS-L7-DRG were observed.
In controls and NFS-L7-DRG, the volume fraction of: a)the blood vessels in the DRG and perineurium ($Vv_{(vessels/DRG\ with\ perineurium)}$), b) the blood vessels in the DRG without counting the blood vessels in the perineurium ($Vv_{(vessels/DRG\ without\ perineurium)}$) and c) the blood vessels in the perineurium ($Vv_{(vessels/perineurium)}$) were, respectively, a) 0.028±0.007 and 0.036±0.015; b) 0.026±0.006 and 0.036±0.016; c) 0.043±0.017 and 0.036±0.029. No significantly differences were seen between the two groups (Table 2).

Marker expression

Expression of VEGF

Independent of the similarity of vascular density, there were significant differences regarding VEGF expression.

In controls, a total of 3.702 neurons were counted and 792 expressed VEGF-IP, which represent the 21.3% of neurons (Figure 1). The expression levels were mostly very mild (grade 1: 11.5%) and mild (grade 2: 16.8%) (Figure 2).

In NFS-L7-DRG, a total number of 3.960 neurons was evaluated, 2.106 (53%) of which stained immunopositive for VEGF. The expression pattern was somatic and diffusely occupied the entire perikaryon, with a mild axoplasmic staining (Figure 3 B). Furthermore, 9/15 (60%) showed a distribution pattern localized in the subcapsular zone (data not shown).

The immunopositivity (IP) was significantly increased in NFS-L7-DRG neurons compared to the NA ($p<0.01$) (Figure 1), as the former showed more moderate (grade 3: $p=0.02$) and extensive labeled neurons (grade 4: $p<0.001$) (Figure 2).

In addition to the neurons, also satellite cells showed a significant increase of IP ($p=0.03$) and there were a significant more mild (grade 2: $p<0.001$), moderate (grade 3: $p<0.001$) and extensive immunolabeling (grade 4: $p<0.001$) (Figure 4).

Concerning the vascular expression, a NFS-associated increase of medial and endothelial VEGF expression was observed in both endoneurial ($p=0.007$) and perineurial ($p=0.01$) blood vessels (Figure 5).

Expression of NGB

In the control group, a total of 3.126 neurons were counted and 2.572 (82.2%) showed NGB-IP (Figure 6). The expression levels were mostly very mild (grade 1: 38.2%) and mild (grade 2:42.7%) (Figure 7).

In NFS-L7-DRG, a total of 3.819 neurons were counted. Of those, 3.437 (89.4%) showed NGB-IP (Figure 6). The expression pattern of NGB was observed diffusely in the cytoplasm, with a mild axoplasmic staining (Figure 8 B). Occasionally, a mild nuclear expression was observed.

Direct comparison of the two groups did not show a significant difference in the proportion of NGB expressing cells ($p=0.15$) (Figure 6) however, NFS-L7-DRG neurons more frequently reached moderate expression levels (25.1%) compared to controls (1.4%) (grade 3: $p<0.001$) (Figure7).

Compared to controls, in NFS-L7-DRG, satellite cells showed a significant more mild (grade 2: $p<0.001$) and moderate labelling (grade 3: $p<0.001$) (Figure 9) but without difference in the proportion of IP ($p=0.02$).

Concerning the vascular expression of NGB, no differences were seen in endoneurial ($p=0.4$) and perineurial ($p=0.6$) blood vessels expression between NFS-L7-DRG and controls.

Expression of CA-IX

In NA-L7-DRG, a total of 3.345 neurons were counted and 1.765 (52.7%) showed CA-IX-IP (Figure 10). The expression levels were mostly very mild (grade 1: 17.4%) and mild (grade 2: 27.5%) (Figure 11).

In NFS-L7-DRG, a total of 4.450 neurons were counted. Of those, 4.058 were IP for CA-IX, which represent the 91.15% (Figure 10). The expression pattern for CA-IX was cytoplasmic and diffusely occupied the entire perikaryon with a moderate axoplasmic staining (Figure 12 B).
Compared with controls, NFS-L7-DRG showed an overall significant increase of neuronal IP ($p<0.01$) (Figure 10). Furthermore, NFS-L7-DRG showed an higher moderate (grade 3: $p<0.001$) and extensive neuronal immunolabeling (grade 4: $p<0.001$) (Figure 11).

In addition to the neurons, also the satellite cells in NFS-L7-DRG showed a significant increase of IP ($p=0.02$). Furthermore, a significant more moderate (grade 3: $p<0.001$) and extensive (grade 4: $p<0.001$) satellite cells labeling was observed (Figure 13).

An increased medial and endothelial CA-IX expression of perineurial ($p=0.005$) blood vessels was observed in NFS-L7-DRG (data not shown).

Expression of MCT-1

In controls, a total of 4.104 neurons were counted. Of those, 520 (12.6%) showed MCT-1 IP (Figure 14). The expression levels were mostly very mild (grade 1: 7.3%) and mild (grade 2: 4%) (Figure 15).

In NFS-L7-DRG,a total of 2.966 neurons were MCT-1 immunopositive, which represent the 70.6% of all counted neurons (n = 4.199) (Figure 14). The expression pattern for MCT-1 was diffuse cytoplasmic witha moderate axoplasmic staining (Figure 16 B).

Compared to controls, NFS-L7-DRG neurons showed a significant proportion of MCT-1 IP ($p<0.001$) (Figure 14). Direct comparison of intensity grades between NFS-L7-DRG and controls showed a substantially higher proportion of mild (grade 2: $p<0.001$), moderate (grade 3: $p<0.001$) and extensive (grade 4: $p<0.01$) neuronal labeling (Figure 15).

Satellite cells in NFS-L7-DRG showed a significantly higher proportion of IP in comparison to controls ($p=0.02$) including increases in mild (grade 2: $p<0.001$), moderate (grade 3: $p<0.001$) and extensive (grade 4: $p<0.001$) satellite cells immunolabeling (Figure 16).

Furthermore, a significant increase of adventitial MCT-1 expression of endoneurial blood vessels was observed in NFS-L7-DRG ($p=0.005$) (Figure 17).

Expression of MCT-4

In controls, a total of 4.222 neurons were counted. Of those, 1.182 (27.9%) were MCT-4 IP (Figure 18). The expression levels were mostly very mild (grade 1: 9.8%), mild (grade 2: 10%) and moderate (grade 3: 6.6%) (Figure 19).

In NFS-L7-DRG, a total of 4.179 neurons were MCT-4 immunopositive, which represent the 83.9% of the total counted neurons (n = 4.979) (Figure 18). The expression pattern for the MCT-4 was somatic and diffusely occupied the perykarion, with a mild axoplasmic staining (Figure 20 B). In scattered neurons, a pointed cytoplasmic staining was seen (data not shown).
Despite controls, NFS-L7-DRG showed an increased neuronal IP ($p<0.001$) (Figure 18). Furthermore, direct comparison between the two groups showed a significant increased in mild (grade 2: $p=0.01$), moderate (grade 3: $p<0.001$) and extensive (grade 4: $p<0.001$) neuronal immunolabeling in NFS-L7-DRG (figure 19).

Satellite cells of NFS-L7-DRG were significantly IP compared to controls ($p<0.05$) with increased mild (grade 2: $p<0.001$) and moderate (grade 3: $p<0.001$) labeling (Figure 21).

A significant increased medial MCT-4 expression of endoneurial ($p=0.02$) and perineurial ($p=0.03$) blood vessels was seen (Figure 22).

Discussion

Previous studies have shown that the DRG plays a key role in generation of low back pain and sciatica in patients suffering from recruudescent pain in disc herniation and LCS.[25-28] Following peripheral and central injuries, cellular and molecular changes occur in the DRG, including proliferation of satellite glial cells and invasion by macrophages.[25,29-31]

Although the direct contribution of vascular dysfunction to NP has not been fully explored, evidence of microvascular disturbances has been reported in both humans suffering NP and in NP animal models.[18,32,33] Furthermore, in vivo studies showed that a direct compression of the DRG results in reorganization of their vascular architecture, providing the structural conditions for changes in the microenvironment that alter the DRG neuron physiology and may induce NP.[18,26] Similarly, a vascular remodelling within the DRG can be hypothesised to occur due to farther proximal NRs compression in an L7-NFS setting.

To identify possible changes to DRG vascularity, we evaluated the endoneurial density of blood vessels in DRG from L7-NFS-affected dogs using stereological algorithms. Thus, an increase in the ganglionic vascular density would be indicative of a neoangiogenesis in entrapped NRs. On unbiased investigation, however, no alterations to the microvascular supply of DRG in L7-NFS could be established. This basically reflects the emperical impression of normal vascularity on routine sections. On the other hand this not necessarily reflects normal perfusion.

Histological inspection of endoneurial arteries showed changes to the arterial wall thickness in 46.6% of entrapped NRs. This may be indicative of an increased arterial perfusion pressure, required to overcome increased endoneurial vascular resistance in compressed roots. Notably, the VEGF signal was most severe in the

subcapsular aspects of the DRG. As the centre of the roots is supplied by the central radicular artery, it may be assumed that nerve root compression may interfere with the transcapsular supply by obliteration of small penetrating arteries. Alternatively and very likely, there is also a compression of epiradicular veins with reduction of the venous outflow from the endoneurium. This way, congestive endoneurial oedema may trigger the observed fibroplasia in compressed roots and the vascular resistance would be elevated, leading to the above described arterial changes.
Credence to a malperfusion concept indirectly is given by increased expression of neuronal stress markers as VEGF, NGB and CA-IX.

VEGF, as well as NGB and CA-IX are some of defined *hypoxia-related markers*, since they are associated to the oxygen status of the tissues.[34-41] Those factors are up-regulated by the Hypoxia-Inducible Factor 1-α (HIF-1α), which is a transcription factor with the subunit α that is sensitive to oxigen. Upon activation in hypoxic conditions, HIF-1α binds to the Hypoxia Responsive Elements (HRE) promoting the transcription of numerous genes, including VEGF, NGB and CA-IX.[38] Despite the relation between HIF-1α and hypoxia, HIF-1α is rapidly degraded in the presence of oxygen and its quantification as chronic hypoxia-related assay remains questionable.[38,42] Thus, the mesurement of VEGF, NGB and CA-IX, in which the increased expression has been already demonstrated in chronic hypoxic conditions, represent a more reilable investigation.[43-45]

VEGF enhances vascular permeability and represents a neurotropic factor, promoting neuronal regeneration and reducing neurodegeneration.[46-48] Several in vitro and in vivo studies have documented effects of ischemia and hypoxia on the expression of VEGF.[49] For example, a comparison of transient and permanent Middle Cerebral Artery (MCA) occlusion in rat showed elevations of VEGF levels in neurons and astrocytes, which were detectable at 1-3 days and were generally more prominent after permanent MCA occlusion.[50] Furthermore, in a different rat model called “global cerebral ischemia”, which is often likened to the hypoxic-ischemic

encephalopathy following cardiac arrest in humans, showed VEGF mRNA induction within hours in neurons and within days in astrocytes.[51]

Since, in our study, nearly 53% of DRG neurons showed increased VEGF immunoreactivity, it can be concluded to be indicative as hypoxic stress marker in L7-NFS-affected DRG.

Despite the percentage of NGB-expressing neurons was not different between affected and controls, the higher grade 3 immunoreactivity in L7-NFS-DRG is indicative for a local increase of neuronal NGB. Under physiological conditions all, or at least most neurons of the central nervous system, express a low NGB concentration.[52] Neuronal NGB is a stress-inducible protein and the primary functions are to store and transport oxygen.[53] Under hypoxia and ischaemic conditions, NGB expression is up-regulated, confering preotection against oxidative stress.[52,54] Those features may explain the overall IP in both affected and controls and, on the other hand, the increased intensity of NGB expression in L7-NFS-affected-DRG is indicative of adaptive survival mechanism to hypoxic conditions through increased neuronal oxygen binding capacity. The nuclear NGB localization, observed in scattered neurons, is a finding not previously described. A previous in vitro study published by Geuens et al. (2003) showed a nuclear localization of the cytoglobin, which is a member of the globin family as well as the NGB.[55] The nuclear localization of NGB in few neurons remain an aspect to be elucidated and may suggest new possible functions.

CA-IX is a transmembrane enzyme which is involved in the respiratiory gas exchanged and acid-base balance mantaining the intracellular and lowering the extracellular pH. Usually it is only limitedly present in normal tissues and its expression increases during hypoxia.[56] Hypoxia stimulates a distinctive set of cellular adaptative processes that include extracellular acidosis and a shift to glycolytic metabolism.[57] Thus, the averall L7-NSA-DRG neuronal immunopositivity for CA-IX is again indicative for hypoxic condition and established extracellular acidosis.

On the other hand, a reversed pH gradient affects ion fluxes. Both MCT-1 and MCT-4 can mediate lactate export, as well as import, resulting in an upregulation in hypoxic conditions.[57] An increasing in vitro and in vivo studies suggested that lactate represents the major neuronal aerobic energy substrate for tissue surviving an ischemic/hypoxic insult and for the recovery of synaptic function, showing a further neuroprotective function.[58-60] Hence, the increased expression of neuronal CA-IX and MCT-1 and -4 are indicative of hypoxia and related glycolytic metabolism and are up-regulated to prevent intracellular acidosis.

Interestingly, satellite cells and blood vessels showed an overall immunoreactivity for those markers, compared to controls. These findings suggest a more connected network. In the DRG of adult animals, each nerve cell body is usually enveloped by its own satellite cell sheath, which is in turn completely surrounded by connective tissue.[61] However, Pannese et al. (2003) highlighted how satellite cells racted to axonal injury of the neurons with which they were associated forming bridges, connecting previously separated perineuronal sheaths and forming new gap junctions with more extensive cell coupling.[62] Thus, our findings may represent a further demonstration that satellite cells react to direct injury of the DRGs' neurons with which they are associated.

Although a cohort of previous studies showed the involvement of the vascular system in both human having neuropathic pain and in neuropathic pain animal models, very little is known about the role of the vascular dysfunction in the development and maintenance of chronic pain conditions.[18,63,64] Evidence of microvascular disturbances has been reported in diabetes, nerve compression and traumatic models of painful neuropathy.[32,64,65] Previous in vivo studies pointed out that ischemic conditions can lead to microcirculatory changes as thrombosis, capillary endothelial cell swelling, intersitial edema, oxidative stress with nerve fibers degeneration, as well as endoneurila fibrosis, increased lactate level and increased metabolic neuronal requirements.[18,63] In those studies these findings were

related to spontaneous pain behaviuours and animals had benefits to hyperbaric oxygen and to administration of free radical scavengers.

Thus, during hypoxia, microcirculatory changes established lead to fibrosis, enlargement of capillaries, further reduction in oxygenation and subsequent increase of metabolic comsumption, acidosis and reduction of N^+/K^+ ATPase levels and possible increase of neuronal excitability, contributing to the onset and maintenance of NP.

Conclusions

These findings highlight a significant neuronal distress in NFS, that is likely to contribute to aberrant electral activity, neuronal drop out and persistence of NP.

Furthermore, taken together, the DRG provide a large body of evidence to consider malperfusive events contributing to the amplification and proximodistal spread of degenerative NR changes in L7-NFS. Thus, restoration of vascular equilibrium could be an interesting target for disrupting the cascade of pathological events in compressed roots.

Figures and Tables

Antisera	Host species	Serum code	Dilution	Source	Pretreatment
factor VIII	rabbit polyclonal	A0082	1:600	Dako, Glostrup, Denmark	proteinase K (Ready-to-use, Dako, Glostrup, Denmark) for 20 minutes
VEGF	rabbit polyclonal	PAK0036	1:50	Linaris, Dossenheim, Germany	0.1% trypsin (Sigma-Aldrich, Saint Louis, Missouri, USA) at 37°C for 20 minutes
NGB	rabbit polyclonal	N7162	1:500	Sigma-Aldrich, Saint Louis, Missouri, USA	citrate buffer solution at boiling temperature for 20 minutes
CA-IX	rabbit polyclonal	MBS616120	1:1000	MyBioSource, San Diego, California, USA	citrate buffer solution at boiling temperature for 20 minutes
MCT-1	rabbit polyclonal	AB3538P	1:200	Millipore, Temecula, California, USA	no pretreatment was required (Cortes Campos et al., 2011) the ab was diluted in 1% bovine serum albumin (BSA)
MCT-4	rabbit polyclonal	AB3316P	1:300	Millipore, Temecula, California, USA	no pretreatment was required (Eilerstein et al., 2014) the ab was diluted in 1% bovine serum albumin (BSA)

Table 1. Primary antibodies used in this study.

Volume fractions	NA-L7-DRG	NFS-L7-DRG	P values
$(Vv_{(vessels/DRG\ with\ perineurium)})$	0.028 ± 0.007	0.036 ± 0.015	p=0.3
$(Vv_{(vessels/DRG\ without\ perineurium)})$	0.026 ± 0.006	0.036 ± 0.016	p=0.2
$(Vv_{(vessels/perineurium)})$	0.043 ± 0.017	0.036 ± 0.029	p=0.5

Table 2. Stereological assessment of vascular density in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs.

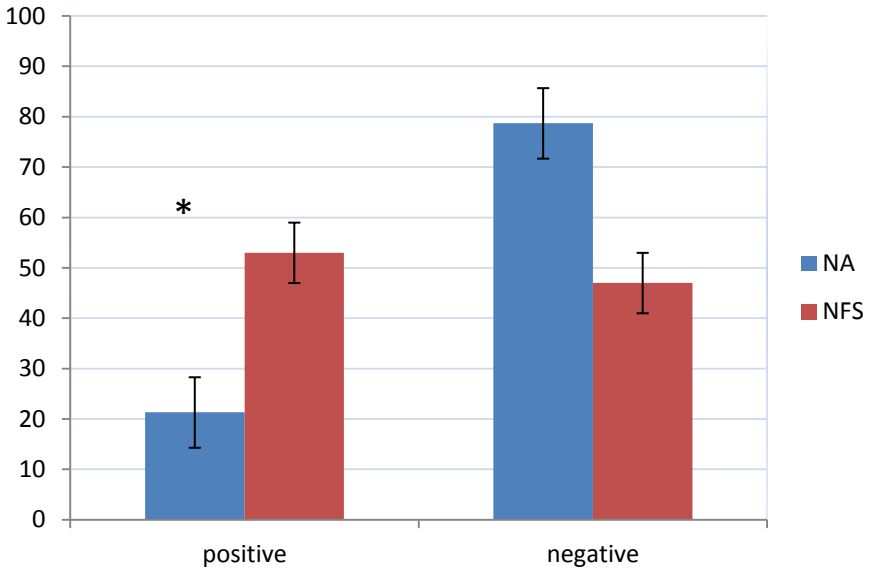

Figure 1. Bar chart showing the percentage of VEGF positive and negative neurons for non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) in positivity are highligthed by the asterisk.

