Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Histochemical Analysis of Herniated Disc Tissue Surgically Removed from 27 Dogs

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ARTICLE HISTORY (15-450)

Received: October 31, 2015
Revised: July 06, 2016
Accepted: July 13, 2016
Published online: March 07, 2017

Key words:
Clinical and neurological signs
Disc herniation
Dog
Histology
Scoring system

ABSTRACT

Hernias of intervertebral discs are a common canine disease that is usually treated surgically. Recently, a histological scoring system for surgically removed canine intervertebral herniated discs has been developed by scoring the lesions of both the anulus fibrosus (AF) and the nucleus pulposus (NP). Since the proportion of AF and NP in the surgical samples may vary, depending on the surgical approach, the aim of this study is to grade separately the lesions in AF and NP and to modify the previously described scoring system adding three parameters: inflammation, mineralization and neovascularization. Possible association of the modified grading system with clinical parameters were statistically assessed. Herniated disc material was collected from 27 dogs. AF was present in 10/27 cases and was classified as grade 2 in 4 cases, grade 3 in 5 cases and grade 4 in 1 case. The NP was present in all 27 cases and was classified as grade 2 in 1 case, grade 3 in 5 cases, grade 4 in 9 cases and grade 5 in 12 cases. A statistically significant association was evidenced between short pre-operative period and higher grade of both the NP and of the AF ($P<0.01$). Separating the grades of the AF and NP can be useful for a fair assessment of degeneration, and circumventing the limitation of the qualitative and quantitative variability of samples.

To Cite This Article: Beha G, Pisoni L, Bombardi C, Sarli G, Magno SD, Tolla LD, Avallone G, Cinti F, Mandrioli L, Gandini G, Foglia A and Benazzi C, 2017. Histochemical analysis of herniated disc tissue surgically removed from 27 dogs. Pak Vet J, 37(2): 150-154.

INTRODUCTION

The literature from the last two decades has focused on the incidence, clinical signs, cytological and histological features (Royal *et al.*, 2009; Fadda *et al.*, 2013), diagnosis and regenerative treatment of canine intervertebral disc herniation (IVDH) (Jeffery *et al.*, 2013), which is a common degenerative disease in dogs (Bergknut *et al.*, 2013a). Histologically, degenerative compositional changes in the nucleus pulposus (NP) and anulus fibrosus (AF) predispose to disc herniation, which has been classically divided into 2 main types: Hansen type I and Hansen type II (Hansen, 1952). Hansen type I hernia refers to explosive extrusion, in which herniation of the NP occurs through the annular fibers of the disc into the vertebral canal and it is associated with chondroid metaplasia (Brisson, 2010). Hansen type II hernia is described as annular bulging protrusion into the vertebral canal, caused by movement of the NP and it is associated with fibrous metaplasia (Chang *et al.*, 2007). In chondroid metaplasia, the NP dehydrates, notochordal cells degenerate, and the whole structure becomes dystrophically calcified (Jeffery *et al.*, 2013), while the AF lamellae are partially or completely ruptured and separated (Bergknut *et al.*, 2013b). In fibrous metaplasia, the AF is the focus of the degenerative process with fibers splitting and dorsal thickening of the AF (Jeffery *et al.*, 2013). The two types of metaplasia and their occurrence were also reported in two groups of dog breeds: chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds (Olby *et al.*, 2004; Parker *et al.*, 2009), associated with Hansen type I and II hernias, respectively (Hansen, 1952). However, recent studies suggest that the NP and AF degenerative changes seen in CD breeds are similar to those seen in NCD breeds (Jeffery *et al.*, 2013; Kranenburg *et al.*, 2013). A histological grading system for surgically removed intervertebral disc tissue has been recently developed and provided useful information about the severity of degenerative changes in the IVD but no

correlation was found between the clinical signs (Kranenburg *et al.*, 2013).

The aim of this study was to score separately the NP and the AF, in order to circumvent the limitation of the variability of surgical samples and to assess possible correlations with clinical variables.

