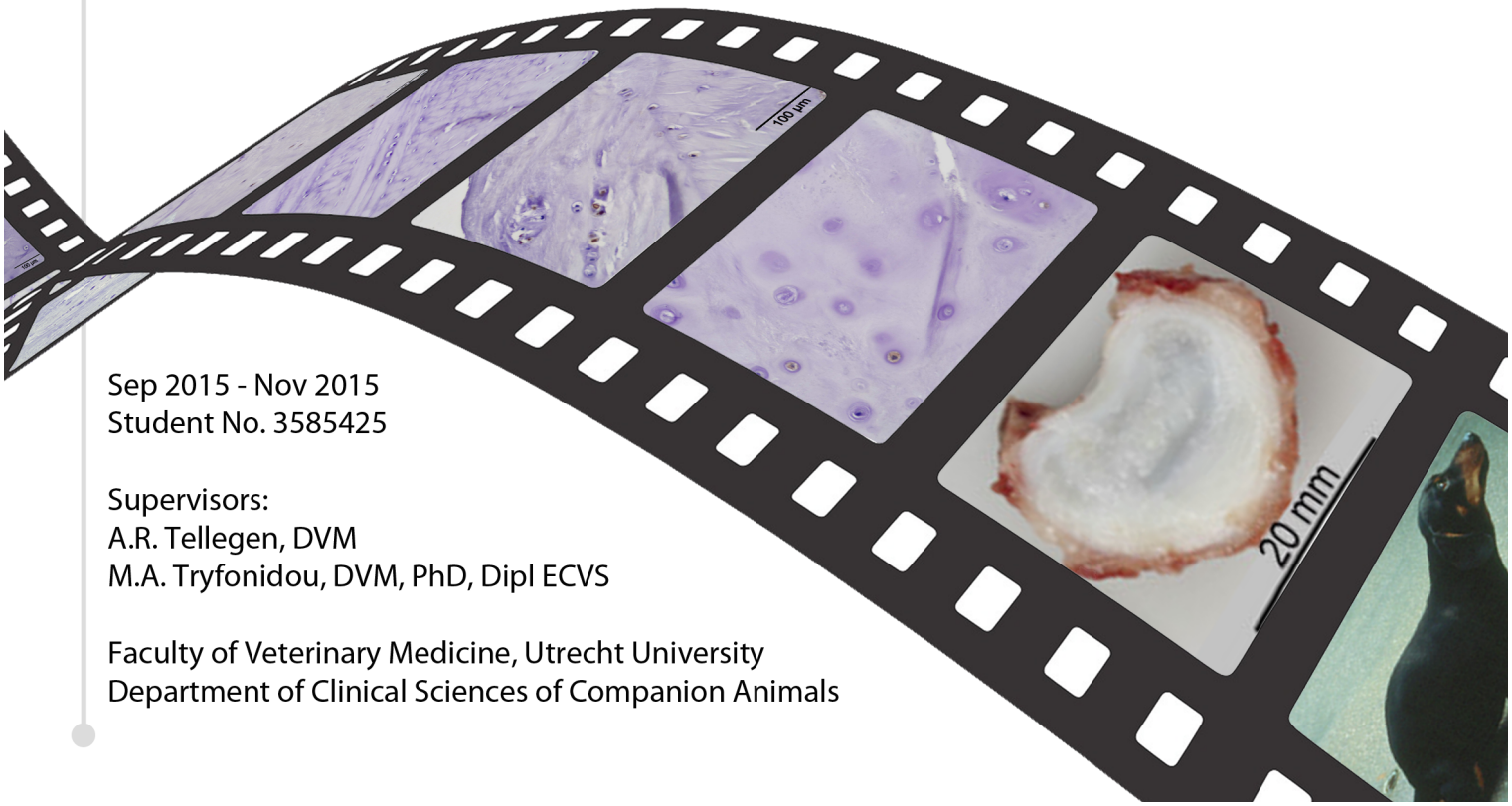
COX-2 expression in canine degenerated intervertebral discs

Anouk Verbeek



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Contents

Abstract

Back pain is a common symptom within the canine population. One of the major causes of back pain is intervertebral disc (IVD) degeneration. Prostaglandins (e.g. Prostaglandin E_2 (PGE_2)) play an important role in the process of IVD degeneration as they have an overall negative effect on the disc matrix homeostasis and induce centrally generated pain. It is known that cyclooxygenase-2 (COX-2) is a rate-limiting step in the synthesis of PGE_2. COX-2 inhibition could be a good therapeutic strategy to reduce pain and inhibit the process of degeneration. Besides this, it might also facilitate regeneration of the IVD.

The aim of this research was to investigate whether the COX-2 expression in IVDs of dogs with clinical signs of degeneration are correlated with higher grades of disc degeneration and clinical neurological score. The expression was investigated in canine surgical samples using immunohistochemical techniques. A distinction was made for expression in the nucleus pulposus (NP) and in the annulus fibrosus (AF). The degree of disc degeneration was previously determined by use of the Pfirrmann-score (based on magnetic resonance imaging (MRI) findings) and a histological score (Boos-score). The canine clinical neurological score was given by a doctor of veterinary medicine (DVM).

Dogs with clinical signs of IVD degeneration showed higher levels of COX-2 expression in the NP with increasing levels of degeneration, according to the Pfirrmann-score. This is in line with former research that was performed on IVDs of dogs without clinical signs of degeneration. No significant difference was found for Boos-score and the clinical neurological-score on the expression of COX-2 in the NP in the current study. COX-2 was significant more expressed in the NP than in the AF, which could indicate that the production of inflammatory mediators is more pronounced at NP level than at AF level.

There was no significant difference between COX-2 expression in the AF and degeneration grade, according to Boos-score and Pfirrmann-score. Dogs with symptoms such as paraparesis/tetraparesis (but still ambulatory) expressed significant more COX-2 in the AF than dogs with only pain symptoms.

With this knowledge, intradiscal application with a COX-2 inhibitor should have the most effect in dogs with later stages of degeneration, according to Pfirrmann-score. To obtain regenerative effects, treatment of grade III discs is the most ideal score, since no structural changes have developed yet. COX-2 inhibition will provide effective analgesia for discogenic pain in all patients with increasing levels of degeneration.

Introduction

A dog has 7 cervical, 13 thoracic, 7 lumbar, 3 sacral and a variable amount of caudal vertebrae (figure 1) (Dyce *et al.,* 2010). All the vertebral bodies, except the first two vertebrae, are connected by an intervertebral disc (IVD). The main biomechanical function of the IVD is contribution to the flexibility of the spine and the distribution of pressure over the vertebrae.