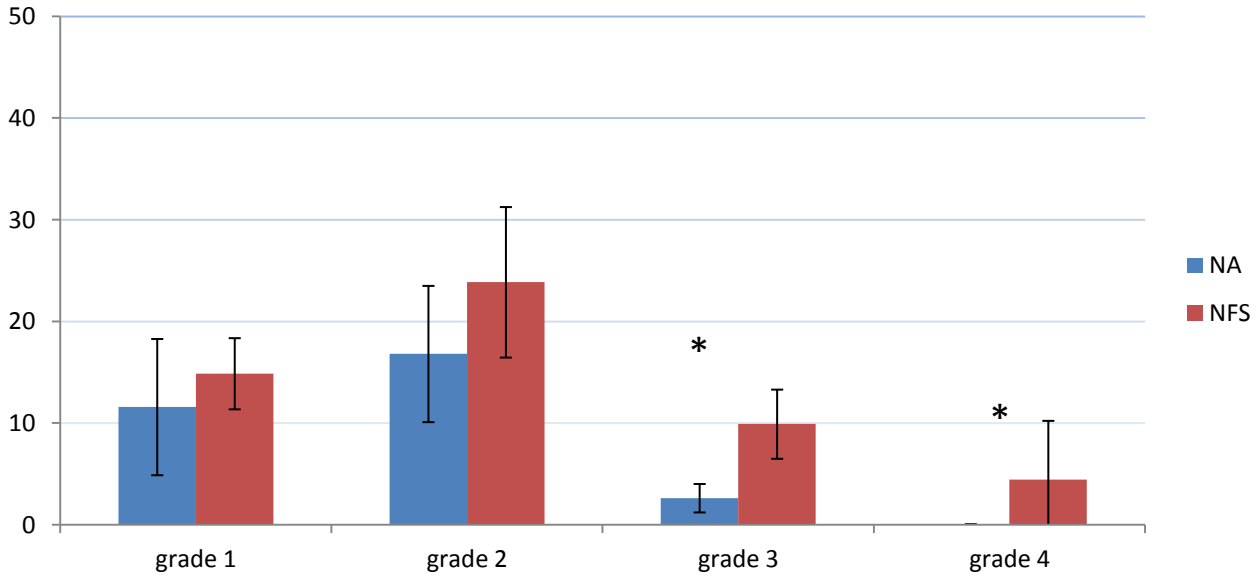

Figure 2. Bar charts showing the percentages of VEGF-immunopositive neurons expressing different levels of intensity in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) are highligthed by the asterisks.

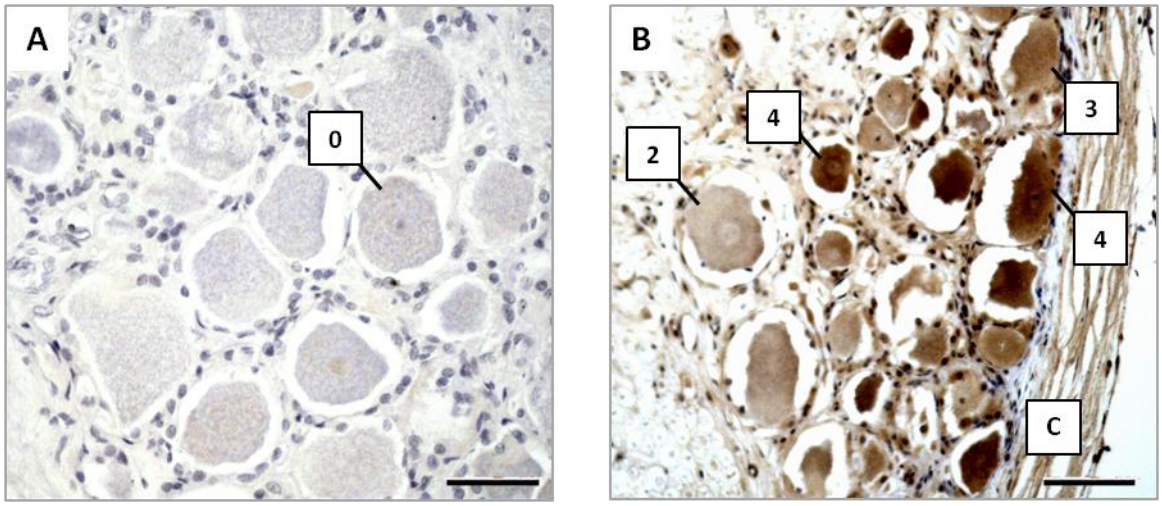

Figure 3. Immunohistochemical staining for VEFG expression in DRG slides. Immunopositive ganglion neurons broadly exhibit three different grades of staining intensity: mild (2), moderate (3) and extensive (4). A: non-affected; B: painful Neuro-Foraminal Stenosis (NFS) affected dogs. 0= negative; C= capsule. Chromagen: DAB. Scale bar= 45µm.

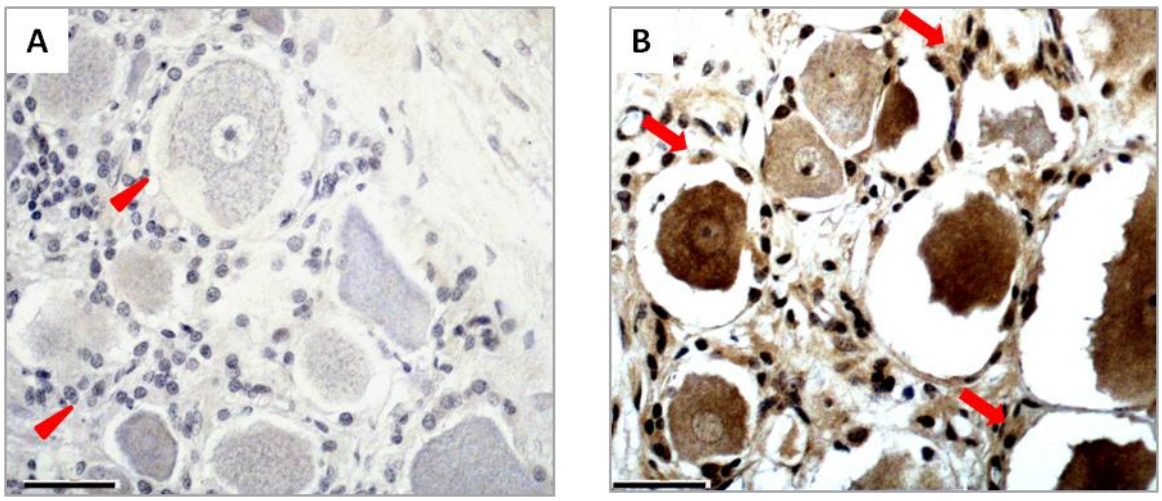

Figure 4. Immunohistochemical staining for VEGF expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed a significant increase of satellite cells- immunopositivity compared to non-affected dogs (A). Arrows indicate extensive (grade 4) intensity; arrowheads indicate negative satellite cells. Chromagen: DAB. Scale bar= 45µm.

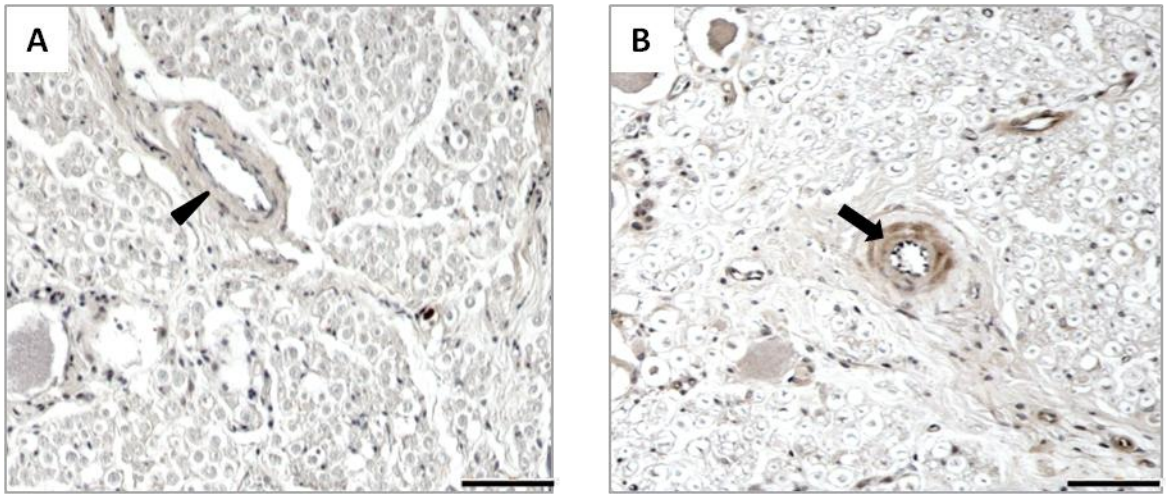

Figure 5. Immunohistochemical staining for VEGF expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed an increase of medial and endothelial expression in blood vessels (arrow) compared to non-affected (A, arrowhead). Chromagen: DAB. Scale bar= 45µm.

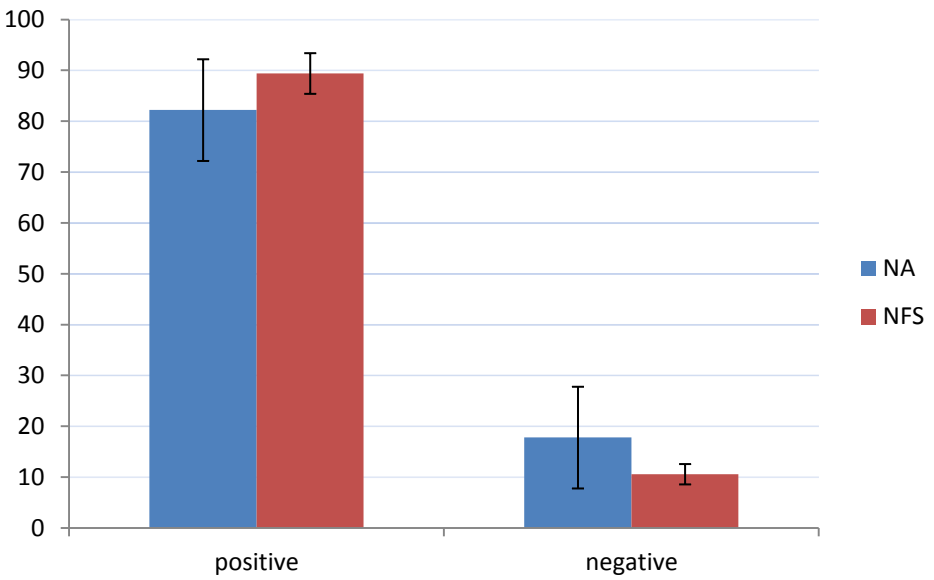

Figure 6. Bar chart showing the percentage of NGB positive and negative neurons for non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. No difference in the percentage of NGB expressing neurons was seen.

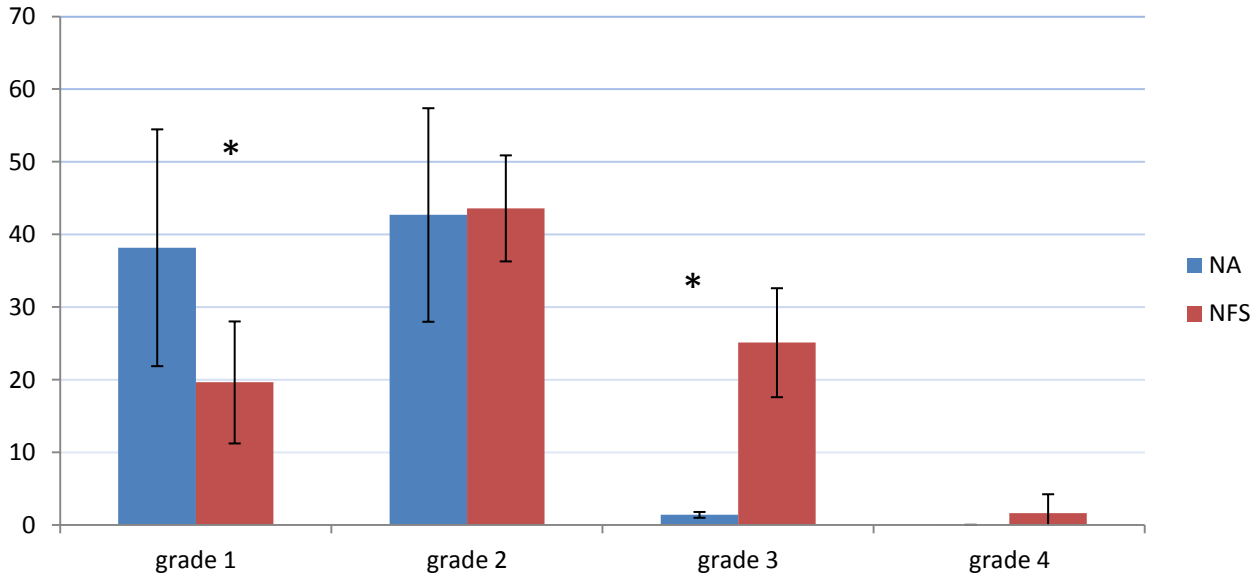

Figure 7. Bar charts showing the percentages of NGB-immunopositive neurons expressing different levels of intensity in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) are highligthed by the asterisks.

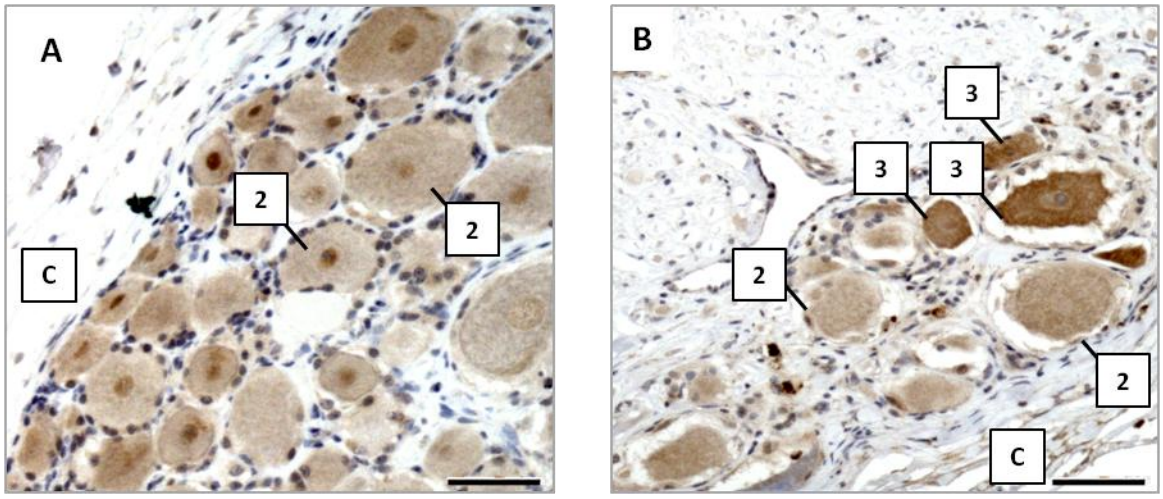

Figure 8. Immunohistochemical staining for NGB expression in DRG slides. Immunopositive ganglion neurons broadly exhibit two different grades of staining intensity: mild (2) and moderate (3). A: non-affected; B: painful Neuro-Foraminal Stenosis (NFS) affected dogs. C= capsule. Chromagen: DAB. Scale bar= 45µm.

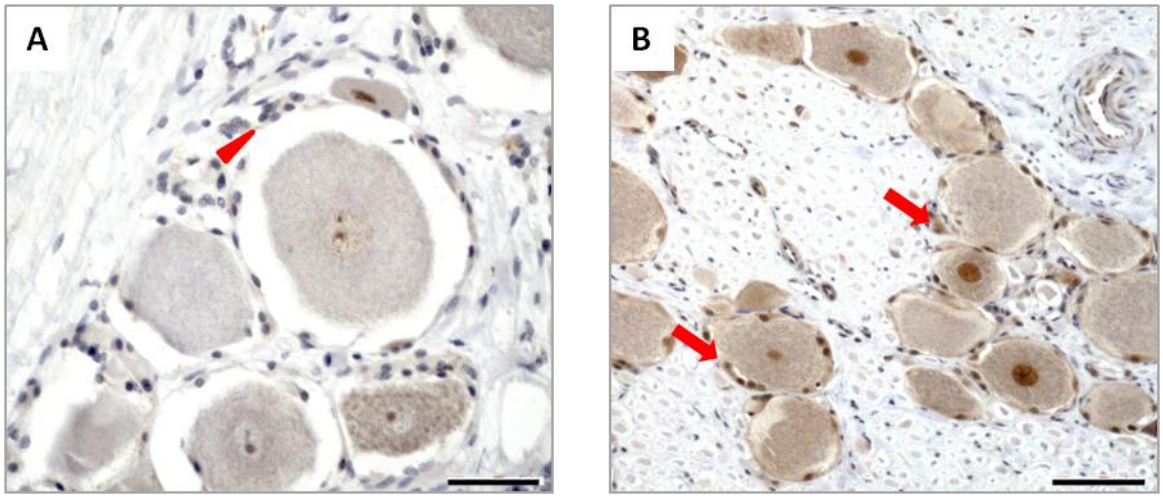

Figure 9. Immunohistochemical staining for NGB expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed an increased moderate saellite cells-immunopositivity compared to non-affected dogs (A). Arrows indicate moderate (grade 3) intensity; arrowhead indicate negative satellite cells. Chromagen: DAB. Scale bar= 45µm.

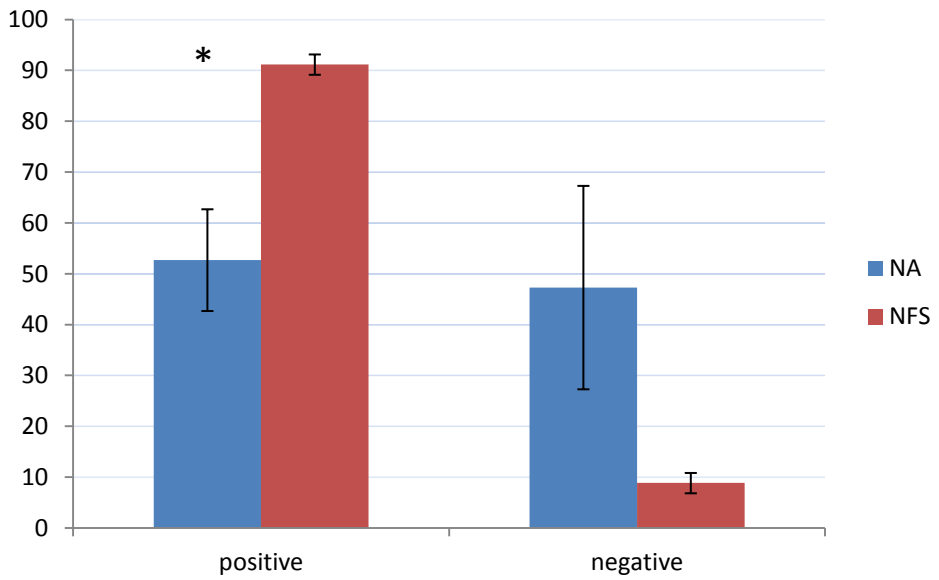

Figure 10. Bar chart showing the percentage of CA-IX positive and negative neurons for non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) in positivity are highligthed by the asterisk.