MATERIALS AND METHODS

Signalament and clinical history: The clinical records of dogs diagnosed with a IVD extrusion or protrusion were retrospectively evaluated.

After neurological localization, the clinical diagnosis of suspected disc herniation was confirmed using advanced diagnostic imaging techniques (Magnetic Resonance Imaging [MRI], and/or Computed Tomography [CT]).

Surgical technique was performed according to the location of the disc herniation: ventral slot (cervical spine) or hemilaminectomy (cervical or thoraco-lumbar spine). For disc herniation in the thoraco-lumbar region, the herniated material present in the vertebral canal was collected after hemilaminectomy. Conversely, in case of herniation in the cervical region, the ventral part of both the AF and NP were collected during discectomy before performing the ventral slot, as well as the disc material present in the vertebral canal.

The period, in terms of days, from the occurrence of the pain to the day of surgery was also considered (length of pre-operative period) and has been grouped as follow: group 1 (≤10 days); group 2 (11 to 20 days) and group 3 (≥ 21days).

Histology: The material collected at surgery was formalin fixed (10%), routinely processed, embedded in paraffin, cut into serial sections (4-µm thick) and stained with H&E and with Gruber stain (Gruber, 2002). The Gruber stain was used to identify changes in the intercellular matrix composition with Weigert's hematoxylin, alcian blue and picrosirius red combined to produce distinctive staining of collagen (red), proteoglycans (blue) and cellular elements (black) of the intervertebral disc (Gruber, 2002).

The histological samples were graded using the validated Kranenburg *et al.,* (2013) histological grading system modified as follows: considering separately NP and AF and adding 4 parameters in the evaluation of NP. The new parameters were: the degree of mineralization, newly formed blood vessels, amount of inflammation, all scored 1 when present or 0 when absent. The differences in the intercellular matrix composition were visualized with Gruber stain, scored 0 when blue stain dominates, 1 mixture of blue and red and 2 when red dominates.

Histological grades were from 1 to 5 for the NP and 1 to 4 for the AF, based on the sum of each parameter score as reported in Table 1.

IHC: The presence of newly born blood vessels was confirmed by Von Willebrand Factor VIII immunostaining. Five-micron thick sections were dewaxed and rehydrated. Endogenous peroxidase was quenched by immersion in 1% hydrogen peroxide in phosphate buffered saline (PBS) for 30 minutes at room temperature. Sections were then rinsed in Tris buffered saline, immersed in citrate buffer (pH 6.0), incubated in a microwave oven at 750 W for two cycles of 5 min, and cooled at room temperature for approximately 20 min. Antibody specific for Von Willebrand Factor VIII (Rabbit polyclonal, Dako, Dilution 1:100), were applied overnight at 4°C. Sections were incubated for 30 minutes at room temperature with the appropriate biotin-linked secondary antibody (Dako, dilution 1:200). Reaction was amplified with the avidin-biotin method (ABC kit elite, Vector) and visualized with 3.3′-diaminobenzidine (0.04% for 4 min). The sections were counterstained with Papanicolaou's stain, rinsed in tap water, dehydrated and cover-slipped.

Statistical analysis: All the statistical analysis was performed with CSS statistics software. The Kendall's tau (τ) coefficient was used to estimate the association between type and site of herniation with the different histological parameters or clinical and neurological variables with the histological scoring. Comparisons between categorical data were conducted with the Chi-square test. Comparisons of continuous data (age) were conducted with one-way ANOVA. $P<0.05$ values were considered significant.

RESULTS

Individual data: Twenty-seven dogs were included in the study, 10 of which were CD dogs, and 17 were NCD dogs. The specific breed of each case is listed in Table 2. Ages ranged from 3.3 to 13.11 years (mean 8). Seventeen dogs were male, 7 females and 3 spayed female. Out of the 27 disc degeneration, 22 were Hansen type I (extrusion) and 5 Hansen type II (protrusion). Regarding the location and the site of the hernia data are reported in Table 2.