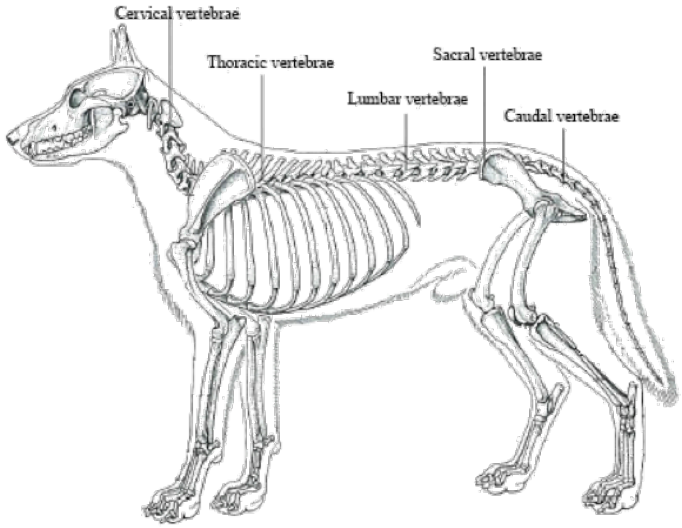

Figure 1. Skeleton of the dog (König and Liebich, 2004).

Anatomy of the intervertebral disc

In figure 2 the position of the IVD in the vertebral column is shown. Each IVD consists of four distinct components (figure 3a). The healthy nucleus pulposus (NP) is a mucoid, translucent, bean-shaped structure and is located in the centre of the IVD (Bergknut *et al.*, 2013b). It is mainly composed of notochordal cells, which produce a matrix rich in proteoglycans and collagen type 2 (Freemont *et al.*, 2002). The proteoglycan molecules are made of a protein backbone with negatively charged glycosaminoglycan side chains. These side chains attract water into the NP; as a result the healthy NP consists of 80% water (Bergknut *et al.*, 2013b).

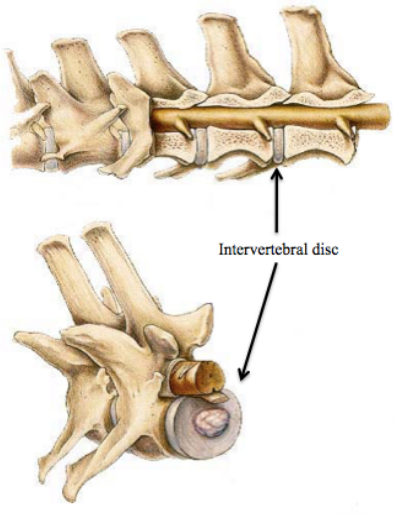

Figure 2. Position of the IVD in the vertebral column.[1]

The NP is surrounded by the transition zone (TZ), also known as the inner annulus fibrosus (AF). The structure of the IVD changes at this point from fibrous to a more cartilaginous/mucoid structure. The TZ forms the connection between the NP and the AF. The main cells in the TZ are

chondrocyte-like cells and fibrocytes embedded in a loose fibrous matrix network, predominantly collagen type 2 (Bergknut *et al.*, 2013b).

The AF is a dense network of multiple, organized, concentric fibrous lamellae. The lamellae are composed of collagen fibrils, mostly collagen type 1 (Freemont *et al.*, 2002). They are aggregated with elastic fibres and are coated by proteoglycans. The AF cell population consist mainly of fibrocyte-like cells (Bergknut *et al.*, 2013b).

The fourth component of the IVD is the cartilaginous endplates (EP), which form the cranial and caudal border of the IVD and attach the IVD to the adjacent vertebrae. The fibers of the TZ are strongly connected with the EPs (Freemont *et al.*, 2002). The EPs play a critical role in supplying nutrients to the IVD. The EPs consist of cranial-caudally oriented layers, on average five cell layers, of matrix and chondrocyte-like cells. The matrix is highly hydrated and is composed of proteoglycans interconnected with hyaluronic acid and link proteins, as well as collagen (mainly type 2) (Bergknut *et al.*, 2013b). The composition of the four components; NP, TZ, AF and EP is essential for maintaining the biomechanical function of the IVD.

Pathophysiology and pathogenesis of intervertebral disc degeneration

Back pain is a common clinical entity within the canine population. One of the causes of back pain is IVD degeneration. IVD degeneration may lead to cervical and thoracolumbar disc herniations, degenerative lumbosacral stenosis and cervical spondylomyelopathy. The most important clinical symptoms are pain and a variety of neurological signs (paraparesis/tetraparesis, paraplegia/tetraplegia, loss of deep pain perception) (Bergknut *et al.*, 2013b). IVD degeneration is also a common incidental finding in dogs without clinical signs of disease. Different causative factors for IVD degeneration have been described, such as physiological ageing, trauma, genetic origin, inadequate nutrition and loading history (Bergknut *et al.,* 2013a).

Degeneration of the IVD is a complex, multifactorial process that is characterized by changes in the composition of the cells as well as the extracellular matrix of the NP, the AF, the TZ and the EP. Figure 2 shows the different zones of the IVD in both a healthy disc (A) as well as in a degenerative disc (B).

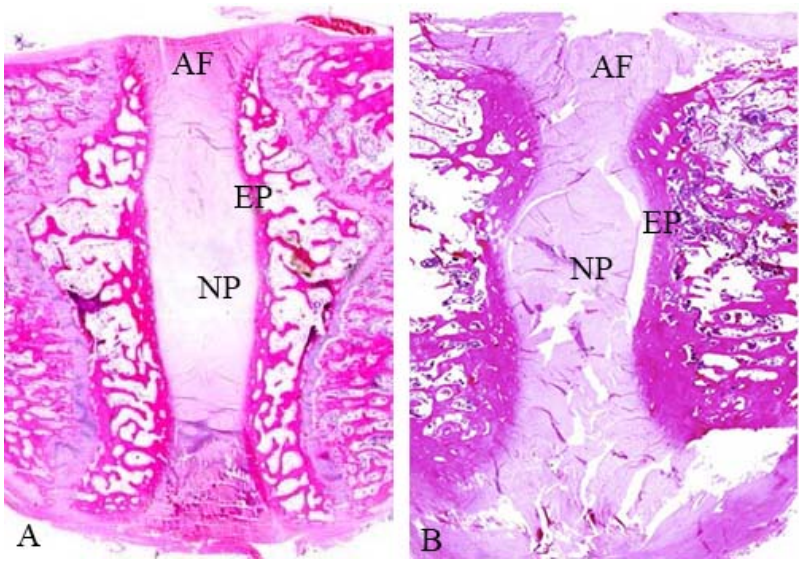

Figure 3. (A) Mid-sagittal histological section (H&E) of a healthy canine IVD. The different sections of the IVD are shown: annulus fibrosus (AF), nucleus pulposus (NP) and the cartilaginous endplates (EP). (B) Mid-sagittal histological section of a canine degenerated IVD with the different sections marked (Bergknut et al., 2013b).