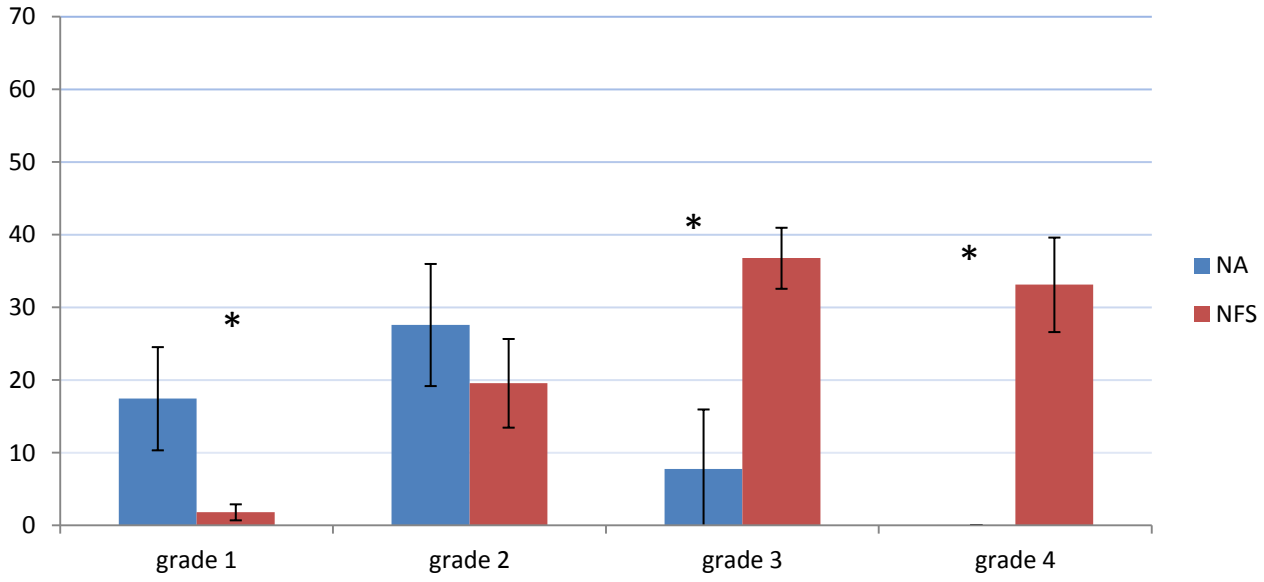

Figure 11. Bar charts showing the percentages of CA-IX-immunopositive neurons expressing different levels of intensity in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) are highligthed by the asterisks.

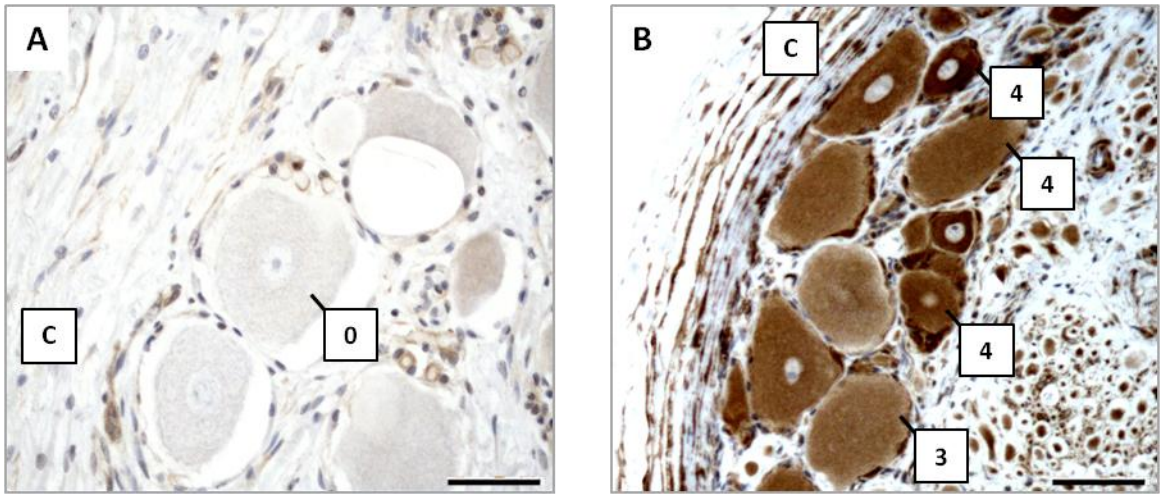

Figure 12. Immunohistochemical staining for CA-IX expression in DRG slides. Immunopositive ganglion neurons broadly exhibit two different grades of staining intensity: moderate (3) and extensive (4). A: non-affected; B: painful Neuro-Foraminal Stenosis (NFS) affected dogs. 0= negative ; C= capsule. Chromagen: DAB. Scale bar= 45µm.

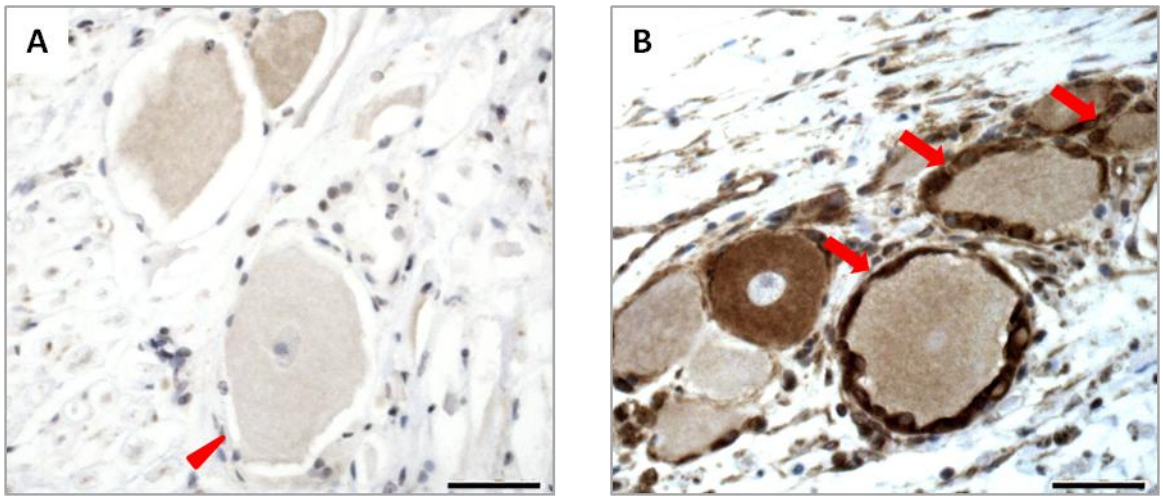

Figure 13. Immunohistochemical staining for CA-IX expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed a significant increase of satellite cells- immunopositivity compared to non-affected dogs (A). Arrows indicate extensive (grade 4) intensity; arrowhead indicate negative satellite cells. Chromagen: DAB. Scale bar= 45µm.

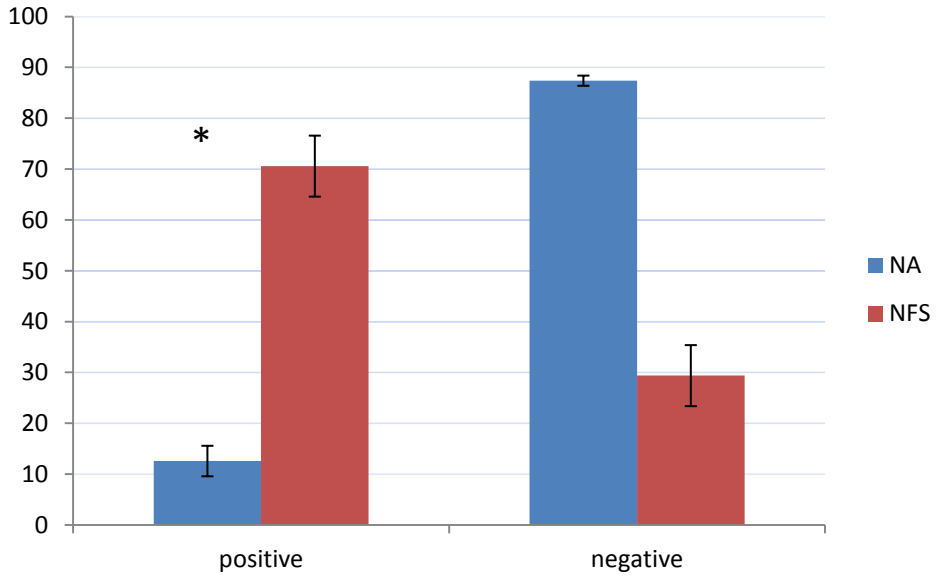

Figure 14. Bar chart showing the percentage of MCT-1 positive and negative neurons for non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) in positivity are highligthed by the asterisk.

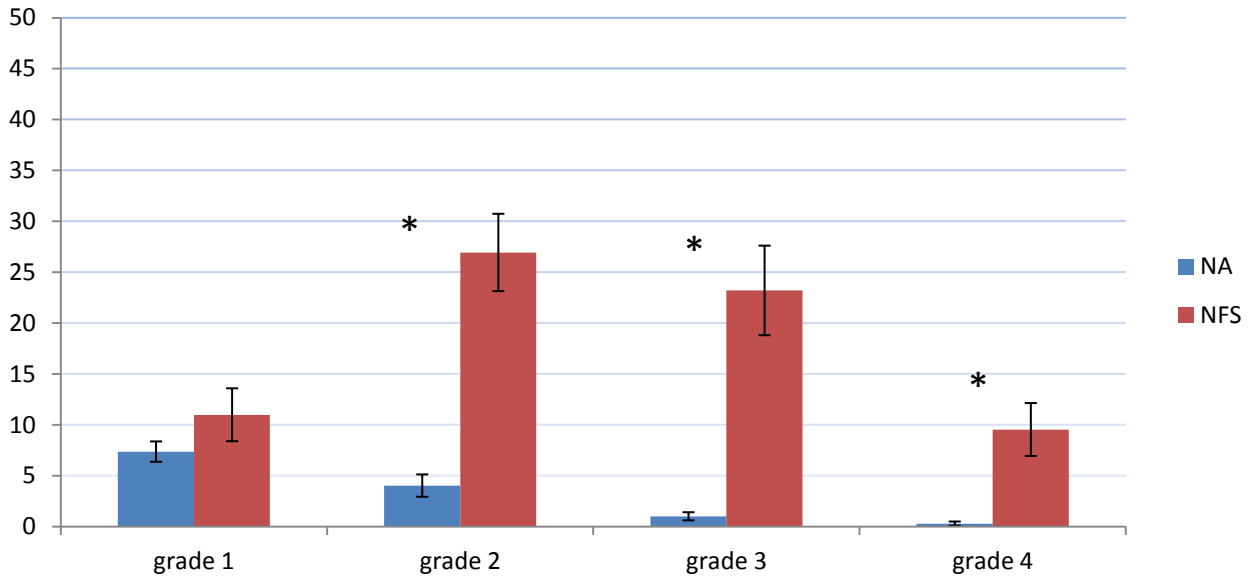

Figure 15. Bar charts showing the percentages of MCT-1-immunopositive neurons expressing different levels of intensity in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) are highligthed by the asterisks.

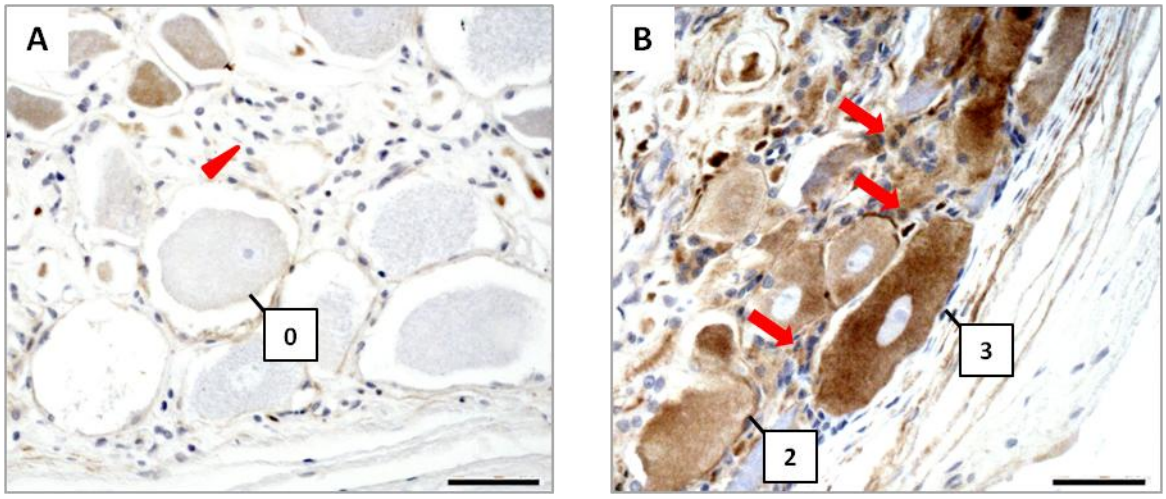

Figure 16. Immunohistochemical staining for MCT-1 expression in DRG slides. Immunopositive ganglion neurons broadly exhibit two different grades of staining intensity: mild (2) and moderate (3). (NFS) affected dogs (B) showed a significant increase of satellite cells- immunopositivity compared to non-affected dogs (A). Arrows indicate moderate (grade 3) intensity; arrowhead indicate negative satellite cells. A: non-affected; B: painful Neuro-Foraminal Stenosis (NFS) affected dogs. 0= negative. Chromagen: DAB. Scale bar= 45µm.

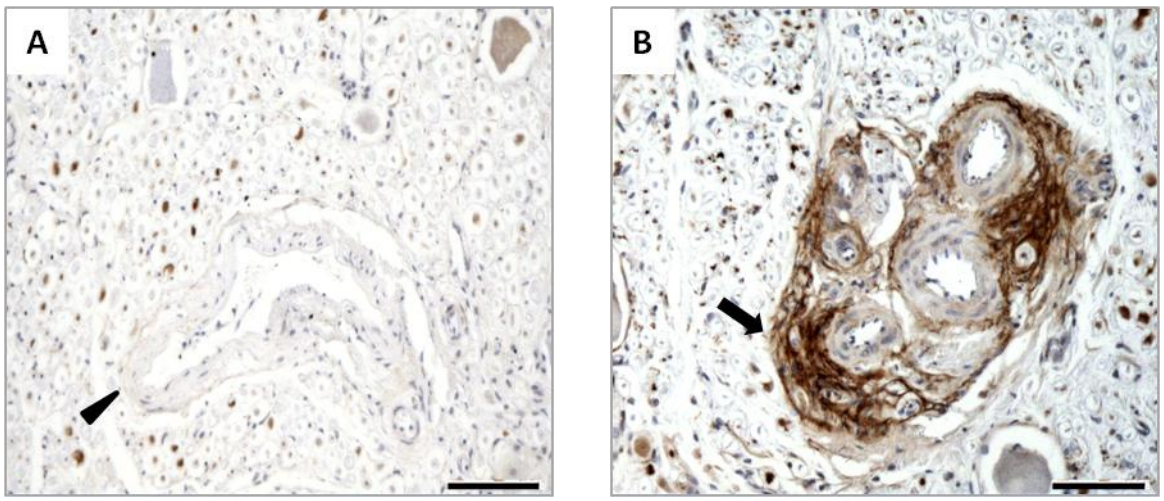

Figure 17. Immunohistochemical staining for MCT-1 expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed an increase of adventitial expression in blood vessels (arrow) compared to non-affected (A, arrowhead). Chromagen: DAB. Scale bar= 45µm.

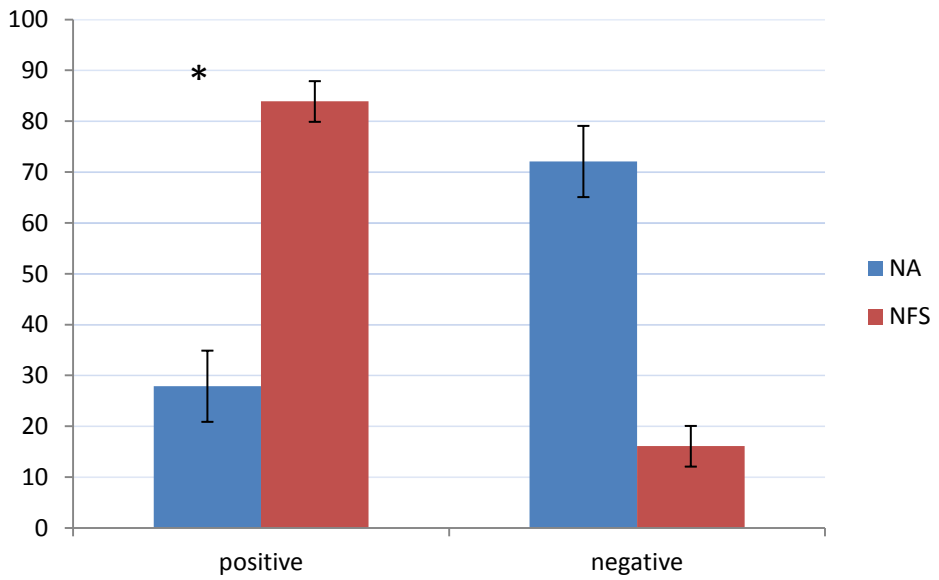

Figure 18. Bar chart showing the percentage of MCT-4 positive and negative neurons for non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) in positivity are highligthed by the asterisk.