Histology: Of the 27 tissue samples, 10 presented with both the NP and the AF and 17 the NP only. No notochordal cells (score 2) were found in any of the NP specimens, and a variable degree of chondrocyte proliferation (chondroid metaplasia) was identified (Figure 1). In the NP, the extracellular matrix was highlighted with the Gruber stain: 4 samples had a prevalence of blue staining proteoglycans (score 0), 10 samples had a mixture of red staining collagen and blue staining proteoglycans (score 1), and 13 samples had a prevalence of red collagen fibers (score 2) (Figure 1). Fifteen cases presented mineralization (1 Hansen type II and 14 Hansen type I hernias) in 7 thoracic, 3 cervical and 5 lumbar herniations (Figure 1). Lymphoplasmacytic and macrophagic inflammation was identified in 11 cases (all Hansen type I hernias) in association with neovascularization (10 cases) (Figure 1), and chondrocytic proliferation (10 cases). NP degeneration was grade 2 in one case (3.7%), grade 3 in five cases (18.6%), grade 4 in nine cases (33.3%), and grade 5 in twelve cases (44.4%) (Table 3). Samples of AF were graded as grade 2 in four cases, grade 3 in five cases and grade 4 in one case (Table 3).

Clinical and neurological signs: Regarding the length of the pre-operative period, 12 dogs (44.4%) were inserted in group 1 (≤10 days), 7 dogs (27.9%) in group 2 (11 to 20 days) and 8 dogs (29.6%) in group 3 (≥ 21days) (Table 4). Considering the CD and NCD breeds, 6/10 CD dogs were assigned to group 1 (60.0%), 3 to group 2 (30.0%) and 1 to group 3 (10.0%). Six out of the 17 NCD dogs were included in group 1 (35.3%), four in group 2 (23.5%) and seven in group 3 (41.2%) (Table 4).