In the process of IVD degeneration, there is a decrease in the synthesis of the normal IVD matrix and an increased production of degradative enzymes (Le Maitre *et al.*, 2007). Because of these alterations, the NP changes from an extracellular matrix rich in proteoglycans and collagen type 2, to a tissue containing mainly collagen type 1. As a consequence, the water-binding capacity of the NP is reduced. In the AF there is a loss of lamellar organization (Seguin *et al.*, 2005; Roughly, 2004). As a result of these changes, the matrix of the IVD becomes more rigid and loses its hydrostatic properties to function as a hydraulic cushion. The IVD matrix is not able to accomplish its biomechanical function. Structural failure of the matrix results in a changed environment of the IVD cells within the matrix. Another consequence of the changes of the IVD matrix is that the diffusion of nutrients and the fluid flow in and out of the disc become constrained. This worsens the health of the IVD cells and the synthesis of the matrix. The weakened IVD is more sensitive to lesions (Bergknut *et al.*, 2013b). Consequently, a vicious cycle of continued damage and inadequate repair and regeneration is triggered, resulting in degeneration rather than healing (Bergknut *et al.*, 2013b).

Types of herniation

There is a difference in the types of herniation that can occur, due to degeneration, between different groups of dog breeds. Dog breeds can be classified into two groups on the basis of predisposition to chondrodystrophy: chondrodystrophic (CD) and non-chondrodystrophic (NCD). CD dogs are characterized by their short limbs and are predisposed to explosive extrusion of the NP, also known as Hansen type I herniation (figure 4). They mostly develop IVD degeneration in the craniocervical- or thoracolumbar region. Clinical signs in CD dogs occur mainly between 3 and 7 years of age. In contrast, NCD dogs are predisposed to protrusion of the AF (Hansen type II) (figure 4). They often generate protruding discs in the lumbosacral and caudal cervical region within the age of 6 to 8 years old (Smolders *et al.,* 2012). An explanation for the difference found between the two groups could be that in NCD dogs the notochordal cell remains the predominant cell type in the NP throughout life, while in CD dogs these cells are replaced at three months of age by chondrocyte-like cells embedded in a large amount of dense extra-cellular matrix (Smolders *et al.,* 2012).

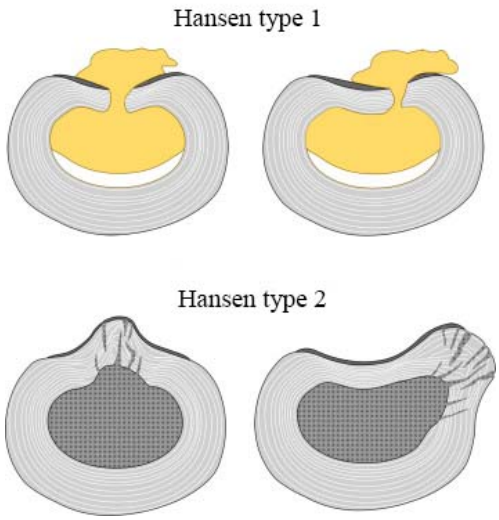

Figure 4. Schematic picture of the two types of herniation: Hanson type 1 and Hanson type 2. CD dogs most commonly have a type 1 of herniation in contrast to NCD dogs who mostly have a type 2 herniation (Smolders et al., 2013).

During the last decades, several research groups have focussed on IVD disc degeneration. Research is still continuing with the aim to enhance the understanding of the process of IVD degeneration, to improve the imaging techniques and search for new therapeutic options for clinical patients (Brisson, 2010). Effective treatment of IVD degeneration, besides surgery and conservative treatment, is an unfulfilled medical need that requires strategies for regeneration of the disc. In the early stages of degeneration minimal invasive repair strategies could help IVD regeneration.

COX-2

A number of inflammatory mediators play a role in the catabolic processes of IVD degeneration. Discs, which are in a degenerative process, produce inflammatory agents, such as TNF-α and IL-1 (Hamamoto *et al*., 2012; Le Maitre *et al.*, 2007). These pro-inflammatory cytokines have multiple actions that may contribute to disc degeneration. For instance, IL-1 induces an imbalance between catabolic and anabolic events, with a shift to catabolism, a change which is seen during disc degeneration (Le Maitre *et al.,* 2006). It appears that both cytokines also induce cyclooxygenase-2 (COX-2) (Vo *et al.,* 2010; Samad *et al*., 2001).

COX-2 is the key rate-limiting enzyme for prostaglandin biosynthesis via the arachidonate cascade (figure 5) (Miyamoto *et al.*, 2002). Prostaglandin E_2 (PGE_2) is the most common prostanoid. It has an important role in physiological and in pathological conditions. COX-1 is another isoform of the COX-2, but has a different physiological function. COX-1 is constitutively expressed in almost all cell types, and is more important for PGE_2 production for homeostatic function (Miyamoto *et al.*, 2002; Samad *et al.,* 2001). On the contrary, COX-2 is highly restricted under physiological processes and in terms of inflammation there is a quick upregulation whereafter PGE_2 is highly expressed (Miyamoto *et al.*, 2002; Samad *et al.,* 2001).

COX-2 converts arachidonic acid into prostaglandin H_2 (PGH_2) and PGH_2 is isomerized to PGE_2 by prostaglandin E synthases (Park *et al.,* 2006; Willems *et al.*, 2015a).

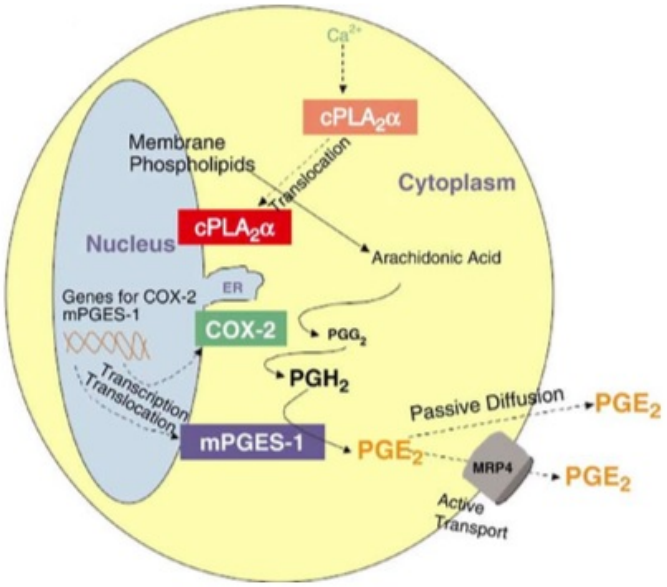

Figure 5. Arachidonate cascade (Park et al., 2006).