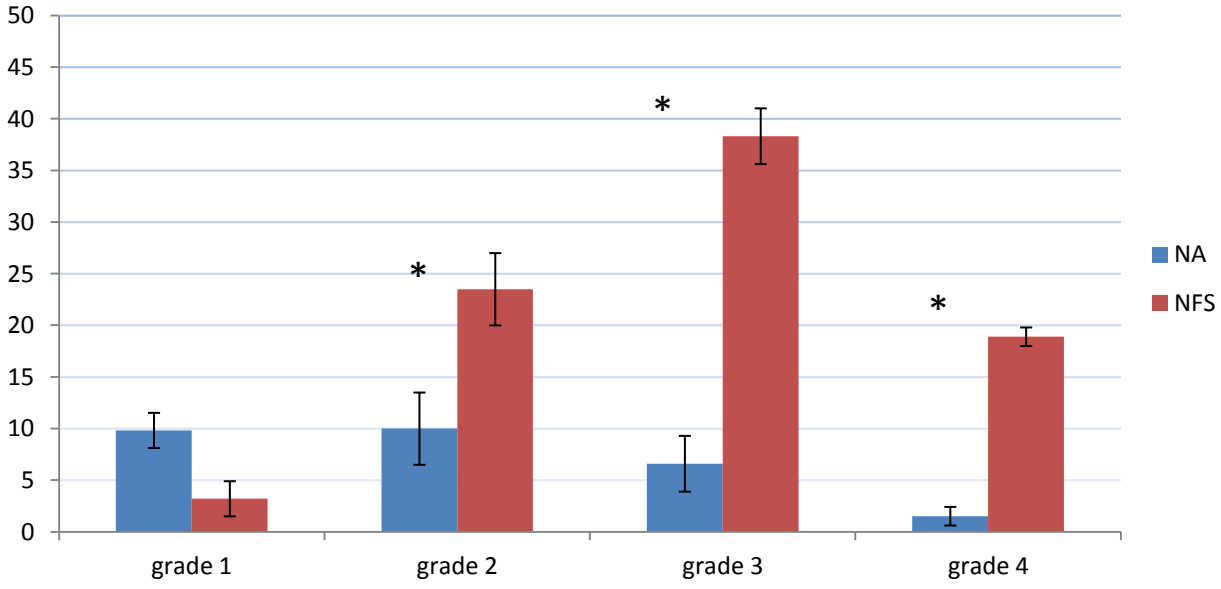

Figure 19. Bar charts showing the percentages of MCT-4-immunopositive neurons expressing different levels of intensity in non-affected (NA) and painful Neuro-Foraminal Stenosis (NFS) affected dogs. Significant differences ($p<0.05$) are highligthed by the asterisks.

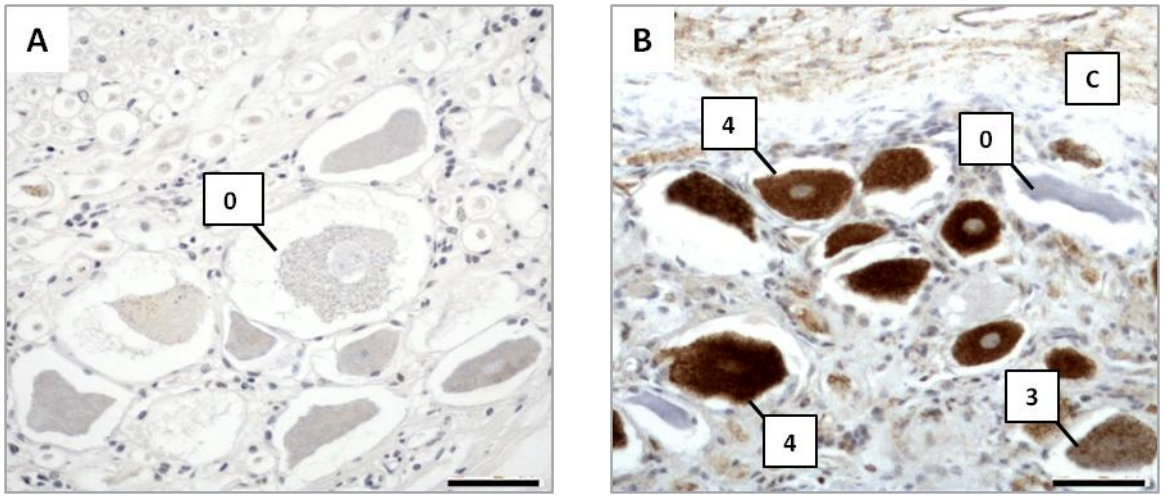

Figure 20. Immunohistochemical staining for MCT-4 expression in DRG slides. Immunopositive ganglion neurons broadly exhibit two different grades of staining intensity: moderate (3) and extensive (4). A: non-affected; B: painful Neuro-Foraminal Stenosis (NFS) affected dogs. 0= negative ; C= capsule. Chromagen: DAB. Scale bar= 45µm.

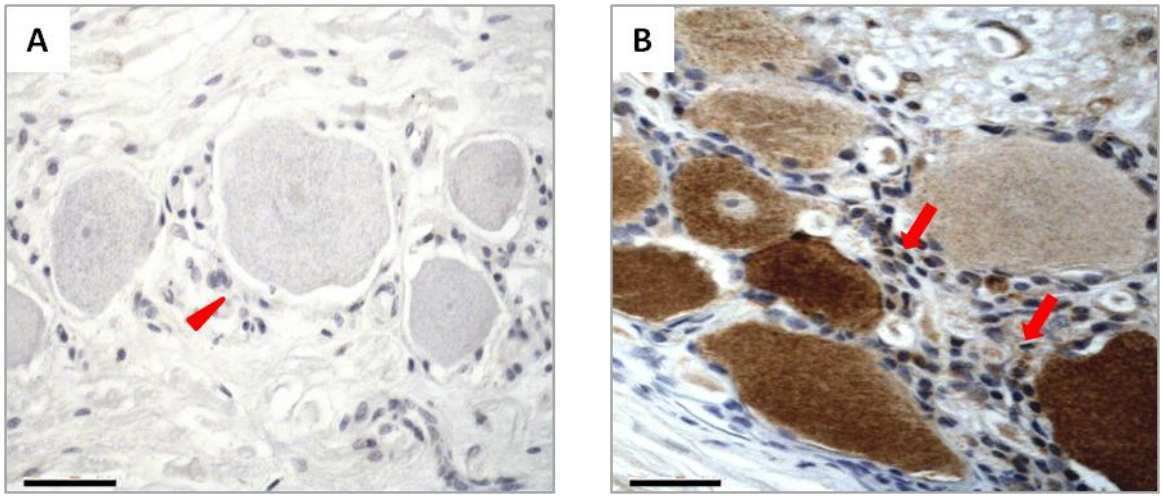

Figure 21. Immunohistochemical staining for MCT-4 expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed a significant increase of satellite cells- immunopositivity compared to non-affected dogs (A). Arrows indicate extensive (grade 4) intensity; arrowhead indicate negative satellite cells. Chromagen: DAB. Scale bar= 45µm.

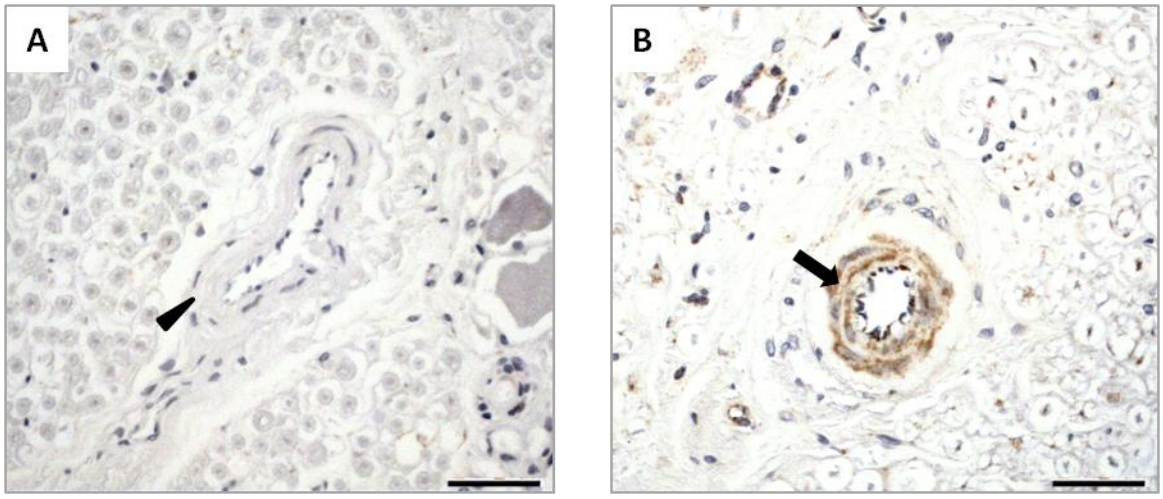

Figure 22. Immunohistochemical staining for MCT-4 expression in DRG slides. Painful Neuro-Foraminal Stenosis (NFS) affected dogs (B) showed an increase of medial expression in blood vessels (arrow) compared to non-affected (A, arrowhead). Chromagen: DAB. Scale bar= 45µm.

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Neurochemical features of canine lumbar spinal ganglion neurons

Introduction

Neuropathic pain (NP) is a pathological, multifactorial acute or chronic pain state, generally accompanied by tissue injury, injured nerve fibers caused by disease, injury, or a lesion of the peripheral (PNS) or central nervous system (CNS).[1] Various mechanisms can initiate and upheld the NP, causing nervous system malfunction or directly by nervous system lesions.[1] In painful neuropathic conditions, there is a spontaneous response to noxious and/or innocuous stimuli triggered by lesions to the somatosensory nervous system altering its structure and function. [1-3] Hence, the nerve fibers begin to misfire and send the wrong pain signals to various pain centers. Those changes in actual nerve function comprise the ectopic generation of action potentials, enabling disinhibition of synaptic transmission and/or loss of synaptic connectivity.[1, 4]

Previous studies showed that following nerve injury, the electrical properties of peripheral and central neurons undergo changes. The excitability of primary afferents increases, as shown by marked enhancements in the level of ongoing spontaneous activity from afferent fibers.[5-8] Studies focused on ectopic firing pointed out that those ectopic afferent discharges originate at the nerve injury site and in axotomized primary sensory neurons in the spinal ganglia (SG), which act triggering and maintaining spinal "central sensitization".[9-13]

The "central sensitization" is a CNS condition which magnifies sensory input in multiple organ systems, increasing the overall sensitivity to future stimuli. The

increased sensitivity produces allodynia (a greater than normal response to nonpainful stimuli) and/or hyperalgesia (increased response to painful stimuli). Thus, the SG, containing the primary sensory neurons, play a key role in triggering and sustaining the NP.[9,14,15]

SG contain the nociceptors' cell bodies, Including those of other somatovisceral sensory receptors, such as neurons responsible for mechanoception, nociception, theroception and sensation of itch.[16-18] Nociceptors comprise most of the subset of SG neurons that have small cell bodies and can be neurochemically subdivided into peptidergic and nonpeptidergic classes.[17] Peptidergic nociceptors express neuropeptides, usually substance P (SP) and calcitonin gene-related peptide (CGRP) and project mainly, but not only, to the lamina I of the spinal dorsal horn. [19] The CGRP, a member of the calcitonin family of peptides, is produced in both peripheral and central neurons. This neuropeptide works as a powerful vasodilator and can be involved in the transmission of pain. CGRP represents the best marker for the neuropeptidergic subpopulation, comprising mostly small neurons with unmyelinated axons (C fibers) and innervating mainly polymodal nociceptors. In this neuronal category also falls most of the SP- expressing SG cells. CGRP and SP are also expressed by a group of medium-sized cells with finely myelinated axons (Aδ fibres), most of which are nociceptors of the high-threshold mechanoreceptor type.[19,20] The Substance P (SP) is an undecapeptide allocated in the peripheral and central nervous system. Previous studies showed the participation of SP in the transmission of pain and its upregulation following pain conditions.[20]

The calbindin D-28k (CALB), a calcium-binding protein, is a well known marker of specific subpopulation of GABAergic neurons. CALB may perform distinct functions in the CNS and plays an active role in nociceptive sensory transmission.[21] CALB has been demonstrated in SG, where is predominantly localized in large neurons whose peripheral processes innervate muscle spindles, representing a marker for muscular proprioception in the DRG.[21] Within the CALB-immunoreactive distribution in the

spinal cord, this was predominant in the superficial dorsal horn.[21] Recent immunohistochemical studies revealed that small distinct subsets of neurons in the SG and spinal cord are immunoreactive for neuronal nistric oxide synthase (nNOS). The Nitric Oxide Synthases (NOS) are a family of key enzimes in Nitric Oxide (NO) biosynthesis and comprise neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The nNOS has been shown to contribute to spinal nociceptive processing in several pain models. Previous studies demonstrated that the nNOS expression in the spinal dorsal horn contributes to hyperalgesia induced by chronic compression of SG.[22]

Despite different animal models have been intensively used to investigate the mechanisms involved in the development of neuropathic pain, there is a lack of studies focused on the expression, distribution, and immunocytochemical characterization of pain-related molecules in SG of healthy dogs.

Hence, the aim of this preliminary study was to describe and characterize the expression of CGRP, SP, CALB, nNOS and the colocalization of those molecules in lumbosacral SG neurons of dogs without pathologies related to the nervous system.

Materials and Methods

Ethics Statement

This study was performed on formalin-fixed canine DRG delivered to the necropsy service of our Department of Veterinary Medical Science of the University of Bologna (Italy) and therefore no approval from ethics committee was needed.

Tissue sampling and preparation

The investigation was conducted on post-mortem tissues from three non-neurological dogs without lumbosacral pathologies, orthopaedic disorders and history of back or limb pain, delivered to the necropsy service of the Department of Veterinary Medical Sciences of the University of Bologna for diagnostic procedures unrelated to this study. The owners explicitly consented to the use of the tissues for scientific purposes related to the animal welfare.

The tract of spinal cord from thoracic (T)13 to caudal (Ca)2 spinal segments, surrounded by the dural sac, was immediately exposed along its full lenght through a dorsal laminectomy. During the procedure, we carefully avoided cutting the spinal roots to ensure that the several spinal segments of and the collection of the SG could be accurately identified at a subsequent stage. Segmental boundaries were localized by means of the the spinal roots and by counting them from the last thoracic spinal nerve located just caudal to the 14^{th} rib. SG and spinal segments were fixed for 24 hours in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) at 4°C. Tissues were subsequently rinsed overnight in phosphate-buffered saline

(PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4° in PBS containing 30% sucrose and sodium azide (0.1%). The following day, the tissues were transferred to a mixture of PBS-30% sucrose-azide and Optimal Cutting Temperature (OCT) compound (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) at a ratio of 1:1 for an additional 24 hours before being ambedded in 100% OCT in Cryomold (Sakura Finetek Europe). The sections were prepared by freezing the tissues in isopentane cooled in liquid nitrogen. Serial longitudinal sections (16 µm thick) of right L6 SG and corresponding spinal cord segments were cut on a cryostat and mounted on gelatin-coated slides. The sections (not coverslipped) were stored at -80°C, and selected for immunofluorescence.

Immunofluorescence

Double-labeling studies were performed by using the indirect immunofluorescence method. To reduce background staining, a high concentration (20%) of the appropriate normal serum was used in the preincubation stage and also in all antibodies solutions. Tissues were incubated in a solution containing 20% of normal serum and 1% of Bovine Serum Albumin (BSA) in PBS, for 1 hour, at room temperature (RT). Sections were then incubated overnight at 4°C in a humid chamber in a mixture of two primary antibodies (Table 1) diluted in PBS with addition of 20% normal serum, 1% BSA and 1% Triton X-100. After washing in PBS (3 X 10 minutes), the tissues were incubated for 1 hour at RT in a humid chamber in a mixture of two secondary antibodies (Table 2). The cryosections were then washed in PBS (3 X 10 minutes) and mounted in buffered glycerol, pH 8.6.

For each animal at least 100 neurons for each marker used in each double-labeled combination were evauated and recorded.

To determine the proportions of each neuronal population (SP, CGRP, CALB and nNOS) to the total ganglion neuronal population, the sections single-stained with each neuronal marker studied were counterstained with blue fluorescent Nissl stain solution (NeuroTrace, Molecular Probes, Eugene, OR, USA).

Tissue Analysis

Preparations were examined on a Zeiss Axioplan microscope (Axioplan epifluorescence microscope, Carl Zeiss, Oberkochen, Germany). The microscope was equipped with the appropriate filter cubes to distinguish between the fluorochromes employed: FITC and Alexa 488 (filter set with 450-490-nm excitation filter and 515-565-nm emission filter) and Alexa 594 (530-585-nm excitation filter). Images were recorded wwith a Polaroid DMC digital camera (Polaroid, Cambridge, MA) and DMC 2 software. Slight adjustements to contrast and brightness were made by using Adoobe (San Jose, CA) Photoshop CS, and the figure panels were prepared by using Corel Draw (Mountain View, Ottawa, Canada).

Results

Immunofluorescence on SG

T13-Ca2 spinal segments and SG were harvested from three dogs (mean age of 10 years) without a history of neurological signs, pain, spinal disease or orthopedic disorders. Immunofluorescence was performed on to L6-SG and corresponding spinal cord segments in order to perform a preliminary study.

Respect to the total neuronal population,CGRP-immunoreactive (IR) neurons were 15.3 ± 8% (Figure 1A), SP-IR neurons were 3.3 ± 2.3% (Figure 1B; Figure 2B; Figure 3B), CALB-IR neurons were 5.1 ± 2.4% (Figure 2A) and nNOS-IR neurons were 14.6 ± 8.3% (Figure 3A) (Table 3). In two cases the SG neurons showed an high amount of lipofuscin (Figure 4B).

About half of SP-IR neurons coexpressed CGRP immunoreactivity (44.4 ± 29.3%), whereas only 4.4 ± 3.3% of the CGRP-IR neurons were also SP-IR (Figure 1D) (Tab 4).

SP-IR neurons slightly coexpressed CALB immunoreactivity (11 ± 11%) and fewer CALB-IR neurons showed SP immunoreactivity (7.3 ± 7.3%) (Figure 2D) (Tab 4).

SP-IR neurons also expressed nNOS immunoreactivity (45.5 ± 27.9 %), whereas nNOS-IR neurons coexpressing SP immunoreactivity were 17.9 ± 12.1% in L6 DRG (Figure 3D) (Tab 4).

Spinal cord staining

To determine spinal cord lamination, we emloyed the NeuroTrace/SP double staining method. Nissl fluorescent staining seems to be the most reliable lamination marker (Lorenzo 2008).

SP-IR and CGRP-IR neurons showed the same distribution in spinal cord laminae (Figure 5). Particularly, SP- and CGRP-labeled fibers were localized predominantly in laminae I and II, with a moderate presence in lamina III and X, around the central canal (Figure 6A). Sattered and isolated neurons showed weekly SP-IR and CGRP-IR and were distribuited in deeper laminae (data not shown).

CALB-IR neurons and fibers were distribuited throught lamina I, II and III (Figure 7A). In lamina I and II, many small neurones and fibers exhibited intense immunoreactivity for the CALB, forming a dense band upon the inner portion of lamina II (Figure 7A, 7C)

nNOS-IR neurons were concentrated in lamina I-II (Figure 8A). A weak nNOS-IR was also observed in lamina X (Figure 6B). The overall expression of nNOS-IR neurons and fibers was weekly than SP, CGRP and CALB.

Discussion

Before discussing the results obtained in this research, it is important to underline some technical limitations occurred during microscopical observation. In particular, the presence of lipofuscines in two of three of dogs enrolled in the study may have represented a problem in masking the IR of neurons. Lipofuscin is a brown-yellow, electron-dense, autofluorescent material that accumulates progressively over time in lysosomes of postmitotic cells such as neurons.[23] The accumulation of lipofuscin in nervous tissue contribuites to the emission of fluorescence. As found in this study, previous researches in nervous tissue of the horse showed that autofluorescence due to lipofuscin pigment was generally limited to few cells and mainly to their edges.[24] Despite it usually does not represent a critical point, it can make more difficult to objectively observe neuronal IR.