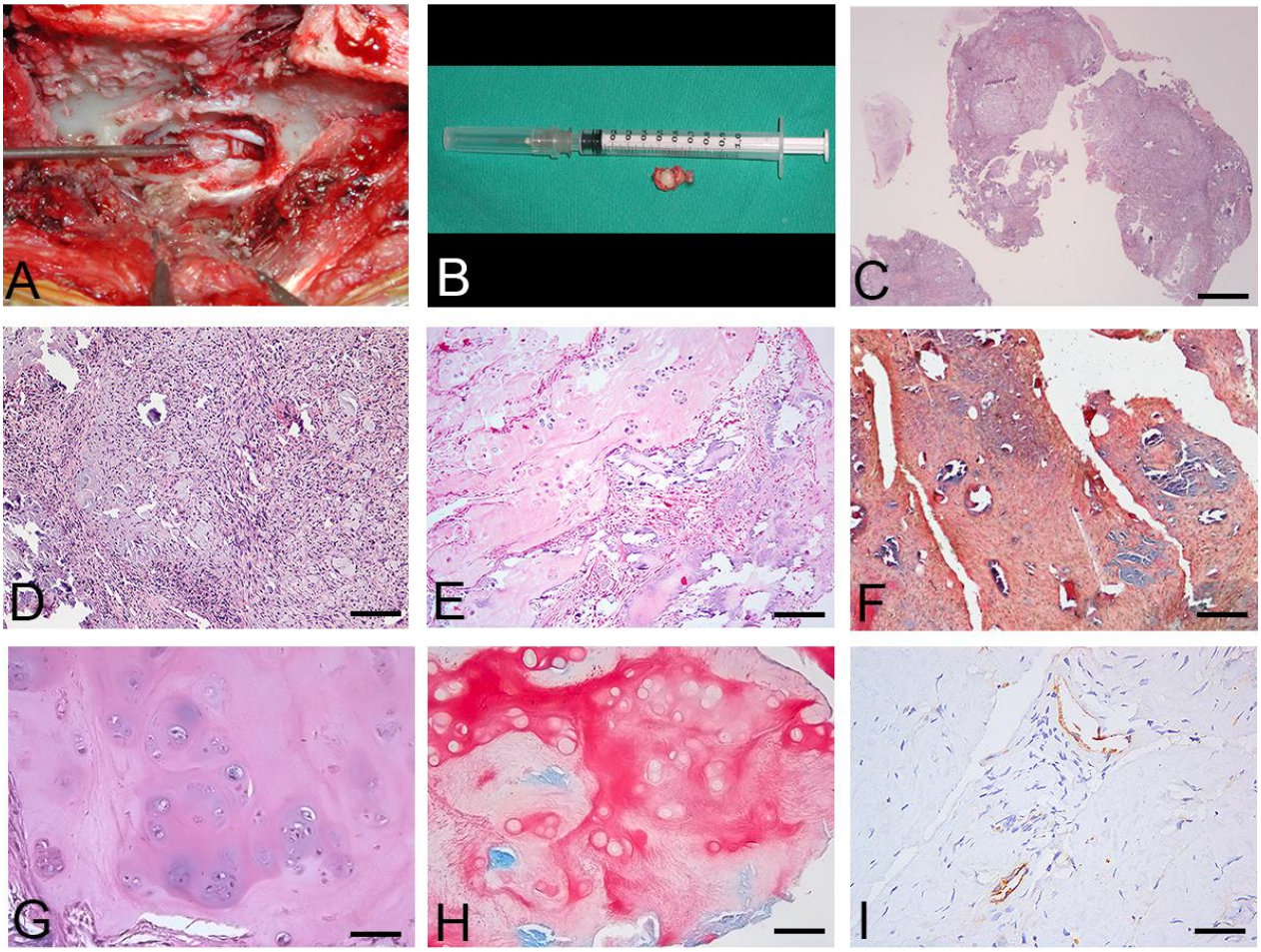

Fig. 1: Nucleus pulposus (NP) degeneration. A) Intraoperative image of a thoracolumbar hemilaminectomy, B) Surgically removed extruded disc material, C) Low magnification view of extruded disc material. The sample is composed of multiple fragments. HE. Bar=2mm, D) Degenerated NP. Scar/tissue defects, areas of mineralization and inflammation. HE. Bar=500µm, E) Degenerated NP. Mineralization areas and increased chondrocyte-like cells density with connection of two and more chondrocytes. HE. Bar=500µm, F) Degenerated NP. Prevalence of red stain indicating high amount of collagen. Gruber stain. HE, Bar=500µm, G) Degenerated NP. Chondroid metaplasia and formation of chondrocyte groups. HE. Bar=100µm, H) Degenerated NP. Chondroid metaplasia and formation of chondrocyte groups. Prevalence of red stain indicating high amount of collagen and highlighting chondrocyte groups. Gruber stain. Bar=100µm, and I) Degenerated NP. Neovascularization demonstrated by IHC brown endothelium stain. Factor VIII, IHC. Bar=100 µm.

Table 1: New additional parameters of the degenerated nucleus pulposus and histological grading system

Nucleus pulposus (NP) (points)	Histological grades	
Gruber Matrix staining	Nucleus pulposus (NP)	Anulus fibrosus (AF)
0 Blue stain dominates	grade 1 (0-1 points)	grade 1 (0-1 points)
1 Mixture of blue and red staining	grade 2 (2-4 points)	grade 2 (2-4 points)
2 Red stain dominates	grade 3 (5-7 points)	grade 3 (5-7 points)
Mineralization	grade 4 (8-10 points)	grade 4 (8-10 points)
0 absent	grade 5 (11-13 points)	
1 present		
Inflammation		
0 absent		
1 present		
New born vessels (IHC Von Willebrand Factor VIII)		
0 absent		
1 present		