Cyclic mechanical stress, a predisposing factor that can disrupt the disc structure, is another factor that directly increases COX-2 and PGE_2 synthesis and so initiates the degenerative cascade (Miyamoto *et al.*, 2006; Dijk *et al.,* 2015). Vo *et al.* found an overall negative effect of prostaglandins (PGE_2 and $PGF_{2\alpha}$) on disc matrix homeostasis: PGE_2 reduces the disc cell proteoglycan synthesis; both PGE_2 and $PGF_{2\alpha}$ decrease mRNAs for anabolic factors; and $PGF_{2\alpha}$ increases mRNA levels for catabolic factors (Vo *et al.,* 2010).

Besides the negative effect of PGE_2 on the disc matrix homeostasis, it also is a major factor in the genesis of a painful IVD (Burke *et al.*, 2001; Kawabata, 2011; Samad *et al.,* 2001). It is associated with sensitization of proprioceptive neurons, can produce localised pain hypersensitivity and plays part in the irritation of nerve roots (O'Donnell and O'Donnel, 1996; Muramoto *et al.*, 1996; Samad *et al.,* 2001). Selective antagonists of the PGE_2 receptors may be useful as analgesics. The amounts of PGE_2 appear to be dependent of the presence of macrophages. Degenerated discs produce significantly higher amounts of PGE_2 in presence of macrophages (Hamamoto *et al.*, 2012).

Selective inhibition of COX-2 could decrease inflammatory prostaglandin synthesis and thereby inhibit the process of degeneration and reduce centrally generated inflammatory pain (Birbara *et al.,* 2003). Oral COX-2 inhibiters are already used in human clinical setting. However administering COX-2 inhibitors directly into the IVD has been suggested as an alternative route (Birbara *et al.,*

2003; Willems *et al.*, 2015b). This will enhance the local efficacy and will minimize the systemic side effects such as cardiotoxicity. These side effects retain widespread use of oral COX-2 inhibitors. Willems *et al.* showed inhibition of PGE_2 production in experimental CD dogs by intradiscal application of a controlled released hydrogel loaded with a selective COX-2 inhibitor (Willems *et al.*, 2015a). Since the dog shows similar pathophysiological aspects in IVD degeneration as humans, these therapeutic strategies are also of interest for human medicine (Bergknut *et al.,* 2012). The amount of COX-2 expression in different stages of disc degeneration would help to maximize the clinical effect of this therapy. Knowledge about this expression patterns in human IVD degeneration has substantially increased over the last years (Kang *et al.,* 1996; Miyamoto *et al*., 2002; Vo *et al.,* 2010), whereas in dogs still little is known. COX-2 expression is significant higher in degenerated IVDs compared to non-degenerated IVDs (Miyamoto *et al.,* 2002; Willems *et al.,* 2015b). In a research population of dogs without clinical signs, higher levels of COX-2 expression were found with increasing levels of degeneration, according to Pfirrmann-score (Willems *et al*., 2015b). To our knowledge no clinical data are available about the level of COX-2 expression in dogs with clinical signs of IVD degeneration. If the COX-2 expression is already high before the irreversible stage of IVD degeneration is reached, COX-2 inhibitors could decrease the inflammatory process and make regeneration perhaps possible (Willems *et al.,* 2015a).

CCL5

As discussed in the general introduction, there are numerous cytokines and degradative enzymes that contribute to the inflammation associated with disc degeneration. Chemokines, small chemoattractant cytokines, are involved as well. They act as important secondary inflammatory mediators, which are released by cells in response to a variety of stimuli (Gruber *et al.*, 2014). A chemokine called "*R*egulated upon *A*ctivation, *N*ormal *T*-cell *E*xpressed, and *S*ecreted/ C-C motif ligand 5" (RANTES/CCL5), and its receptor have clinical significance in the inflammation cascade of different diseases: rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, central nerve system infections, osteonecrosis and asthma (Kepler *et al.*, 2013). Recently Kepler *et al.* have shown an elevation of CCL5 in degenerated discs, which was found to increase with advancing grades of disc degeneration (Kepler *et al.*, 2013; Gruber *et al.,* 2014). Gruber *et al.* also found a significantly upregulation of CCL5 in more degenerated discs compared to less degenerated discs (Gruber *et al.*, 2014). Furthermore, a significant positive correlation has been found in the relationship between IL-1β and CCL5 within human annulus fibrosus tissue from painful discs (Kepler *et al*., 2013). These findings suggest that production of inflammatory cytokines by the tissue may correspond to an increase in chemokine production, in particular CCL5 (Pattappa *et al*., 2014). In vitro studies of production of CCL5 following exposure to TNF-α resulted in elevated levels of CCL5 in conditioned media (Gruber *et al.*, 2014).

Although mediators like CCL5 may lead to further disc damage, they may also stimulate endogenous repair mechanisms, including recruitment of mesenchymal stem cells (MSCs) (Illien-Jünger *et al.*, 2012). Cells migrate to the site of injury due to the presence of chemoattractant signals, such as CCL5. Chemokine immunoprecipitation showed that mesenchymal MSCs had a significantly reduced chemotactic migration towards CCL5-immunoprecipitated and CCL5/CXCL6 co-immunoprecipitated media (Pattappa *et al*., 2014). The positive correlation between IL-1β and CCL5 within human annulus fibrosus tissue from painful discs suggests that the production of inflammatory cytokines by the tissue may correspond to an increase in chemokine production, such as CCL5 (Kepler *et al.*, 2013; Patttappa *et al*., 2014). Little is known about the CCL5 expression in canine IVD degeneration.
The production of inflammatory cytokines may enable chemokine receptors on MSCs to be more sensitive to chemokine produced by the tissue and therefore induce greater cell migration, especially under degenerative conditions (Pattappa *et al.,* 2014). Summarizing, CCL5 may be a key chemoattractant that is produced and released by the degenerated IVD cells. Therefore, these factors could be used in regenerative therapies to improve MSC mobilisation in disc degeneration (Pattappa *et al.*, 2014).