The expression of CGRP, SP, CALB and nNOS in SG neurons of different species was previously described.[22, 25-31] The results obtained in this study indicate a lower percentage of CGRP- and SP-IR neurons, compared with those of other mammals.[24, 26, 31] The percentage of CALB-IR neurons observed in L6 spinal segment was close to that found in the pig[23]. On the contrary, the percentage of nNOS-IR neurons was more than that observed in the pig[25] but less than that reported in sheep and boar.[24, 26]

Considering the colocalization, the percentages of SP-IR neurons coexpressing CGRP-IR are higher in the goat[32] and rodents[33] than in dog. The same is for the percentages of CGRP-IR neurons coexpressing SP-IR. [32, 33] Similarly, the percentages of SP-IR neurons coexpressing CALB-IR is lower in goat [32] than in dog, but higher in rodents[33] than in dog. However, the proportion of CALB-IR neurons which colocalized SP-IR is higher in dog than in goat [32] and rodents.[33] The percentages of

SP-IR neurons coexpressing nNOS-IR is lower in dog than in rodents [33] but is the same as that observed in sheep.[26] Finally, the proportions of nitrergic neurons expressing SP are lower in dog than in sheep [26] and rodents. [33]

Our results showed that canine SP, CGRP, CALB and nNOS labeled fibers at the same spinal cord level terminations as previously described. [21, 31, 34-38]

As previously described, CGRP-, SP-, CALB- and nNOS-IR in SG neurons show a wide variability among different species and SG levels, particularly from thoracic to lumbar segments. [22, 24-31] Furthermore, despite the considerable number of studies focused on the characterization and distribution of peptidergic neurons in DRG, there are few comparative studies launched on canine tissues. Sensory neurons in the SG, with their peripheral and central (spinal) projections, are the "gateway" for painful signals emanating from both somatic and visceral structures. In particular, SP and CGRP are synthesized by nociceptor neurons in the SG.[30] These neuropeptides are important biochemical mediators in somatic pain pathways, which are evidenced by a significant alteration of their expression in spinal cord and SG after somatic stimulation.[30] CALB, which is an intracellular calcium-binding protein, has been associated with excitatory neurons and its potential role in mediating neuropathic pain has been recently investigated. [39] Furthermore, the involvement of nNOS in neuropathic pain has been already investigated.[40] nNOS regulates immune function, blood vessel dilatation as well as transmitter or modulator in the process of nociceptive stimuli.[40] Peripheral nerve injury can cause the excessive expression of nNOS in the spinal dorsal horn neurons, and accompanied by hyperalgesia or even pain disorders.[40]

Despite an extensive employing of rodents in neuropathic pain models has been observed in the last decades, few studies were focused on dogs as natural models of neuropathic pain. Further studies will be required in order to better define the characterization of those markers in canine SG.

Figures and Tables

Antibody	Host species	Serum code	Dilution	Source
CGRP	rabbit	C8198	1:5000	Sigma-Aldrich, St. Louis, MO
SP	rat	10-515A	1:400	Fitzgerald, Concord, MA
CALB	rabbit	CB-38A	1:4000	Swant, Maryl, Switzerland
nNOS	mouse	sc-5302	1:100	Santa Cruz Biotechnology, Santa Cruz, CA

Table 1. Primary antibodies used in the study.

Antibody	Dilution	Source
Donkey anti-rat IgG Alexa 594	1:50	Invitrogen, Carlsbad, CA
Goat anti-rabbit IgG FITC	1:300	Calbiochem-Novabiochem, San Diego, CA
Donkey anti-mouse 488	1:100	Biotium, Hayward, CA

Table 2. Secondary antibodies used in the study.

Marker	L6
CGRP/NT	15.3 ± 8.1 (140 / 855)
SP/NT	3.3 ± 2.3 (76 / 2,033)
CALB/NT	5.1 ± 2.4 (32 / 624)
nNOS/NT	14.6 ± 8.3 (127 / 851)

Table 3. Percentages of neuronal markers of the total population of canine lumbar (L6) spinal ganglia. NeuroTrace (NT) was employed as a pan-neuronal marker. Data are mean ± standard deviation. The numbers of cells counted are in brackets. Counts were all performed on three animals.

Colocalization of markers	L6
CGRP/SP SP/CGRP	4.4 (0-11) (10 / 140) 44.4 (0-100) (10 / 12)
CALB/SP SP/CALB	7.3 (0-22) (2 / 32) 11 (0-33) (2 / 22)
nNOS/SP SP/nNOS	17.9 (0-41.2) (32 / 111) 45.4 (0-96.2) (32 / 42)

Table 4. Colocalization (percent) of markers in canine L6 spinal ganglia. The number of cells counted are in parenthesis.

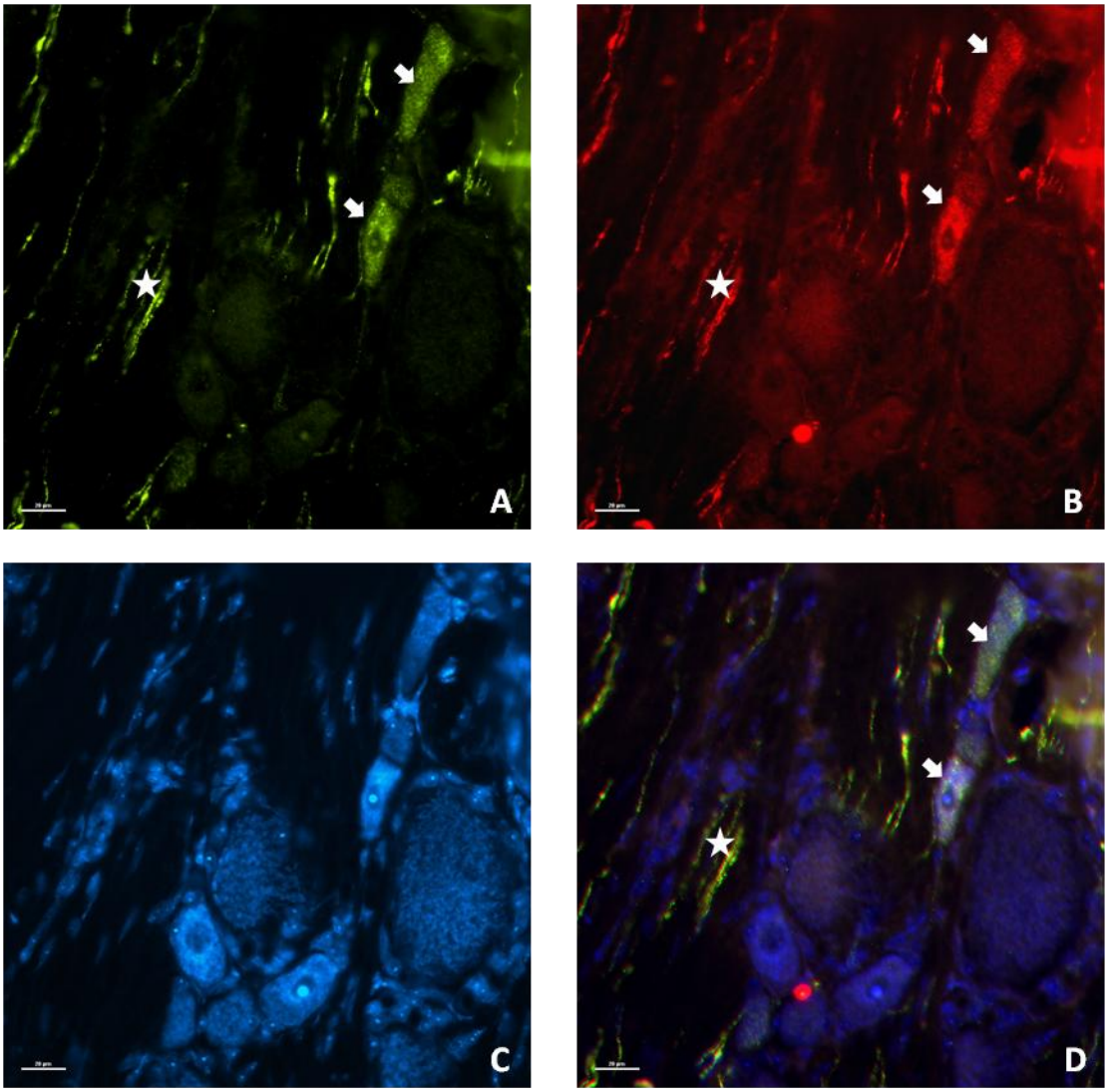

Figure 1. CGRP (A) and SP (B) immunoreactivity in neuronal SG.
D: NeuroTrace (NT) stain. E: merge of CGRP, SP and NT stain.
The arrows indicate neurons labeled for CGRP (A), SP (B) or both (D); the stars indicate fibers labeled for CGRP (A), SP (B) or both (D).

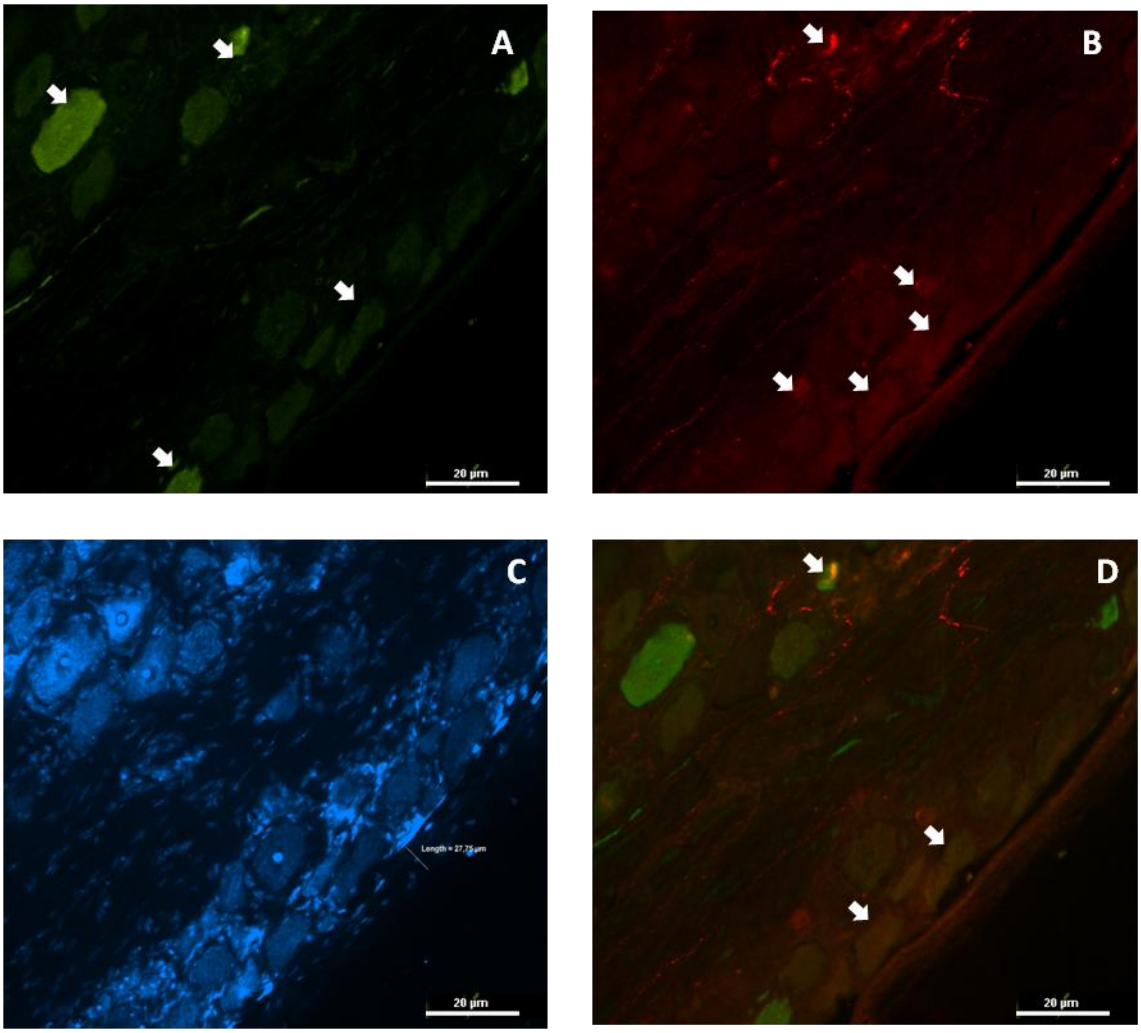

Figure 2. CALB (A) and SP (B) immunoreactivity in neuronal SG.
D: NeuroTrace (NT) stain. E: merge of CALB and SP stain.
The arrows indicate neurons labeled for CALB (A), SP (B) or both (D).

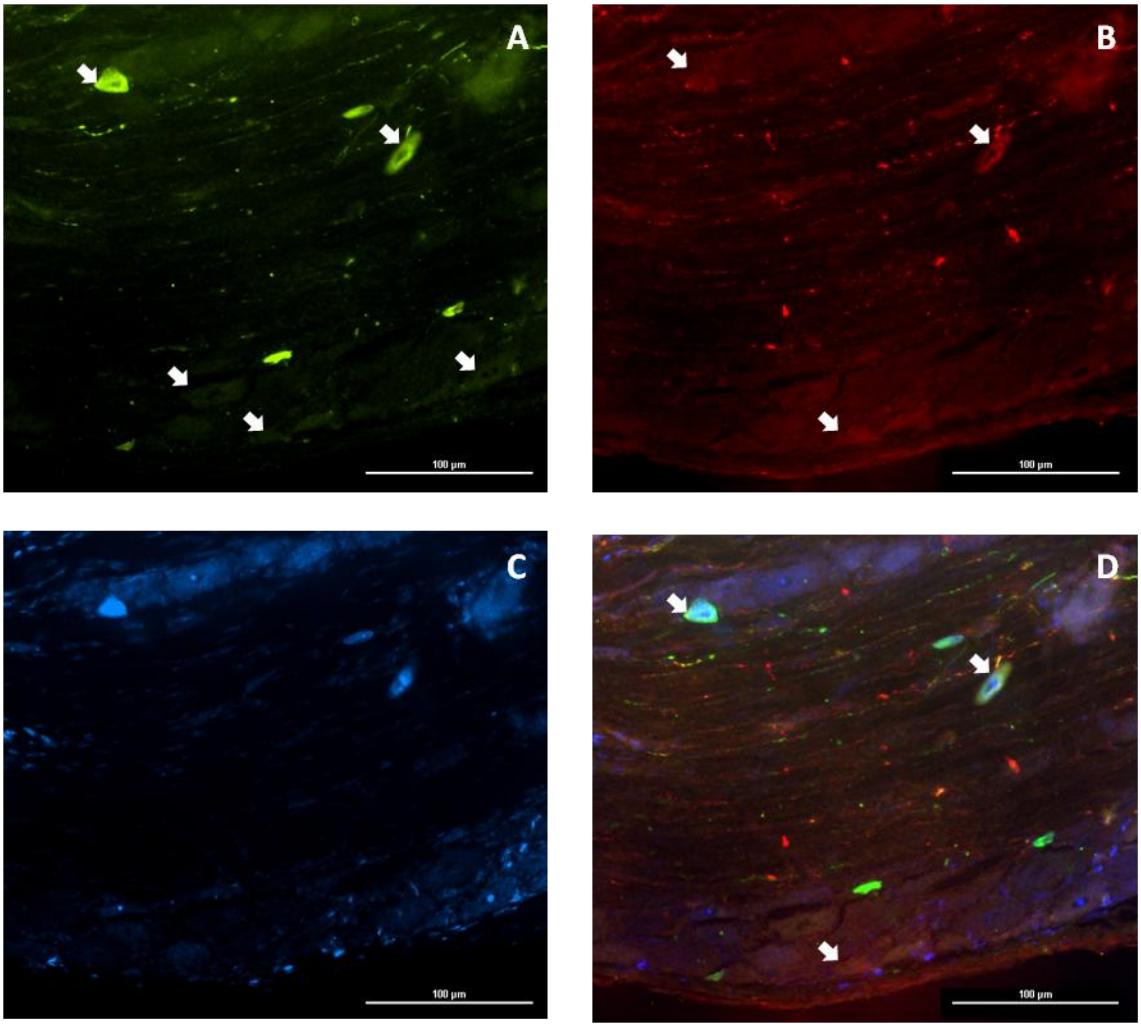

Figure 3. nNOS (A) and SP (B) immunoreactivity in neuronal SG. D: NeuroTrace (NT) stain. E: merge of nNOS, SP and NT stain. The arrows indicate neurons labeled for nNOS (A), SP (B) or both (D).

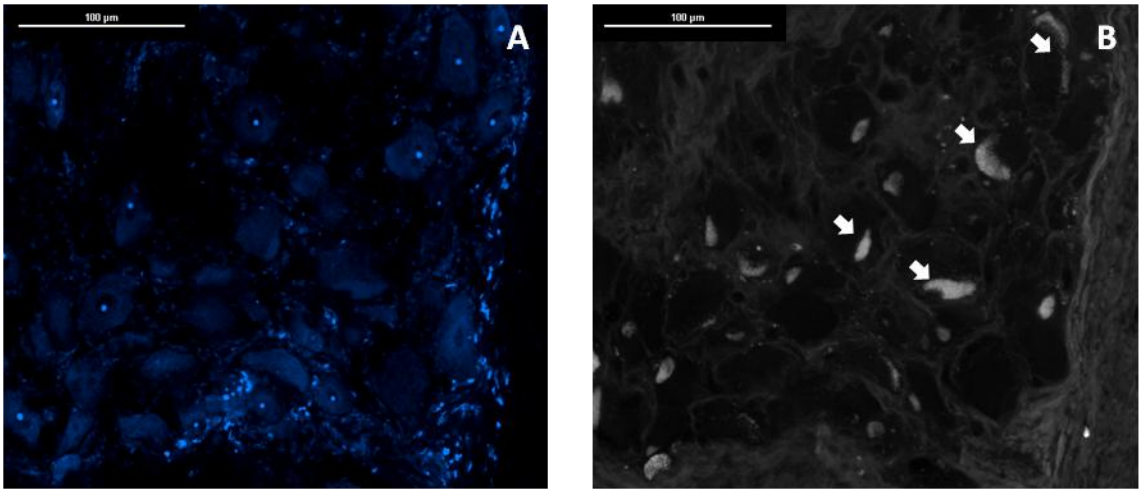

Figure 4. Autofluorescence of lipofuscin expressed in neurons (B) marked with NeuroTrace (A).