Epidemiological factors: The mean age of the CD dogs and the NCD dogs was significantly different (CD dogs 5.9±1.91; NCD dogs 8.54±2.93; ANOVA $P<0.05$). An association (τ-0.36; $P<0.05$) was apparent between the type of breed (CD or NCD) and the type of hernia (Hansen type I or II): all the 10 CD dogs were Hansen type I, while in 17 NDC dogs 12 were Hansen type I and 5 type II. Significant association was found between the type of hernia and chondrocyte proliferation (τ-0.38; $P<0.05$) and the presence of inflammation (τ-0.36; $P<0.01$); the extruded herniation was associated with a greater proliferation of chondrocytes in the NP and inflammation. A short pre-operative period was associated with severe morphological abnormality (τ-0.65; $P<0.01$), severe chondroid metaplasia (τ-0.56; $P<0.05$) of the AF, and with increased proliferation of chondrocytes (τ-0.44; $P<0.01$), extracellular matrix (Gruber red stain) (τ-0.36; $P<0.01$), mineralization (τ-0.43; $P<0.01$) and presence of inflammation (τ-0.34; $P<0.01$) in the NP. A significant association between different pre-operative period and histological grade in both AF (τ-0.65; $P<0.01$) and NP (τ-0.48; $P<0.01$) was identified. A significant association was apparent between CD/NCD breeds and grade in the NP (higher grade in CD dogs) (τ-0.53; $P<0.01$), while no association in AF histological grade was found between CD and NCD dogs. No association was found between the length of pre-operative period and the CD and NCD breeds or the type of hernia. All the above-mentioned data are presented in Table 5.

Table 2: Individual data of the 27 dogs

Id	Type of hernia	Site	Breed	Age (years)	Pre-operative period
1	Type 1	C3-C4	Mixed breed (NCD)	6.5	3
2	Type 1	C4-C5	Pinscher (NCD)	8.3	3
3	Type 1	C5-C6	Mixed breed (NCD)	8.9	2
4	Type 1	C5-C6	German Shepherd (NCD)	11.2	3
5	Type 1	C6-C7	Dalmatian (NCD)	13.6	1
6	Type 1	C6-C7	Dobermann (NCD)	9.5	1
7	Type 1	T11-T12	Shih-Tzu (CD)	3.4	2
8	Type 1	T11-T12	Dachshund (CD)	4.7	1
9	Type 1	T11-T12	German Shepherd (NCD)	6.7	2
10	Type 1	T12-T13	Mixed breed (NCD)	7	1
11	Type 1	T12-T13	Mixed breed (NCD)	4.6	2
12	Type 1	T12-T13	Dachshund (CD)	5.10	1
13	Type 1	T12-T13	Dachshund (CD)	6.7	3
14	Type 1	T13-L1	Poodle (CD)	9	2
15	Type 1	T13-L1	Mixed breed (NCD)	13.11	1
16	Type 1	L2-L3	Shih-Tzu (CD)	3.3	1
17	Type 1	L2-L3	Mixed breed (NCD)	3.3	2
18	Type 1	L2-L3	Cocker Spaniel (CD)	8	2
19	Type 1	L3-L4	Maltese (NCD)	10.11	3
20	Type 1	L4-L5	Beagle (CD)	5.1	1
21	Type 1	L5-L6	Dachshund (CD)	6.2	1
22	Type 1	L6-L7	Dachshund (CD)	7.5	1
23	Type 2	C4-C5	Mixed breed (NCD)	10.6	3
24	Type 2	C5-C6	Italian Mastiff (NCD)	11.7	3
25	Type 2	C6-C7	Dobermann (NCD)	5.4	1
26	Type 2	T11-T12	German Shepherd (NCD)	8.10	3
27	Type 2	T13-L1	Italian Mastiff (NCD)	6.7	1

Type 1=Extrusion; Type 2=Protrusion; C=Cervical; T=Thoracic; L=Lumbar; pre-operative period: group 1 (≤10 days); group 2 (11 to 20 days) and group 3 (≥ 21days).