Aim & hypotheses

The aim of this study was to compare the level of COX-2 and CCL5 expression in dogs with different stages of IVD degeneration and to compare the expression in different clinical severities. Former research of COX-2 expression was done on dogs without clinical signs of IVD degeneration, this study will focus on dogs with clinical signs. As far as we know, no previous research was done on the CCL5 expression in dogs. The degree of disc degeneration was previously determined by a MRI score (Pfirrmann-score) and a histological score (Bergknut-score). The clinical neurological score was given by a doctor of veterinary medicine (DVM). The expression was investigated by immunohistochemical techniques. Since there was no protocol for immunohistochemistry of CCL5, such a protocol needed to be developed.

H_0= There is no significant correlation between degenerative grade and the level of COX-2 and CCL5 respectively.
H_1= There is a significant correlation between degenerative grade and the level of COX-2 and CCL5 respectively.

H_0=There is no significant correlation between neurological score and the level of COX-2 and CCL5 respectively.
H_1= There is a significant correlation between neurological score and the level of COX-2 and CCL5 respectively.

Material & Methods

Surgical IVD samples (NP and/ or AF) were collected from 44 dogs that were suffering from disc degeneration. Before surgery, all dogs underwent clinical examination and a magnetic resonance imaging (MRI) –scan, whereby images were collected of the degenerated area of the vertebral column. From all dogs the IVD was surgically removed at the University Clinic for Companion Animals in Utrecht. All samples were sent in for histopathological examination. The 44 canine IVDs were fixed with formalin, embedded in paraffin, and cut into 5 µm sections.

COX-2 immunohistochemistry

Tissue sections were subjected to deparaffinization and subsequently rehydrated through a series of steps: two rounds of xylene and four rounds of alcohol, with concentrations of respectively 96%, 80%, 70% and 60%. Each step was performed for 5 minutes. After the deparaffinization and rehydration, the nonspecific endogenous peroxidase activity was blocked using Dual Endogenous Enzyme Block (Dako S2003) for 10 minutes at room temperature. Next, two washing steps were conducted, 5 minutes each in Tris- buffered Saline with 1% Tween 20 (TBS-T). The following step in the protocol was to treat the samples with Tris-buffered saline (TBS) bovine serum albumin (BSA) 5% solution (5% TBS-BSA) to reduce antibody cross-reactivity, which prevents false positive staining. The samples were incubated with 5% TBS-BSA for one hour at room temperature. After this blocking, the primary antibody (primary mouse anti-canine monoclonal COX-2 antibody) was added in a solution of 1:50 in 5% TBS-BSA. For the negative control staining, the primary antibody was replaced by a negative-control mouse antibody (Dako X0903) in the same concentration as the COX-2 primary antibody dilution. The samples were incubated overnight at a temperature of 4° Celsius.

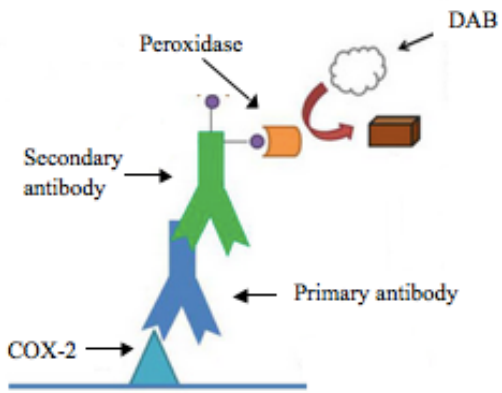

Figure 6. COX-2 Immunohistochemistry principle.

The following day the primary antibody was washed off by TBS-T (two times 5 minutes), whereafter the secondary antibody (Envision anti-mouse K4001, Dako), a peroxidase-labeled polymer, was introduced. The samples were incubated for 30 minutes at room temperature with this antibody.

Subsequently the samples were washed by Tris Buffered Saline (TBS), two times 5 minutes, and the 3.3'-diaminobenzidine substrate (DAB) was added to the samples to visualize the antibody binding. The samples were incubated for 5 minutes with DAB, and were rinsed with milliQ. A visualization of the total reaction is shown in figure 6. The next step in the protocol was to counterstain the background by use of haematoxylin. The haematoxylin was applied for 30 seconds whereafter the slides where washed by running-tap water for 15 minutes. For the last step, the samples had to be dehydrated for 5 minutes for each alcohol concentration, of respectively 60%, 70%, 80%. Next, there were two rounds of 5 minutes alcohol 96%, followed by one round of 5 minutes in 100% alcohol and finally two rounds of 5 minutes in xylene. The final action was to put on a coverslip by use of vectamount, in order to protect the tissue. Figure 7 shows a summarization of the total immunohistochemistry protocol.

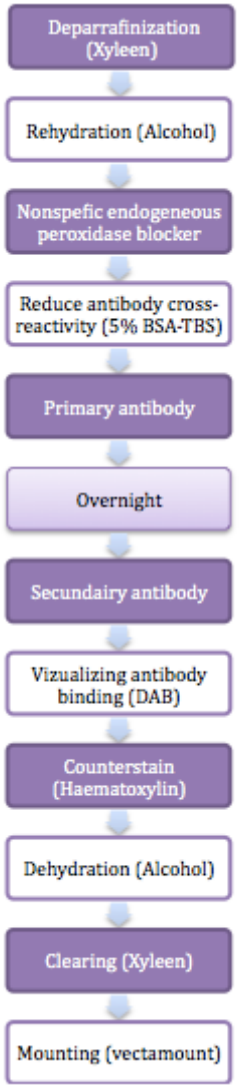

Figure 7. Basic protocol of COX-2 staining.

Quantification of COX-2 expression

The samples were visualised under a microscope. Digital images were obtained using a 10x objective. The percentage of COX-2 positive cells over the total number of cells was counted manually. Differentiation was made between positivity in the NP and in the AF. For this quantification Photoshop CC 2014 was used. For some patients more than one histological section was collected and for some sections more than one digital image was made to have a more objective view. In these cases an average percentage of COX-2 positive cells was determined.

Optimization of the immunohistochemistry protocol of CCL5

Please note that due to the time consuming process of optimization of the immunohistochemical staining protocol of CCL5, and the fact that the research time for this master thesis was ended, further optimization will need to be done. The procedure of optimization is given in appendix I. The results and discussion will focus on the COX-2 protein expression.

Other used variables

The degenerative grade was determined by the canine-modified histological grading scheme of Boos *et al.* and the MRI-grading scheme of Pfirrmann *et al. (*Bergknut *et al.,* 2013a; Phirrmann *et al.*, 2001). Another variable to correlate the COX-2 expressing with, is the presence of the clinical neurological signs the dogs showed before surgery. Please note that the scores of these three variables were determined by professionals (veterinary pathologist for histological score, DVM for Pfirmann- and neurological score) prior to this research.