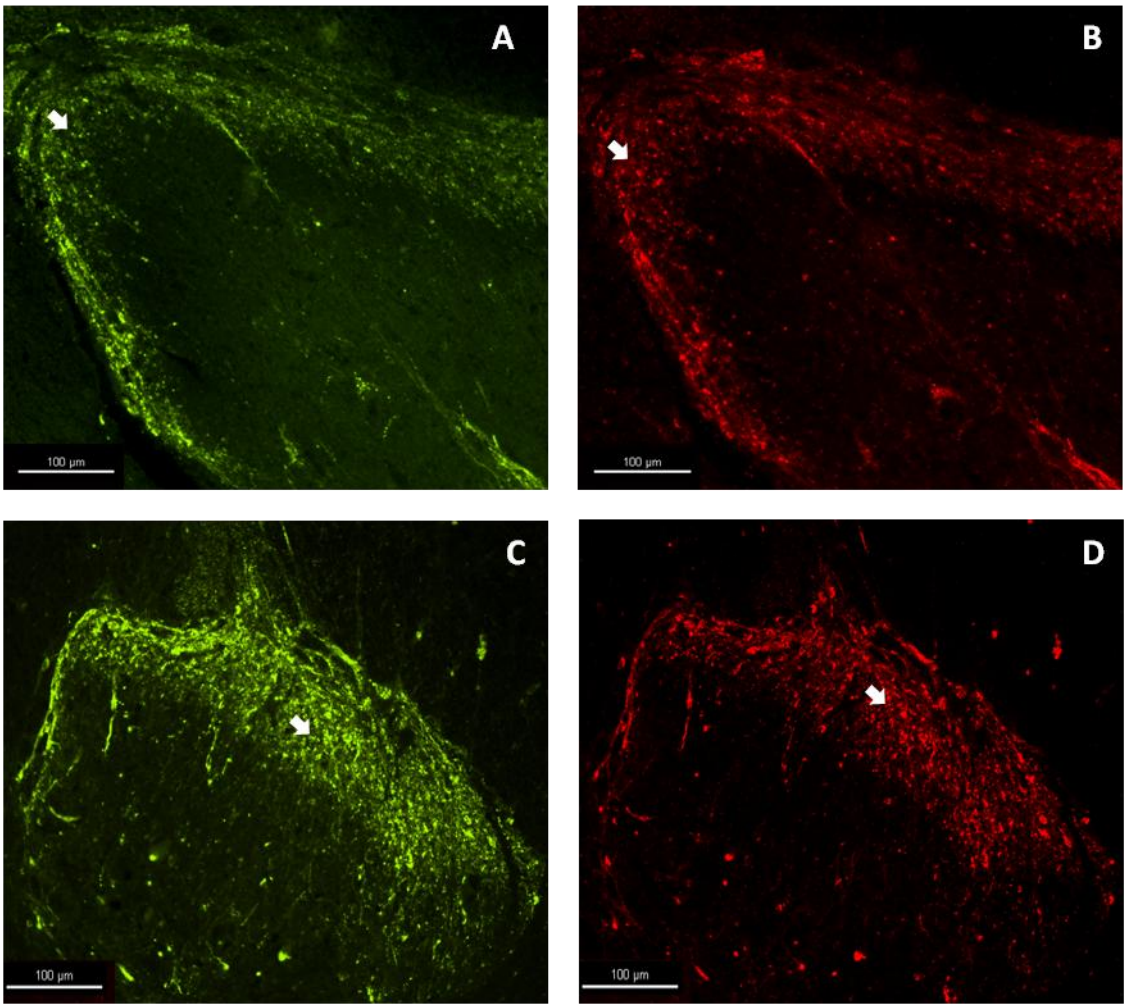

Figure 5. Spinal cord staining for CGRP (A; C) and SP (B; D). The arrows indicate the the markers distribution in lamina I and II.

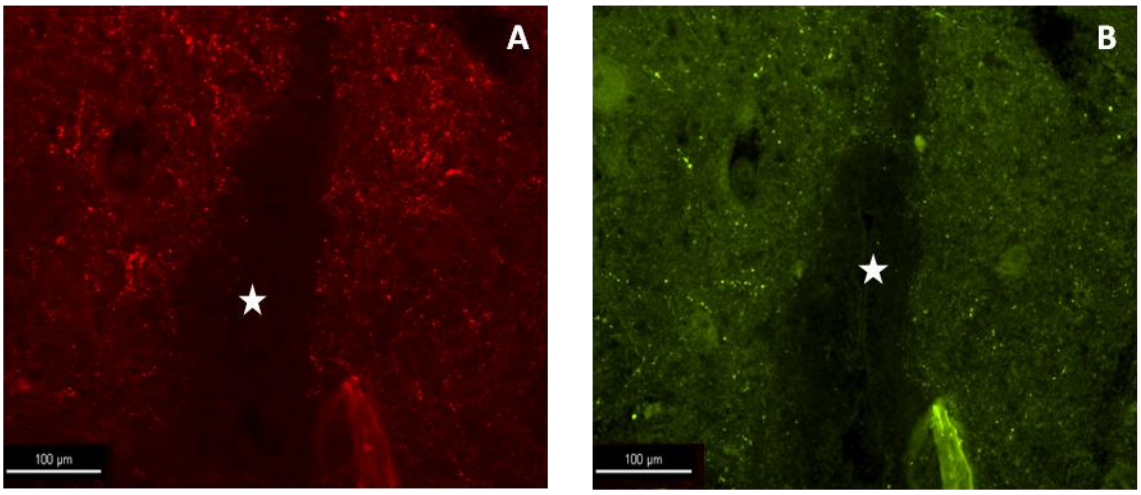

Figure 6. Immunolabeling for SP (A) and nNOS (B) at the level of lamina X. The stars indicate the central canal.

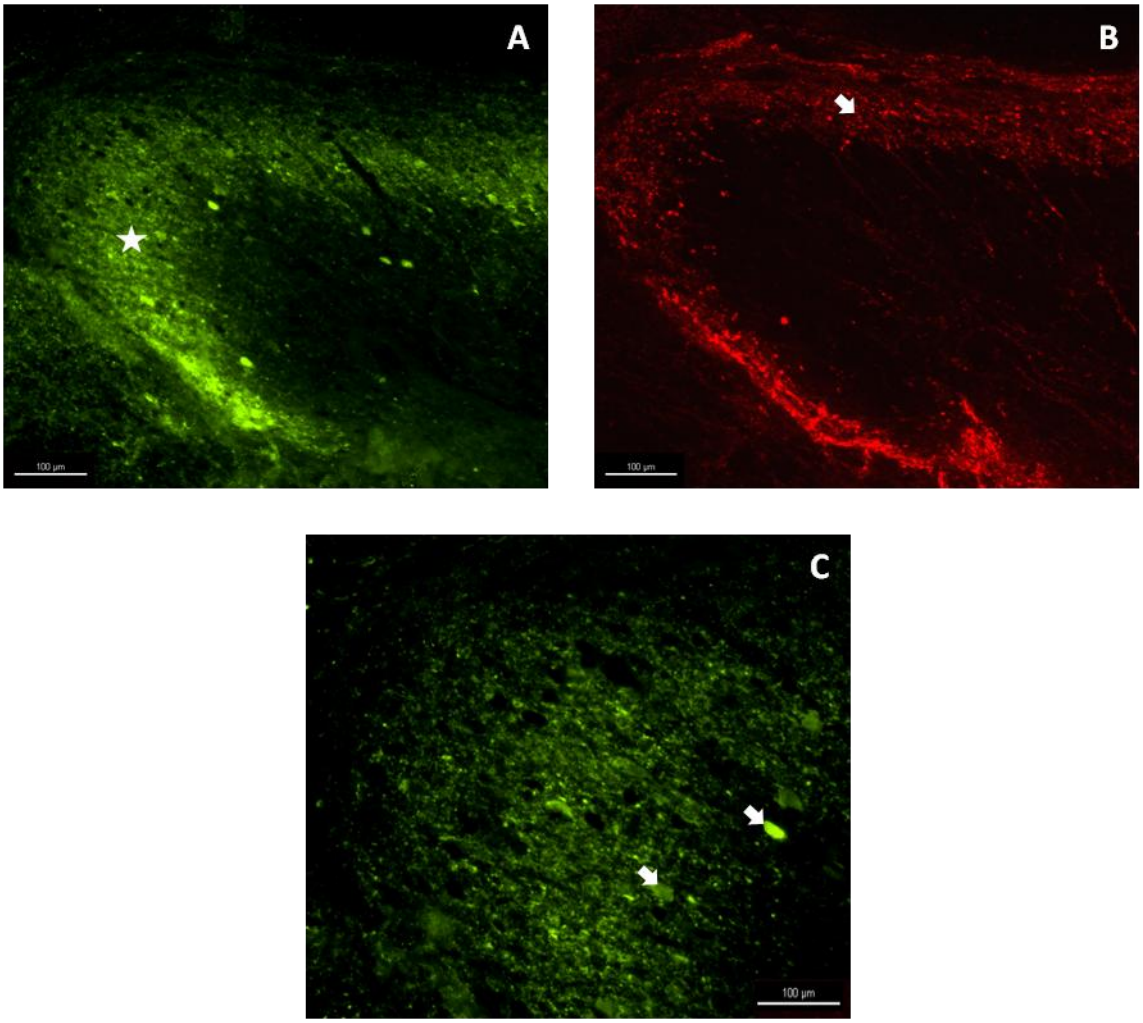

Figure 7. Spinal cord staining for CALB (A) and SP (B). The arrows indicate the the CALB distribution in lamina I, II and III (A) and neuronal labeling (C).The arrow indicates SP-IR in lamina I and II (B).

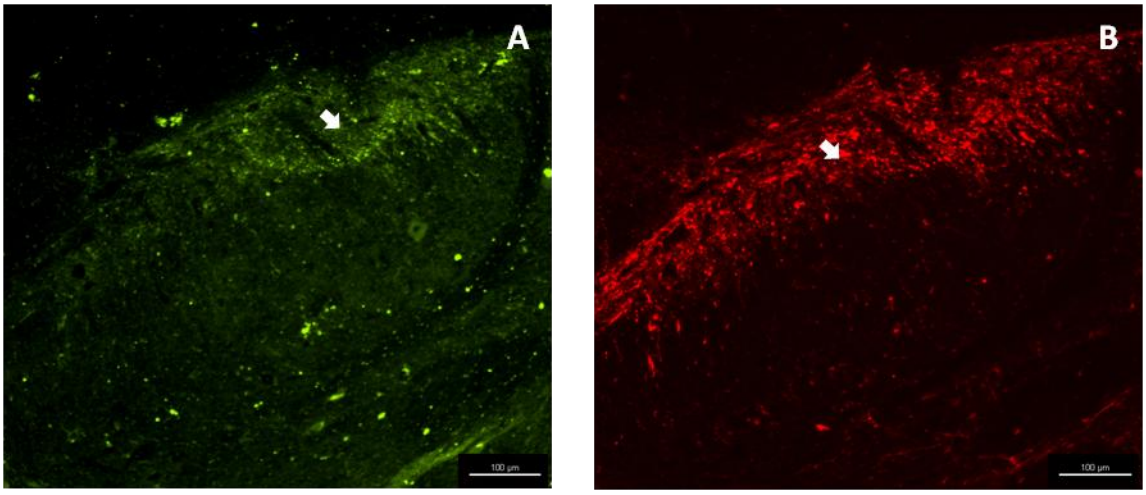

Figure 8. Spinal cord staining for nNOS (A) and SP (B).
The arrows indicate the the nNOS (A) and SP (B) distribution in lamina I and II.

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Assessment of prevalence, quality of life and risk factors associated to Phantom Complex in a client-owned dog population after limb amputation

Introduction

Amputation of one or more body parts in human patients has (since long) been commonly associated to devious sensations arising from the missing body portion. First descriptions of this phenomen date back to 1536 when the french barber surgeon Ambroise Paré described it in soldiers in which he performed surgical amputations. This syndrome, nowadays called “Phantom Limb Pain” (PLP), is described as pain perceived in the body part that is no longer present.[1, 2] PLP is reported to occur in about 60 to 80% of patients within the first 2 years after amputation and in up to 10% of patients PLP persists during life.[3-5] Pain onset is dated early after amputation and several studies have shown that a range from 75% to 85% of PLP patients develops pain within the first few days after surgery.[6, 7]

The PLP is part of a more complex syndrome, called “Phantom Complex” (PC), which includes the Phantom Limb Sensation (PLS), described as any sensation in the absent limb except pain, the Stump Pain (SP) as pain localized in the stump and the PLP. PLP could be confused or overlap with the common acute post-surgical pain or SP, which can be related to factors that may cause pain as infections or bone spurs.However, SP usually subsides with healing, whereas PLP persists in 5-10% of cases and may get worse with time.[6] The pathophysiology of PLP, similarly to other manifestations of neuropathic pain, involves plastic changes of the general somatic

afferent pathways either affecting the peripheral nervous system, at the site of transected nerve, or the central nervous system, within spinal cord and somatosensory cerebral cortex, or most likely both simultaneously.[6, 8]

Different studies have shown a correlation between the presence of PLP and risk factors such as physical and psychological conditions, pre- and post-amputations treatments and the role of pre-amputation pain in the development of long-term PLP.[2, 3, 9]

Amputation of a limb is a commonly performed procedure in small animals, suggested for numerous reasons including unresectable neoplasia, severe fractures, ischaemic necrosis, osteomyelitis or myofascitis and severe disability due to unmanageable arthritis, paralysis or congenital deformity.[10-12] Adaptation of dogs to the amputation of a limb, presence of risk factors associated to a poor quality of life and owners' satisfaction have been the topic of several veterinary studies in the last years.[10, 11, 13-16] However, none of those specifically investigated the occurrence of pain and pain related behaviours after amputation that could account for stump pain from neuropathic origin (neuroma) and/or PLP in veterinary patients. Laboratory animals receiving mechanical injury of a peripheral nerve exhibit behavioral symptoms of neuropathic pain manifested as mechanical and thermal hyperalgesia or allodynia.[17] Neuropathic pain, that is pain caused by a lesion or disease of the somatosensory system, is characterized by abnormal sensations that in a non-verbalizing patient migth be difficult to be clinically identified, delaying its treatment. Furthermore, PLS, SP and PLP often coexist and may be difficult to separate their presence in veterinary patients. In this vein, identification of specific symptoms, behaviour and clinical signs suggestive of PC in dogs would represent a useful tool for PC screening and ideally for response measurement to appropriate neuropathic pain treatments. Hence, we screened a client-owned population of dogs with limb amputation through an online survey aimed to document PC prevalence, through the identification of symptoms and behaviours suggestive of

neuropathic pain, evaluation of risk factors associated to its occurence and owners' perception of the quality of life (QoL) of their three-legged pets.

Matherials and Methods

Project questionnaire design and description

The questionnaire was designed on the basis of the clinical experience of veterinary specialists, the Helsinki Chronic Pain Index (HCPI) (hielm-bjorkmank 2009) and the German Pain Questionnaire for Childrens and Adolescents (DSF-KJ) (http://www.deutsches-kinderschmerzzentrum.de/fileadmin/media/PDF-Dateien/englisch/parents_initial_3.0.pdf). The latter is a pediatric model for pain measurement, giving the indirect evaluation of pain from the owner´s perspective.
A first draft of the questionnaire was initially evaluated by 2 members of ALGOVET (Italian group of veterinary algology), 2 board certified veterinary neurologists (GG and LM), 4 veterinary surgeons and a three-legged pet owner. Feedback regarding appropriateness of the questions as well as relevance of the clinical parameters considered was requested and the survey was modified accordingly. Ethics approval was granted by the University of Bologna Department of Veterinary Medical Sciences ethics committee (ID 664/2016).
The questionnaire included three sections with a total of 75 questions. The first section consisted of 30 questions (26 closed-ended and 4 polar questions) concerning factual data that targeted presence, characterization and factors related to pain before amputation as well as reason for amputation, duration of disease

prior to amputation and use of specific treatments. The second section consisted of 35 questions (33 closed-ended and 2 polar questions) aiming for detection of pain-related behaviours after amputation that could account for abnormal painful sensations, risk factors usually associated to PLP in humans, post-surgical complications and therapies used to control post-amputation pain if present. This section included further questions referred to the degree of adaptation to amputation in terms of mobility, persistence of usual behaviour and/or attitude changes towards other animals or family members and the owner´s perception of their pets' QoL. The last section consisted of 10 closed-ended questions that evaluated the owner's satisfaction as caregiver of a three-legged pet and the evaluation of the effects of limb amputation from the perspective of family- and social life .

Pain was characterized in terms of *prevalence*, as pain observed by the owner before and after amputation; *onset*, as the time in which dogs started showing pain-related behaviours; *frequency*, as pain recorded episodes (several times per day, weekly, monthly or yearly) and *type*, as quality of pain described as persistent, waxing and waning or sudden and transient (referring to a 7-days pre- and a "typical month" post-amputation).

Furthermore, *pain onset before amputation* and *time between diagnosis and amputation* were defined as the moment in which dogs started showing signs of pain and the time elapsed between the diagnosis of the underlying disease and amputation.

With *typical week after* and *typical month after*, authors reffered to a standard time frame of one week or month during the post-amputation phase.

Recruitment of responders

The questionnaire was presented via the online survey software and questionnaire tool SurveyMonkey (https://www.surveymonkey.com) from February to March 2015. Advertisement of the study and enrollement of the cases was announced through the Facebook page and website of the three-legged dogs "tripawds'" owners community (https://www.facebook.com/tripawds; http://tripawds.com/) which were invited to participate to the survey (http://downloads.tripawds.com/2015/02/23/take-the-tripawd-phantom-limb-pain-survey/).

Inclusion criteria for survey participation comprised dogs that underwent surgical amputation of one limb, either proximal or distal, independentlyof the reason for amputation, and that had a minimum of three months follow-up after surgery. This 3 months timeframe was considered an adequate period of time in order to discriminate between development of specific post-surgical pain and/or occurrence of PLP in a non-verbalizing patient.

Owners were free to decide whether to answer or not to all questions. Only complete questionnaires (at least 95% of questions answered) were included. Since a variable number of owners answered to each single question, in order to avoid discrepancies we decided to report the results as percentages instead of numbers. Percentages are therefore related to the number of responders, which was slightly variable among questions.

Statistical analysis

All data were fed into Microsoft Excel® and PAST® software for statistical analysis. The distribution characteristics of the values were checked for each linear parameter by Shapiro-Wilk test and normal probability plotting. Categorical or ordinal data were described as percentages of the total. Association between categorical variables was assessed with chi-squared test or Fisher's exact test. P values ≤ 0.05 were considered significant.

Results

Descriptive data

107 owners of 107 dogs participated to the study; 63% were purebred dogs including 32 breeds, with Golden Retriever (11%) and Labrador Retriever (9%) beeing most commonly represented (Figure 1); mean age at time of the survey of 7.5 years (median 8 years, range 0.2-16 years). 69% were male dogs (39% neutered) and 31% female dogs (40% spayed). At the time of amputation 39% of dogs were 6 to 10 years old, 31% were 1 to 5 years old, 21% were 1 year or less and 9% were 11 to 15 years old. At the time of the study 79% were still alive. Large size dogs (over 25 kg) represented the majority accounting for 59%, while medium size dogs (10-25 kg) acounted for 29% and small size dogs (<10 kg) for 12%.