Table 3: Grading of degenerated NP and AF samples

Grades	NP		AF	
	n° of samples	%	n° of samples	%
Grade 1 (0-1 points)	0		0	
Grade 2 (2-4 points)	1	3.7	0	
Grade 3 (5-7 points)	5	18.6	4	75
Grade 4 (8-10 points)	9	33.3	1	25
Grade 5 (11-13 points)	12	44.4		

NP=Nucleus pulposus; AF=Anulus fibrosus.

Table 4: Clinical and neurological data

Pre-operative period	Total of 27 samples	%	CD samples	%	NCD s samples	%
1	12	44.4	6	60.0	6	35.3
2	7	27.9	3	30.0	4	23.5
3	8	29.6	1	10.0	7	41.2

CD= chondrodystrophic breed; NCD= non-chondrodystrophic breed.

Table 5: Significant Epidemiological factors

	Type of hernia (Hansen type I or II)	Pre-operative Period	CD/ NCD breeds
Type of breed (CD or NCD)	τ 0.36; P<0.05		
Chondrocyte proliferation of NP	τ-0.38; P<0.05	τ-0.44; P<0.01	
Inflammation OF NP	τ-0.36; P<0.01	τ-0.34; P<0.01	
Extracellular matrix in the NP		τ-0.36; P<0.01	
Mineralization of NP		τ-0.43; P<0.01	
Morphological abnormality of AF		τ-0.65; P<0.01	
Chondroid metaplasia of AF		τ-0.56; P<0.05	
Histological grade of AF		τ-0.65; P<0.01	
Histological grade of NP		τ-0.48; P<0.01	τ-0.53; P<0.01

τ=Kendall's tau coefficient; P=Probability; CD=Chondrodystrophic breed; NCD=Non-chondrodystrophic breed; NP=Nucleus pulposus; AF=Anulus fibrosus; empty boxes means that no association was found.

DISCUSSION

Disc disease, including hernias (Jeffery *et al.*, 2013), is typified by changes in the mechanical integrity and biochemical composition of the NP (Hunter *et al.*, 2004). IVDD has been classified into 2 main types: Hansen type I IVDD, most common in CD breeds (Brisson 2010), and Hansen type II IVDD, prevalent in NCD breeds (Royal *et al.*, 2009). In CD breeds, the disease typically occurs in young dogs, mostly in the cervical or thoracolumbar spine (Brisson, 2010) and is more frequent than in NCD breeds (Bray and Burbidge 1998). In contrast, in NCD breeds, IVDD develops later and primarily affects the caudal cervical or lumbosacral spine, although the thoracolumbar spine can also be affected (Rossi *et al.*, 2004; Brisson, 2010; Meij and Bergknut, 2010). In the present study, all the Hansen type II hernias were NCD and there was a significant difference in the age of the affected dogs between CD and NCD breeds, with a lower mean age in CD dogs, consistently in agreement with the literature.

The intervertebral discs (IVD) can degenerate and consequently herniate anywhere along the column but most commonly extrude between vertebral levels T12-L2 (Seiler *et al*., 2002; Penning *et al.*, 2006). The most affected hernia site in the present study was T11-L1, probably due to the absence of the intercapital ligaments in this spine segment, which would prevent dorsal and dorsolateral displacement of the IVD (Penning *et al.*, 2006; Smolders *et al.*, 2013).

The histopathological features of AF degeneration identified in our caseload were the disorganization of the lamellar fibers and the ingrowth of chondrocyte-like cells as previously described (Bergknut *et al.*, 2013a), and were identified in both types of hernias and both CD and NCD.

In the present study, the NP was altered by different degrees of chondrocyte proliferation replacing the notochordal cells. Absence of notochordal cells in all samples may be caused by their differentiation into chondrocyte-like cells and/or to a migration to the transitional zone as previously suggested (Bray and Burbidge 1998). The disc degeneration is associated with loss of water from the NP, secondarily to a lowering of the proteoglycan concentration (Bray and Burbidge 1998). Normal aging, trauma, and abnormal biomechanical stresses affecting the NP are associated with a decrease in the amount of proteoglycan and a shift to collagen II from collagen I (Bergknut *et al.*, 2013b). The degeneration of the extracellular matrix of the NP was evidenced by the Gruber stain, that helped in assessing the proportion of normal proteoglycan-rich matrix (blue) and abnormal collagen-rich matrix (red) components in the same histology section. Our results confirmed that the Gruber stain revealed details that would not be apparent with routine H&E stain.