Pfirrmann-score

When a dog suffers from IVD degeneration, MRI is the most reliable method to imaging the area of interest. All the dogs of this study were examined by MRI before they underwent surgery. This MRI was used to classify the IVD degeneration by the Pfirrmann score (Pfirrmann *et al.*, 2001). Pfirrmann *et al.* developed a human grading system for MRI images. Pfirrmann divided the disc degeneration into five grades with minimal changes in grade I up to severe changes in grade V. Appendix II gives an overview of the characteristics of each grade. In figure 10 four MRI pictures are presented of respectively grade I, grade II, grade III and grade IV.

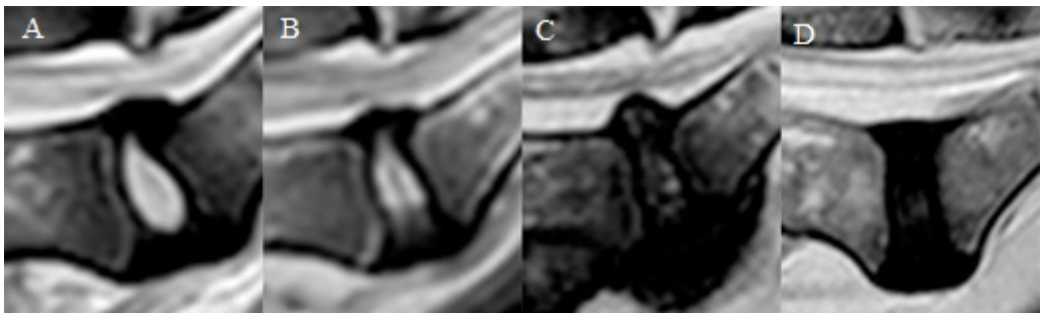

Figure 10. MRI pictures of Pfirrmann-score (A) grade I, (B) grade II, (C) grade III, (D) grade IV of IVD degeneration. Pfirrmann-score grade V of degeneration is not shown.

Boos-score

The 44 IVDs were also graded histologically by a board certified veterinary pathologist using the modified and validated (for the canine IVD) Boos grading system (Bergknut *et al.,* 2013a). This system is the most commonly used histological classification system to classify disc degeneration. This scoring system takes cytological and structural variables into account reflecting changes in the NP, AF, EP and subchondral bone. An overview of the exact parameters, which are considered by this method, are presented in appendix III.

Clinical neurological score

For this score, dogs were classified according to the outcome of the clinical neurological examination. Grade 1 was assigned to dogs with only pain symptoms; grade 2 to dogs with paraparesis/tetraparesis but still ambulatory; grade 3 to dogs with paraparesis/tertraparesis and being no longer ambulatory; grade 4 to dogs with paraplegia/tetraplegia but still having a deep pain perception; grade 5 to dogs with paraplegia/tetraplegia and loss of deep pain perception (Kranenburg *et al.*, 2013; Scott, 1997; Scott and McKee, 1999; Sharp and Wheeler, 2005).

Statistical analysis

Data were analysed using Statistical Package for the Social Sciences (SPSS) v.22.0. Descriptive statistics were used to express medians, means, standard deviations, ranges, percentages and frequencies of the study population characteristics. The dependent variables were 'COX-2 expression in the NP' and 'COX-2 expression in the AF'. Assumptions for normal distribution of the dependent variables were checked by distribution of the residuals and the quantile-quantile (Q-Q)-plots.

Because only one dog had a Pfirrmann grade V of disc degeneration, grade IV and V were combined for statistical analyses. For biological reasons, grade I with only two dogs, was not integrated with grade II.

To analyse the effects of modified Boos-score, Pfirrmann-score and neurological-score on the expression of COX-2 in the NP (normal-distributed), multiple linear regression analyses were used to estimate the regression coefficients. Because only two dogs were scored as a Pfirrmann grade I, grade II was used as the constant and Pfirrmann grade I is not included in the interpretations of the different models. Other variables tested to incorporated into the model were 'age', 'type of herniation' (protrusion, herniation), 'location' (cervical, thoracolumbar, lumbosacral) and 'chondrodystrophy' (CD, NCD). Confounding factors were tested by forward selection, adding one by one. Only one (the most powerful) confounder could be submitted to the model to retain enough power, since for every 10 cases one variable can be submitted to the model (Maxwell, 2000). Confounding was assumed when the regression coefficient of the independent variable changed by more than 10%.

Interaction terms were made to test whether the effect of Pfirrmann-score, Boos-score, and neurological-score on the expression of COX-2 in the NP was different for CD vs NCD dogs, different locations of the herniation (cervical, thoracolumbar, lumbosacral), protrusion vs herniation and different ages. Effect modification was assumed when $p<0.05$. When significant, further stratification was performed.

Due to the skewed distribution of 'COX-2 expression in the AF', the nonparametric Kruskal-Wallis test was used to analyse the effect of Boos-score, Pfirrmann-score and clinical neurological-score on the expression of COX-2 in the AF. When a significant effect was found a Mann-Whitney U-test was performed.

Only categorical determinants can be tested nonparametric, whereby Boos-score was divided into quintiles: group 1 (0-4 points), group 2 (4-8 points), group 3 (8-12 points), group 4 (12-16 points) and group 5 (16-20 points) (Kranenburg *et al.*, 2013). Correcting for effect modification and confounding is not possible with nonparametric testing.

Finally a paired sample t-test was done to compare the overall COX-2 expression in the NP with the overall COX-2 expression in the AF. Assumptions for normal distribution were based on the histogram and Q-Q-plots (rest on the differences between these outcome variables).

Results

IVDs samples (NP and/ or AF) were collected from 44 dogs, which suffered from clinical IVD degeneration. The population characteristics are summarized in table 1.

Table 1. Characteristics of the research population.

Variables	N (%)	Mean (SD)	Median (range)
Chondrodystrophy:			
NCD	26 (59.1)		
CD	18 (40.9)		
Age		6.5(2.6)	
Spinal location:			
Cervical (C1-T1)	18 (40.9)		
Thoracolumbar (T1-L1)	9 (20.5)		
Lumbosacral (L1-S1)	17 (38.6)		
Hansen type of herniation:			
Hansen type 1: extrusion	25 (56.8)		
Hansen type 2: protrusion	19 (43.2)		
Pfirrmann-score:			
Grade I	2 (4.5)		
Grade II	5 (11.4)		
Grade III	14 (31.8)		
Grade IV	19 (43.2)		
Grade V	4 (9.1)		
Histological modified Boos-score			10.25 (3-18)
0-4	1 (2.3)		
4-8	4 (11.4)		
8-12	31 (70.5)		
12-16	3 (6.8)		
16-20	5 (11.4)		
Clinical neurological score:			
Grade I	25 (56.8)		
Grade II	8 (18.2)		
Grade III	6 (13.6)		
Grade IV	4 (9.1)		
Grade V	1 (2.3)		

Note. N=44. The mean and standard deviation and median and range were presented for age and Histological Boos-score to give a more informative overview.