The main reason (53%) for amputation was neoplasia (66% osteosarcoma, 11% soft tissue sarcoma, 7% peripheral nerve sheath tumour, 5% histiocytic sarcoma, 5% mast cell tumour, 2% fibrosarcoma, 2% chondrosarcoma, 2% lipoma), followed by trauma in 41% (70% irreparable fracture, 15% major soft tissue trauma, 10% gunshot, 5% spinal lesion), limb malformation in 3% and infection in 3% (Figure 2). In 75% of dogs the entire limb was amputated while the remaing 25% underwent distal amputation (13% above the knee, 11% above the elbow, 1% below the elbow) (Figure 3). Of the 107 dogs, 61% underwent thoracic limb amputation, and 39% underwent pelvic limb amputation.

Pain before and after amputation

According to the owners' perception, pain was reported in 83% of dogs before surgery and in 85% of dogs after amputation; there was no significant differences in the prevalence of pain before and after amputation (p=0.6) (Figure 4).

Onset of pain before amputation was described more than 1 month in 53% of dogs (68% of oncologic patients), whereas in 35% pain was persent from 2 to 4 weeksbefore ; 12% experienced pain 24 hours to 1 week prior to amputation. The onset of pain before surgery did not represent a risk factor for postsurgically pain development (p=0.09).

However, the time of pain onset before amputation was significantly related to the frequency of pain afterwards, with a significantly higher frequency of pain episodes in dogs which showed an early onset of pain before the amputation (p<0.01) (Figure 5).

Regarding pain experience during recovery after surgery there was an overall steep decrement in pain prevalence over time, with 51% of dogs experiencing pain between 24 hours to 1 week after surgery, 19% between the second and fourth week, 9% between one and three months and 5% between three and six months (Figure 6).

In 34% of dogs the time between diagnosis of the underlying disease and amputation ranged from 48 hours to 1 week, and more than 1 month in 27% of dogs. The shorter the time gap between diagnosis and amputation, the lower was the frequency of pain episodes described after. However, no correlation was seen amongst the time between diagnosis and amputation and the prevalence of pain after amputation (p=0.6).

Regarding the frequency of pre-amputation pain episodes, 57% of dogs experienced pain several times daily, 27% weekly, 12% monthly and 4% yearly. No differences were seen in the frequency reported after the amputation, with pain episodes

described likewise as several times daily in 57%, weekly in 22% , monthly in 14% and yearly in 7% of dogs (p=0.4) (Figure 7).

During the 7 days prior to amputation, owners described the type of pain experienced by their pets as waxing and waning in 45%, persistent in 40% and sudden and transient in 15% of dogs. A significant difference in pain phenotype was seen in a "typical month" after the amputation, where the type of pain was predominantly described as sudden and transient in 53% of dogs experiencing pain ($p<0.01$) (Figure 8).

No breed, sex, weight or reason for amputation were related to the prevalence of pain after the amputation ($p>0.1$).

Complications after surgery occurred in 20% of cases, comprising infection (28%), pain (24%), swelling of the surgical wound (24%) and failure of the suture (24%) with 95% of postoperative problems started during the first week after surgery. However, comparing this subpopulation with that of patients that did not experienced post surgical complications there is no significant difference in terms of prevalence of pain after the amputation ($p>0.1$).

Therapies

79% of dogs received medical treatment before amputation including the following: pain killers (28%), anti-inflammatory drugs (21%) and antibiotics (14%) (Figure 9). In 46% of dogs these therapies were administered for more than 1 month. Medical treatment before amputation did not prevent the occurrence of pain in the post-amputation period (p=0.3)

After amputation, 91% of dogs received treatment for pain relief and 67% of owners felt the need to consult their veterinary surgeon because of pain. The most

frequently administered therapies were pain killers in 35% of dogs, followed by anti-inflammatory drugs (30%) and gabapentin (14%) (Figure 10); in 39% of cases duration of therapies lasted between 2 to 4 weeks.

When specifically asked about satisfaction in view of pain management, 26% of owners chose the option from “not satisfied” (1%) to “partially satisfied”(25%) in the post-amputation period, while 15% described pain control as unsatisfactory before surgery with a clear-cut drop to 1% of unsuccessful treatment after amputation.

Manifestations related to the PC

After 3 to 6 month from the amputation, dogs showed a reduced activity level (67%) and overall playfulness (46%), reduction in mood (44%), decreased participation in family life (31%), appetite loss (30%), sleeping reduction (21%) (Figure 11). Interaction with other animals was also impaired in terms of decreased friendliness with family pets (18%) and strange pets (26%) (Figure 11).

Investigating the precence of possible PLP related behaviours in the timeframe comprised between 3 months to 1 year or more after the amputation; 35% of dogs showed muscular twitching at the level of the stump, 22% licked the stump, 19% expressed whimpers and 17% yelps, 16% were restlessness, 12% looked anxious, 11% chewed the stump and 8% scratched the stump (Figure 12).

Behavioural changes in terms of agression and withdrawal from interactions were described before and after the amputation. In particular, owners described episodes of aggression towards humans (12%) and animals (19%) and the tendency to prevent contacts with humans (17%) and animals (18%) in the pre-amputation phase. The same changes in behaviour were reported in the post-amputation phase regarding aggression towards humans and animals (13% and 17% respectively) and

prevention of human and animal contacts (15% and 22% respectively), without significantly differences between the two phases ($p>0.1$).

Environmental and/or physical stress as judged by the owner was reported in 78% of dogs that experienced pain after amputation. Accessory symptoms that could possibly account for pain were reported in 26% of dogs in the post amputation period. In particular, owners described tiredness (27%), fast breathing (25%), and irritability (12%). Interestingly,, in the 24% of cases these symptoms were evident in a period ranging from 3 months to 1 year or more after amputation. Furthermore, during a "typical week after amputation", defined as a standard week in the post-amputation period, 47% of owners described pain vocalization even though most of these (68%) reported a frequency equal to "hardly never".

Quality of Life

The degree of adaptation after amputation was described from good to very good in 94% of dogs, without relation to which limb was removed, front or hind limb, and the level at which the amputation was performed. 72% of dogs moved adequately within the first week after the amputation. Dogs that showed a better adaptation resulted to have lower chances to experience pain in a "typical month" ($p=0.02$) and adapted quickly ($p=0.005$). Reported movement restrictions in a "typical week" after the amputation were: difficulties in jumping (28%), moving after a major activity (21%), moving after a long rest (15%), rising from a lying position (13%), walking (6%) and galloping (5%).

Owners' satisfaction and perspective

After the amputation, 59% of owners reported an improvement in the quality of their relationship with their pets describing it as "better" and "much better" and in 75% of cases the overall response of the family to the amputation was considered to be "very positive".

However, in the first month following amputation, 62% of owners felt their pet caused conflict with their work, education or daily activities, 52% felt a limited indipencence and 46% felt a limitation in their social life. Nevertheless, 89% of the interviewees did not regret the decision of amputation and 92% felt well informed by their veterinarian during the decision making process.

Discussion

The present investigation is the first attempt to address the presence of neuropathic pain and clinical signs compatible with PC in a client-owned population of dogs that underwent amputation of one limb. Similarly to what previously described in human patients, pain was particularly common in the early post-amputation phase with 51% of dogs experiencing pain in the time-frame comprised between 24 hours to 1 week after surgery.[4, 7, 18-20] The onset of pain in humans is reported to appear with a high frequency during the first week after amputation and usually it subsides with healing of the surgical wound.[6] However, in 5-10% of patients pain arising from the residual stump can persist beyond the stage of post-surgical healing and may get worse with time, leading to the development of a burdening chronic pain.[4, 6, 18, 21] In our study group, we observed a reduction of the incidence of pain over time likewise, with 9% experiencing pain at 1 to 3 months and 5% at 3 to 6 months follow up.[7, 18, 19] These data are in accordance to previous human findings, suggesting that establishment of neuropathic pain in the residual limb may be delayed for months after surgical resection in the canine species.

Investigation of neuropathic pain and PC (comprising PLP) in animals represents a clinical challenge. In human medicine, patients usually describe PLP as intermittent, burning, cramping and stinging pain.[4, 18] These descriptions refer to either peripheral or central abnormal neuronal firing responsible for the neuropathic component of PLP.[6] Veterinary patients unfortunately can not verbalize, therefore information on abnormal painful sensations such as burning, tingling or electrical discharges of one part of the body as well as quality and intensity of pain can be easily missed despite careful behavioural observation. With this inherent limitation in mind we developed a set of behavioural observations that

could directly and indirectly suggest perception of abnormal painful sensations, together with recording of their type and frequency before and after amputation of a limb. Manifestation of discomfort focused on the stump were consistently reported in the period comprised between 3 months and one year after amputation and featured muscular twitching in one third of the dogs, accompanied by episodes of incessant licking or chewing and scratching of the stump. Together with peculiar signs and increased attention towards the stump, more than the half of amputated dogs further displayed a clear overall change of their daily activities comprising reduction of physical activity and playfulness. Interestingly, also activity of daily life was perceived as changed by the owners with modification of the sleeping pattern and loss of appetite. Other aspects of a regular pet life were also affected, as decreased participation in family life and alteration in the mood. Behavioural changes were described both before and after amputation. Those changes were previously described in amputated dogs and varied from aggression, anxienty, descrease in dominance and lack of interest in other dogs.[11] Similarly to the previous study, it was not possible to determine the reason for these changes in behaviour. However, these changes were described both before and after amputation and may reflect the presence and peristence of pain perception. Furthermore, the onset in some cases was reported to be from 3 months to 1 year or more after the amputation and this data, even if we can not rule out other causes, may reflect the occurrence of PLP.

Negative impact of amputation on daily life has been reported in human patients likewise, where amputees' QoL was poorer if compared to the general population.[22] For these patients the biggest matter of complaint was limitation in physical activities followed by low social acceptance.[22, 23]

Pain after amputation, as perceived be the owner, was described as sudden and transient, disclosing yelps and whimpers that would most likely account for the acute character of bursts of abnormal neuronal firing giving credit to the hypothesis

of this pain being neuropathic in origin. Noteworthy, there was no difference of pain frequency before and after surgery. Dogs showing pain several times a day prior to amputation were likely to present with pain with the same frequency after. Furthermore, in the post amputation phase more than two thirds of the owners felt the need to seek for medical advice because of pain.

Although the relationship between the significance of pre-amputation pain on post-amputation pain is still debated, several retrospective studies have pointed to the duration of pre-amputation pain longer than 1 month as a risk factors for development of chronic PLP.[4, 9] Accordingly, dogs experiencing pain for more than 1 month before amputation had a higher chance to develop daily episodes of pain after amputation. The shorter the time between diagnosis and amputation, the lower the frequency of post-amputation pain episodes. Neuropathic pain is defined as pain caused by damage or disease affecting the somatosensory nervous system, hence it is not surprising that long lasting noxae may more likely produce peripheral and/or central sensitization of the sensory pathways and consequently pain.

Previous studies in human medicine suggested that pre-amputation pain might play a role in PLP development[24-26]. For this reason, several authors tried to evaluate the effect of different therapies on to the outcome of amputated patients, but most of them showed that pain control before amputation does not prevent development of PLP.[2, 24] Likewise we did not find any correlation between administration of analgesic therapies before amputation and the incidence of post-amputation pain.

A relationship between PLP and the aetiology of the amputation have been proposed by some authors[6, 19] but their investigations failed to prove interdependency between primary disease and incidence of PLP. Accordingly, in our study the aetiology of amputation was not correlated to the occurrence of post surgical pain. Altogether these observations suggests that indipendently of the

cause of primary damage of the somatosensory pathway, PLP is a complex and slowly developing phenomenon that plunges its roots way head of amputation.

As previously described[10], post-surgical complications can occur in 20% of amputated dogs and cats. We found similar percentages of complications and the most commonly reported was infection of the surgical site in the immediate post-amputation phase. We did not however find any positive correlation with the occurence of pain after amputation and post-operative complications.

Stress during the post amputation phase seems to play a role in the development of pain. A possible relation between stress, accessory symptoms and onset of pain episodes was reported in 24% of patients within the time frame from 3 months to 1 year. This is in accordance with human studies in which psychological stress represents a risk factor for PLP occurence.[24]

As previously described,[11,15,16] dogs adapted very well to amputation in the 94%, -independently fromwhich limb (thoracic or pelvic) was amputated or at which level, (dorsalor proximal) the amputation took place. Interestingly, the better and quicker the recovery of dogs, the lower the occurrence of post-amputation pain. Based on these observations, we can postulate that pain in the post-amputation phase can reduce the ability to adapt to walk on three legs and, therefore, pain detection and control should be considered a crucial goal in the recovery phase.

The overall owners' perception of QoL of their pets was satisfactory and more than a half of the interviewed further described an improvement in the quality of the relationship with their pet together with an overall positive response of the family to their three-legged pet. Within the first month after amputation owners felt limitations in their indipendence and conflict with everyday activities that however appeared to be only transient, and 89% did not regret the decision of amputation.

Our data demonstrate the presence of previously unreported signs and symptoms that may be interpreted as expression of PLP. This is particularly true for those dogs which experienced post-amputation pain after at least one month from

surgery. It is of great interest the owners' recognition of behavioural and specific signs that may be reconducted to the presence of unpleasant sensation due to abnormal and/or ectopic neuronal firing.

While the distribution of frequency of pain episodes is almost identical in the pre- and post-amputation periods, the different type of pain experienced after the amputation may suggest evolution into neuropathic pain.

Significant risk factors associated with the frequency of post-amputation pain episodes are duration of pain before amputation and time between diagnosis and amputation, confirming what is reported in human patients with PLP.[4, 9]

Conclusions

The present study, in the authors' view, represents a first step towards providing new useful information in the recognition of the presence of PLP, a condition to date never reported in dogs which and that requires adequate consideration in the clinical management of these patients.

Figures and Tables

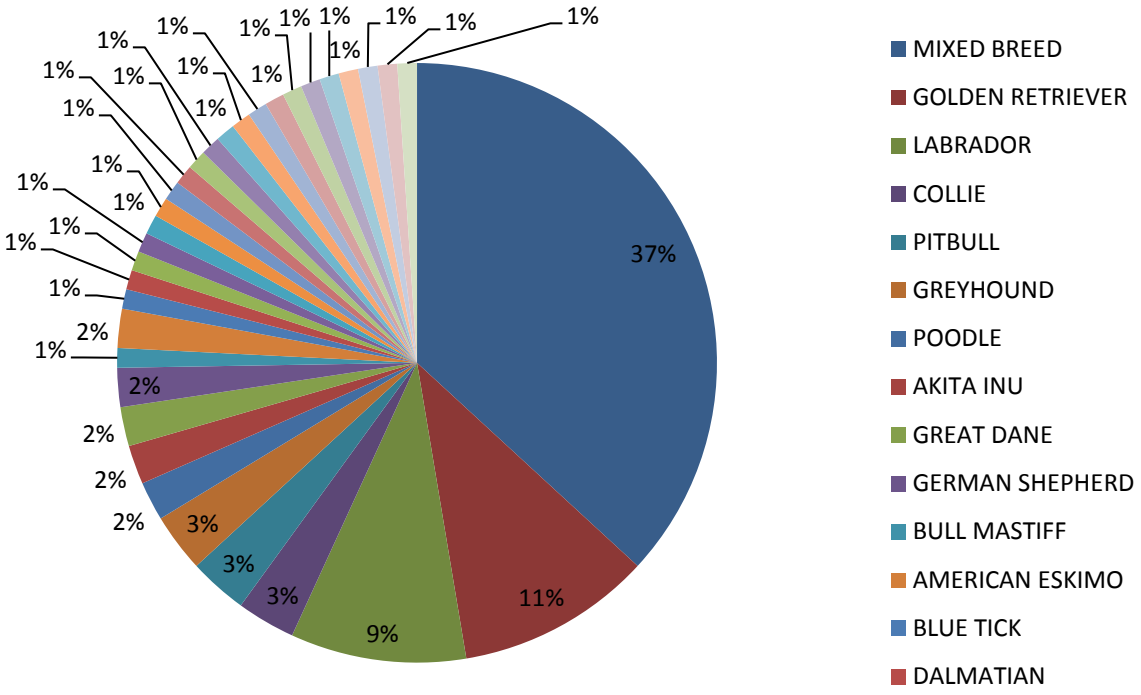

Figure 1. Percentages of dogs' breed described in the study.

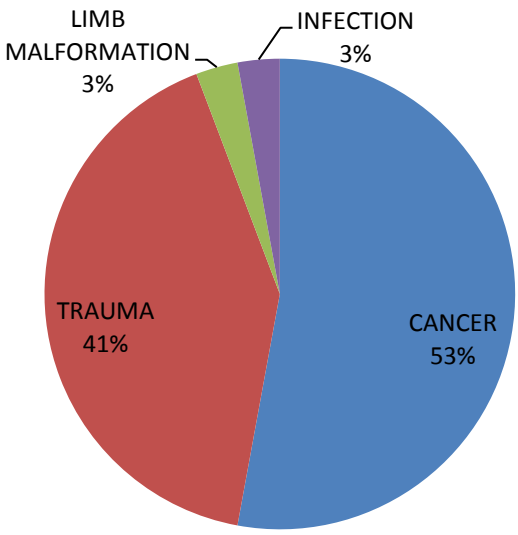

Figure 2. Reasons for amputation

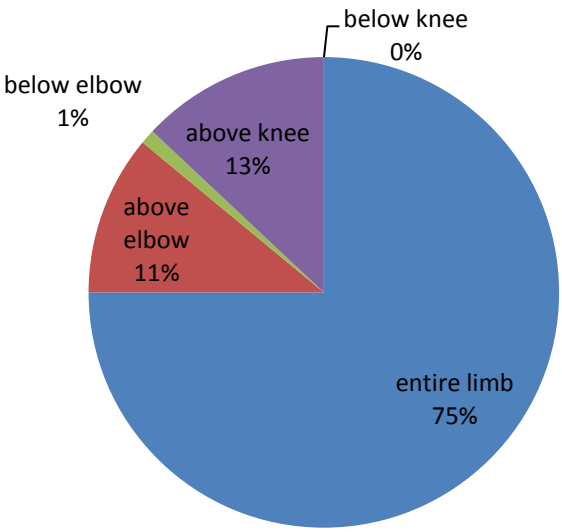

Figure 3. Levels of amputation.