Inflammation was evidenced in 11 cases and was associated with neovascularization and chondrocytic proliferation. The inflammatory response has been hypothesized to be caused by antigenic components of the NP that, once herniated into the vertebral canal, stimulate the response of the immune system (Arai *et al.*, 2000; Fadda *et al.*, 2013). Lymphocytes, fewer plasma cells and macrophages is the most common chronic inflammatory infiltrate evidenced in this study, in agreement with a previous report (Ito *et al.*, 1996; Fadda *et al.*, 2013). Furthermore, it has been hypothesized that the proliferation of small vessels is a consequence of the inflammatory reaction (Ito *et al.*, 1996). Interestingly, it has been suggested that neovascularization present at the

edge of fragments of the NP was the most specific histologic indicator of extrusion (Doita *et al*., 1996; Moore *et al.,* 1996), since, in healthy IVD, the outer layers of the AF have a limited blood supply, and there is no direct blood supply to the inner layers of the AF or in the NP (Bergknut *et al.,* 2013b). The mineralization of the herniated disc material was thought to be dystrophic in nature secondary to tissue necrosis (Smolders *et al.,* 2013); it mainly occurred at the periphery of the NP and is rarely observed in the AF. In accordance with a previous study (Smolders *et al.,* 2013), mineralization was identified more frequently in CD dogs in the thoracic spine T11-T13, probably due to a higher chance of herniation at this level. Presence of mineralization in all Dachshunds was not surprising since they have a locus on chromosome 12, which harbors genetic variations associated with IVDD mineralization (Mogensen *et al.,* 2011). The latter was also associated with presence of chronic inflammation and new born vessels (in nine out of 16 samples). This association can be explained considering that mineralization, as part of disc degeneration, can lead to IVD herniation and consequent neovascularization and inflammation.

The statistical analysis revealed an association between type I hernias and severity of chondroid metaplasia and inflammation. This finding parallels previous studies that indicate chondroid metaplasia and inflammation as typical of this type of hernia (Bergknut *et al.,* 2013a), (Arai *et al.,* 2000; Kranenburg *et al*., 2013). High scores of a single histological parameter as well as in the grade of AF and NP degeneration were associated with a short pre-operative period, indicating increased severity of degeneration and a more acute clinical onset. A shorter pre-operative period and the frequent presence of inflammation, mineralization, neovascularization, and NP chondrocyte proliferation, were seen primarily in CD dogs, data that seems to be in accordance with previous reports (Smolders *et al.,* 2013). During decompressive surgery in the thoraco-lumbar spine a hemilaminectomy has been usually chosen and the NP was frequently found extruded into the vertebral canal. A disc fenestration is not routinely performed in our institution, so the presence of the AF is inconstant in our samples. In the presence of a ventral slot, discectomy is performed and the AF is easily collected. As a consequence, in most cases the NP was collected, while only in some cases it was possible to collect AF. The variable composition of the examined samples can be related, as mentioned before, to the type of surgical approach performed (Kranenburg *et al.,* 2013).

Conclusions: The separation of the AF from the NP grades and the addition to the latter of three parameters (inflammation, mineralization and neovascularization) allowed us to highlight the differential involvement of AF and NP. Considered as two separate grades, the AF and NP can be useful for a fair assessment of degeneration as the major limitation in the evaluation of surgical biopsies is the great variability of quantity and type of material collected. The benefit in using two different histological grading systems, with the suggested modifications, was supported by the evidence of a statistical association between clinical data and the two separate grades as well as single histological variable.

Authors contribution: These authors contributed equally to this work. All authors read and approved the final manuscript.

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