A total of 18 CD and 26 NCD dogs were included with a total mean age of 6.5±2.6 years. Of the collected IVDs, 18 discs originate from the cervical region, 9 from the thoracolumbar region and 17 from the lumbosacral region. The pre-operative neurological status was collected of all 44 dogs. Most dogs were neurologically classified as grade I (56.8%). The overall median Boos-score for the dogs was 10.25 (3-18), with the largest proportion (70.5%) of dogs in the category score 8-12. Most dogs had a Pfirrmann-score of grade III or IV (80%).

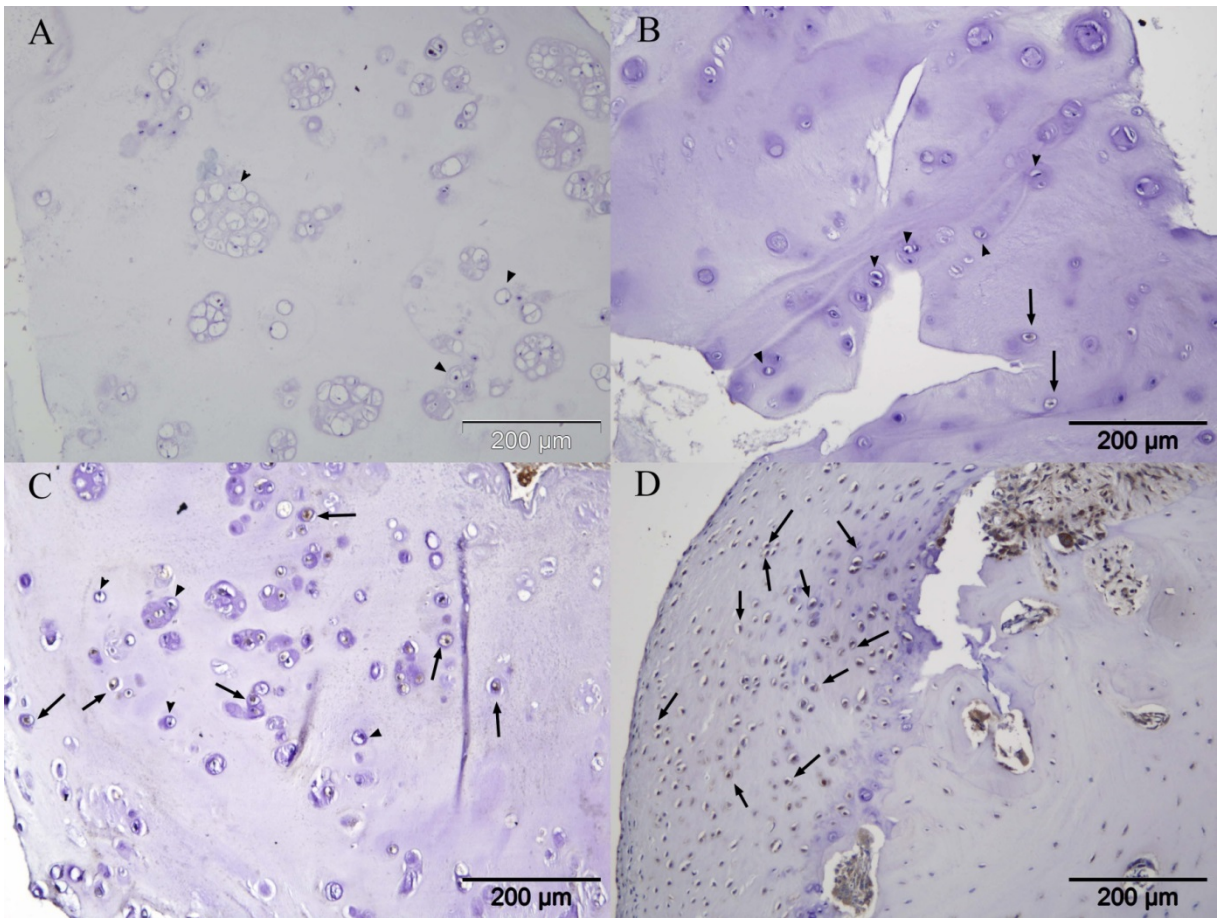

Figure 11. Histological images of the NP of the IVDs, graded according to Pfirrmann-score, stained with a COX-2 antibody and counterstained with haematoxylin. (A) NP of a Pfirrmann-score grade I, (B) NP of a Pfirrmann-score grade II, (C) NP of a Pfirrmann-score grade III and (D) NP of a Pfirrmann-score grade IV. Positive cells are marked by arrows and negative cells by arrowheads.

COX-2 expression in the NP

Surgical NP samples of 7 dogs were missing, one outlier was excluded prior to the performance of the statistical analyses. Thirty-seven dog-samples of the NP were used for analyses. Figure 11 shows histological images of the NP of four different stages of disc degeneration according to the Pfirrmann-score. Grade I had an average (SD) COX-2 expression in the NP of 2.56% (3.26), grade II of 1,9% (3.90), grade III of 22.5% (16.99) and grade IV and V of 22.5% (20.49). A significant difference in COX-2 expression in the NP was found between grade IV+V and grade II of the Pfirrmann-score (B=0.206, 95%CI=[0.003-0.408], $p<0.05$). Dogs with a Pfirrmann-score grade IV+V score had a more than ten time higher expression of COX-2 in the NP than dogs with a Pfirrmann-score grade II score (figure 12a). 16.7% of the variances in COX-2 expression in the NP were explained by the Pfirrmann-score.

None of the interaction terms were significant. The effect of Pfirrmann-score on COX-2 expression in the NP is the same for each of the tested effect modifiers (CD vs NCD, type of herniation, location of herniation and age).

After correcting for location of herniation (the most powerful confounder), the regression coefficient for as grade III (B=0.228, 95%CI=[0.004-0.452], p<0.05) as well as grade IV+V (B=0.239, 95%CI=[0.019-0.459], p<0.05) were statistical significant different from grade II (figure 12b). Outcome of this multiple linear regression analyses are presented in table 2. Dogs with a Pfirrmann-score grade III score have a 22.8% higher expression of COX-2 in the NP than dogs with a Pfirrmann-score grade II score, after correcting for location of herniation. Dogs with a Pfirrmann-score grade IV+V score have a 23.9% higher expression of COX-2 in the NP than dogs with a Pfirrmann-score grade II, after correcting for location of herniation.