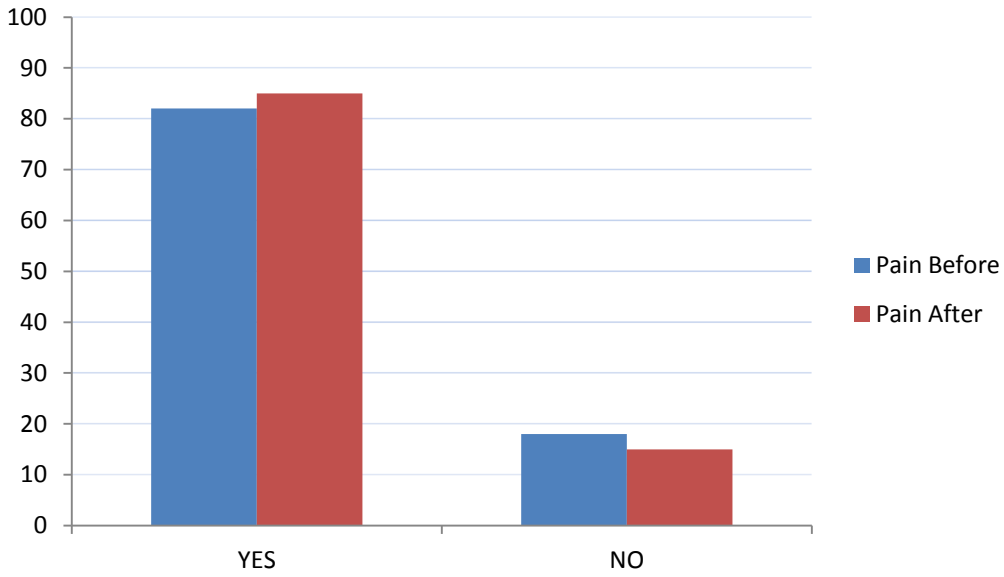

Figure 4. Percentages of dogs showing pain before (blue columns) and after (red columns) the amputation.

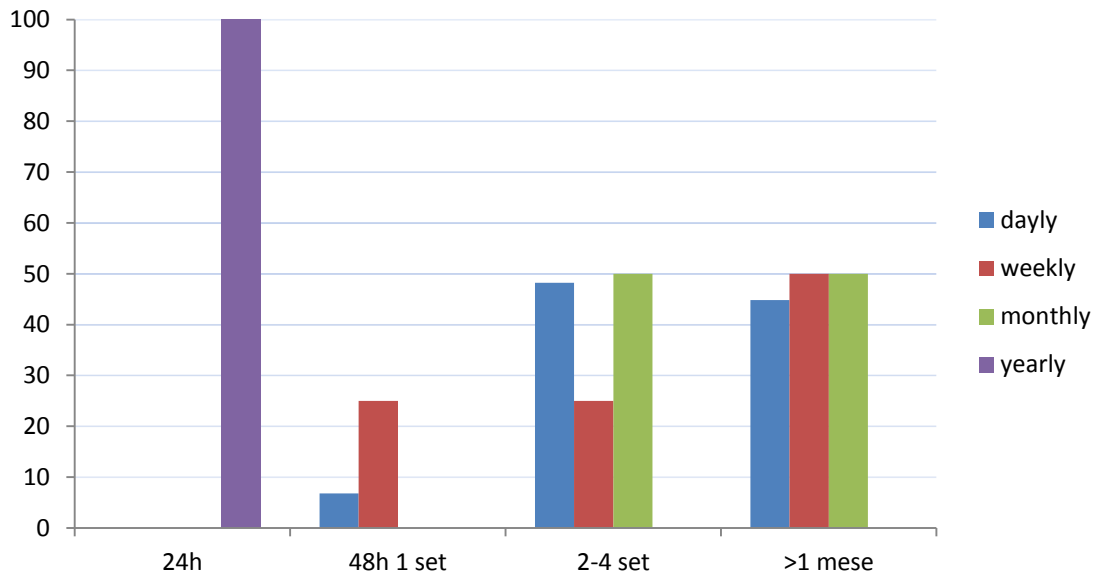

Figure 5. Frequency of pain episodes after the amputation, in relation to the onset of pain before the amputation.

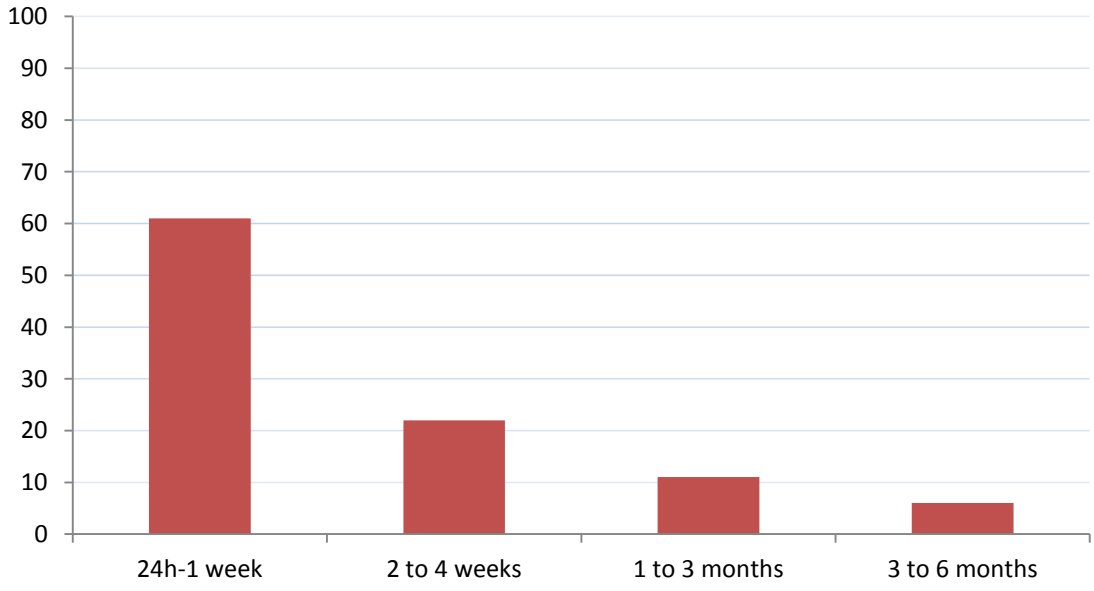

Figure 6. Percentages of dosg showing pain in the post-amputation phase. Percentages are divided in four time frames.

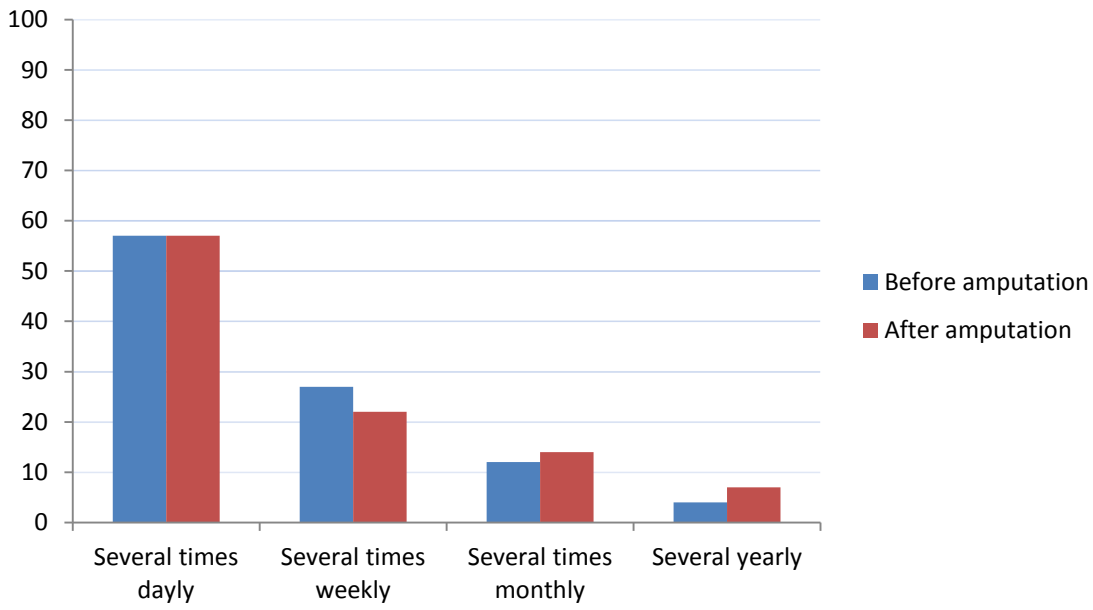

Figure 7. Frequency of pain episodes before (blue columns) and after (red columns) the amputation.

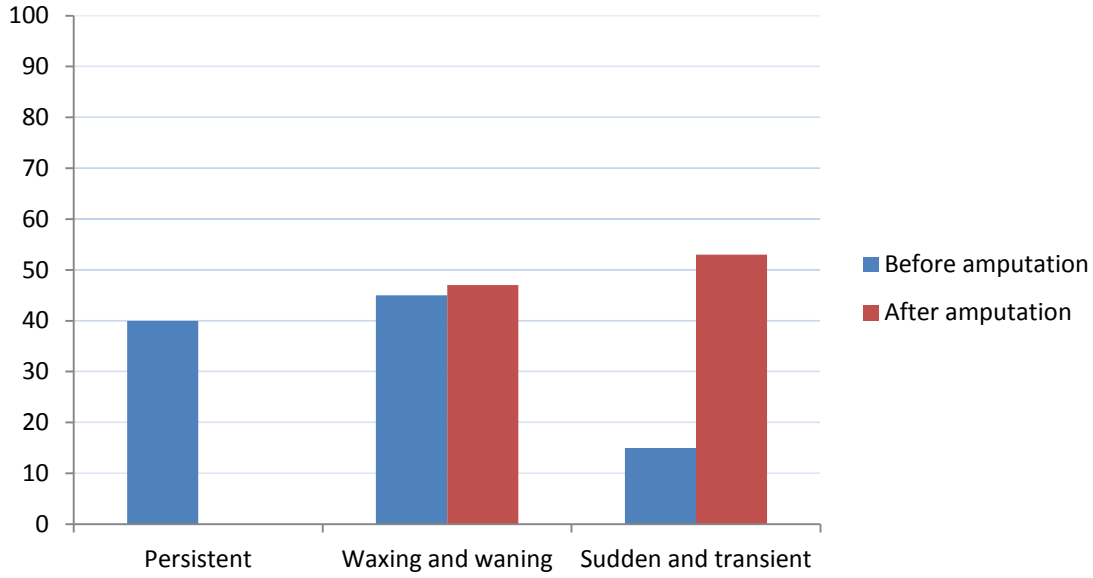

Figure 8. Type of pain perceived before (blue columns) and after (red columns) the amputation.

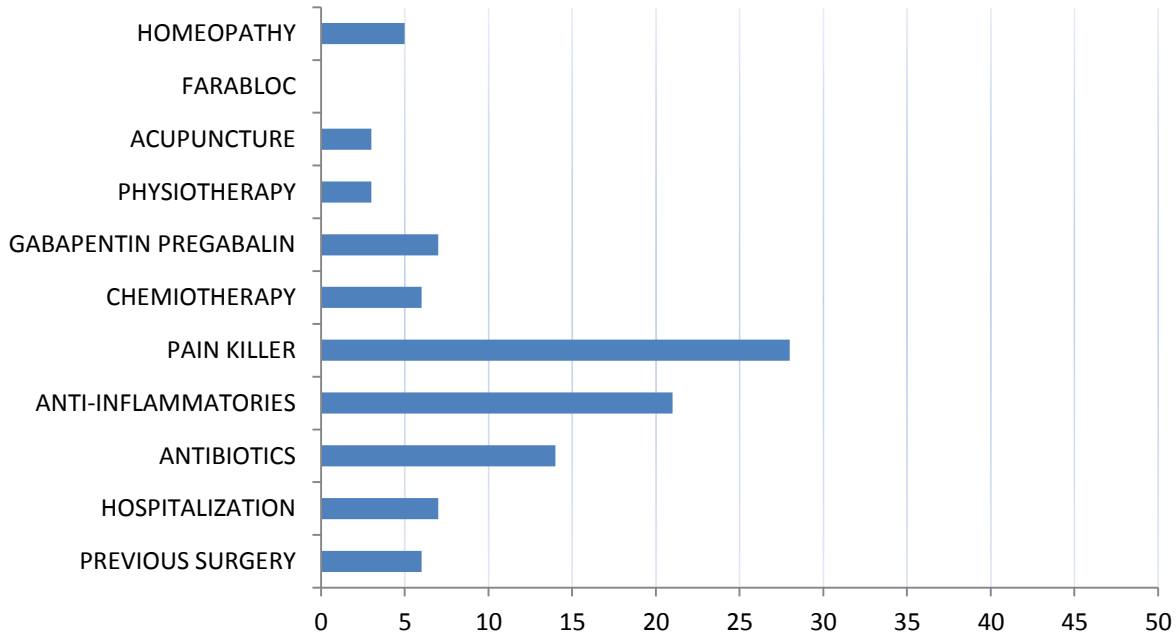

Figure 9. Therapies administered before the amputatio.

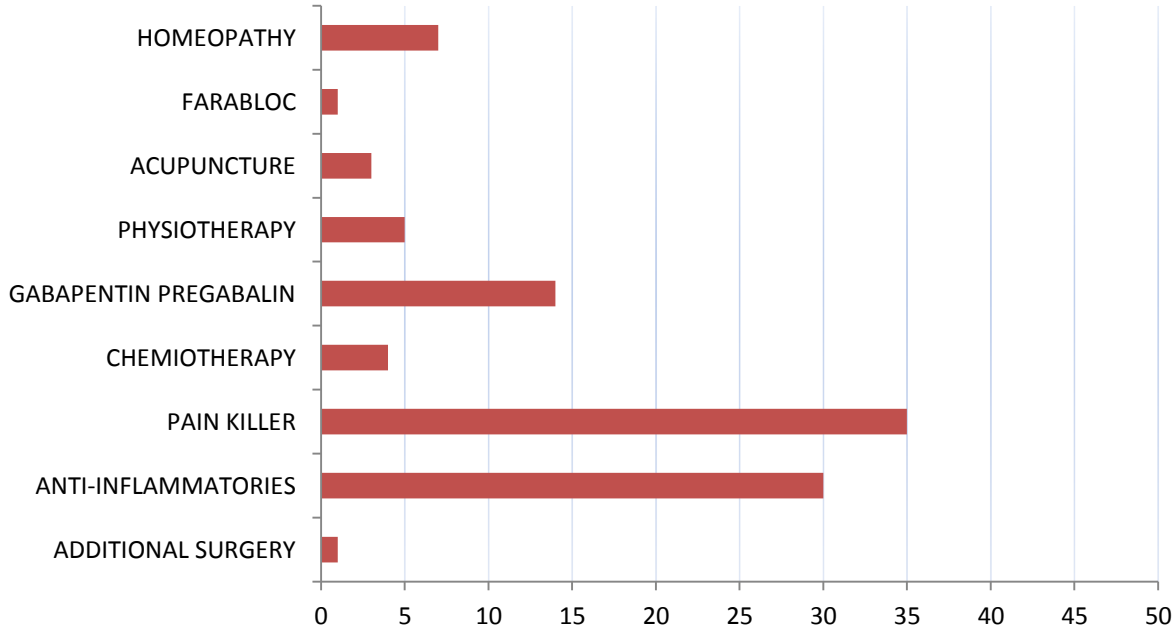

Figure 10. Therapies administered after the amputation.

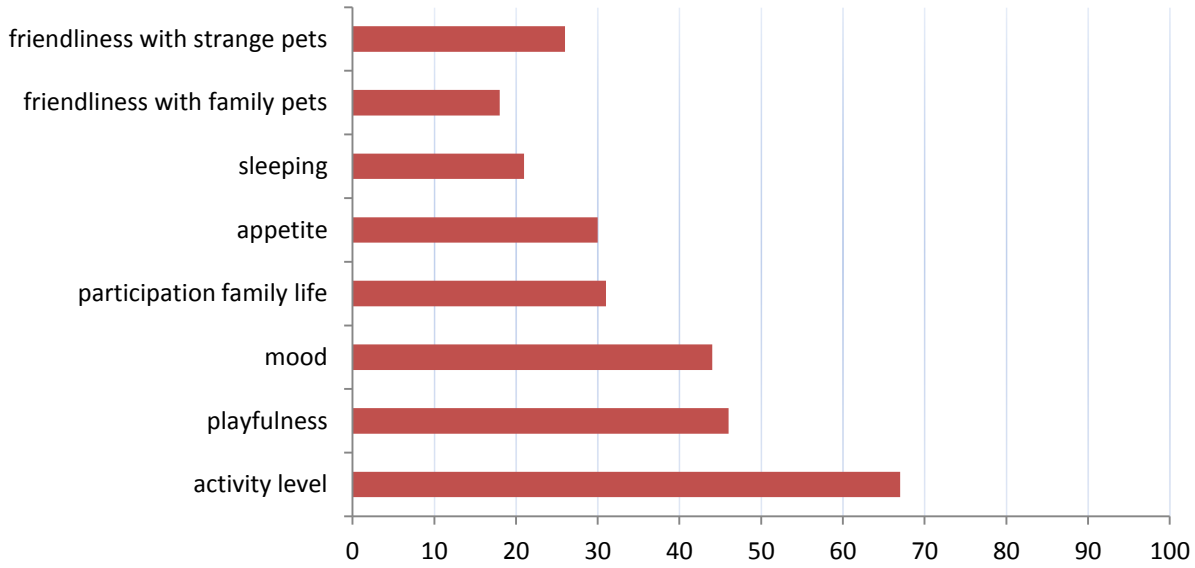

Figure 11. Activities reduced after 3 to 6 months from the amputation

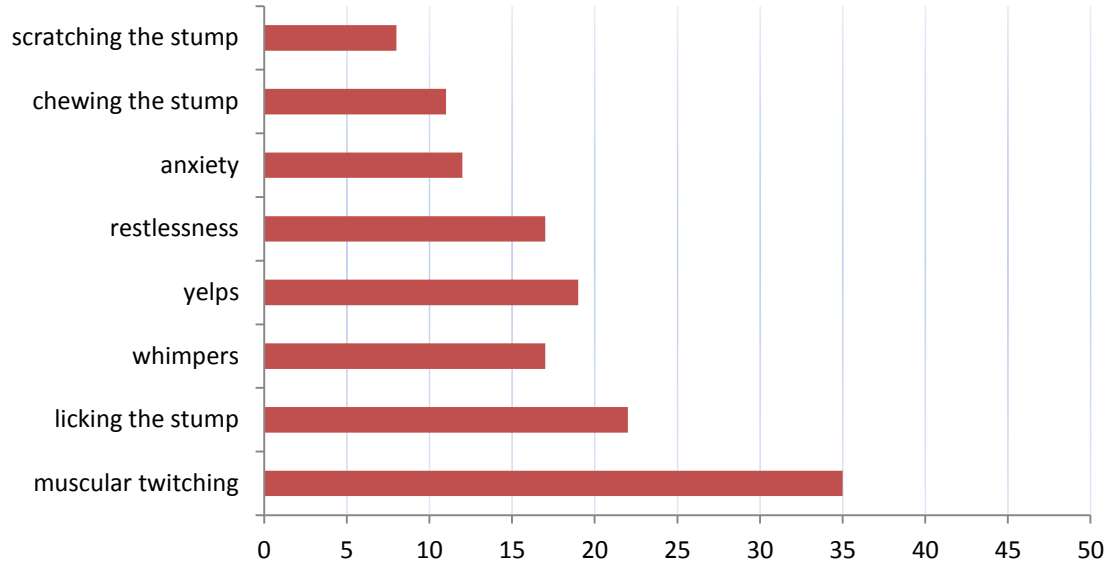

Figure 12. Manifestations related to PC after 3 months to 1 year from the amputation.

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