Table 2. Multiple linear regression for Pfirmann-score on the expression of COX-2 in the NP.

	B	95% CI	p-value	R^2
Model A				0.167
Grade II (constant)	0.190	-0.164-0,203		
Grade III	0.206	-0.006-0.417	0.57	
Grade IV+V	0.206	0.003-0.408	**0.47**	
Model B				0.192
Grade II (constant)	-0.044	-0.274-0.187		
Grade III	0.228	0.004-0.452	**0.46**	
Grade IV+V	0.239	0.019-0.459	**0.34**	

Note. N=37. Outcome of the multiple linear regression analyses. Model A: Unadjusted model. Model B: Model A corrected for the confounder location of herniation.

No statistical significant effect (p=0.13*)* was found between Boos-score and COX-2 expression in the NP, nor between clinical neurological-score and COX-2 expression in the NP. When possible effect-modifiers and confounders were taken into account, the effect between Boos-score and neurological-score on the expression of COX-2 in the NP was neither significant different.

COX-2 expression in the AF

From the surgical AF samples, 11 cases were missing. Therefore, the remaining 33 dogs were taken into account for the statistical analysis. No significant differences in COX-2 expression in the AF were found between the different groups of the Pfirrmann-score (figure 12c). This was similar for the relation of the different grades of the Boos-score on the COX-2 expression in the AF. However, there was a significant difference between COX-2 expression in de AF and the different neurological-scores (figure 12d). Dogs with grade I of the clinical neurological-score had an average (SD) COX-2 expression in the AF of 4.74% (7.96), grade II of 16.15% (7.96), grade III of 5.81% (5.26), grade IV of 2.52% (4.01) and grade V of 64.1%. Because only one dog was suffering from grade V of neurological signs, this grade was excluded for statistical analyses. A significant difference (p<0.01) in COX-2 expression in the AF was found between grade I and grade II of the clinical neurological score (U=14, z=-2.58, p<0.01). Dogs suffering from grade II expressed significant more COX-2 in the AF than dogs suffering from grade I.

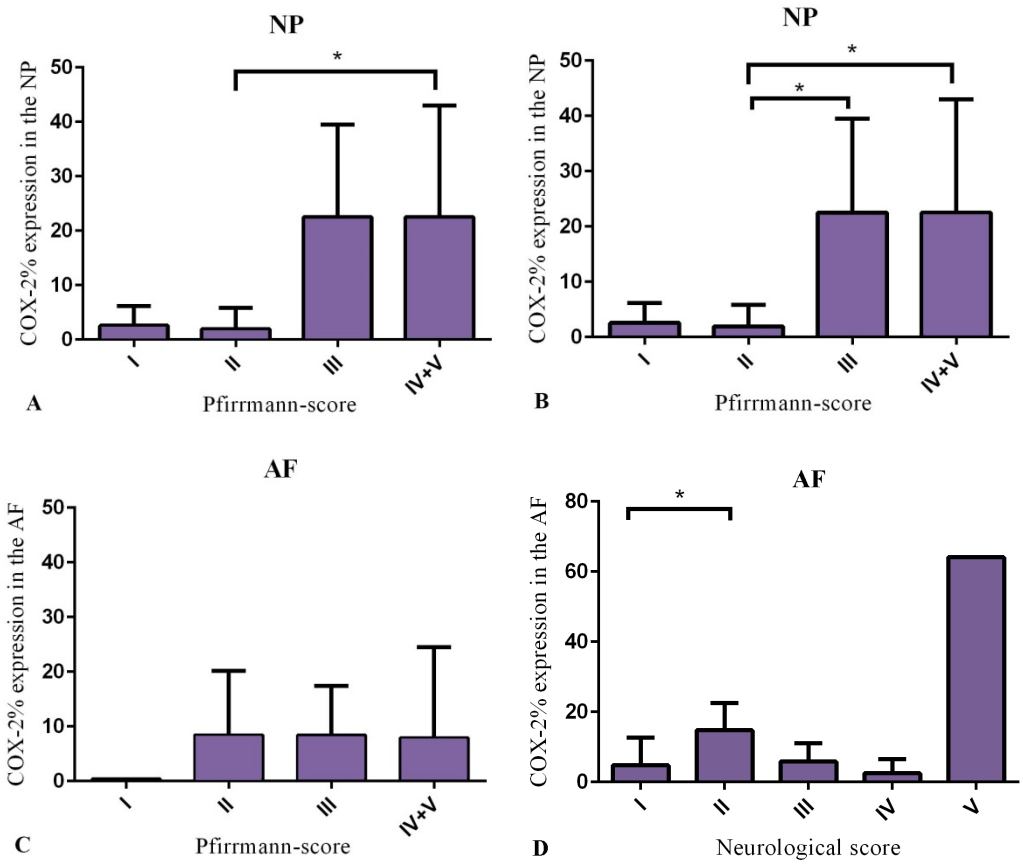

Figure 12. (A) COX-2 expression in the NP was significant different for dogs with a Pfirrmann grade IV+V compared to dogs diagnosed with Pfirrmann grade II of degeneration. (B) After correcting for location of herniation, also Pfirrmann grade III was significant from grade II. (C) No significant differences were found for Pfirrmann-score on the expression of COX-2 in the AF. (D) COX-2 expression in the AF was significant different for dogs with a neurological score grade II compared to dogs with symptoms classified as grade I.

COX-2 expression in the NP versus AF

When the overall COX-2 expression in the NP was compared with the overall COX-2 expression in the AF, a significant difference was observed (t (26)= 12.68, $p<0.05$, r= .29). The COX-2 expression is significant higher ($p<0.05$) in the NP (mean: 20.92%, SD: 19.18) than in the AF (mean: 8.24%, SD: 13.74).

Discussion

Back pain is a common feature within the canine population, and is commonly caused by IVD degeneration. Prostaglandins (e.g. PGE_2) play an important role in the process of IVD degeneration as they have an overall negative effect on the disc matrix homeostasis and induce centrally generated pain (Samad *et al.*, 2001; Vo *et al.*, 2012). COX-2 is a rate-limiting step in the arachidonate cascade, the process in which PGE_2 is synthesized. Knowledge about the expression patterns of COX-2 and PGE_2 in human IVD degeneration has substantially increased over the last years (Kang *et al.,* 1996; Miyamoto *et al*., 2002; Vo *et al.,* 2010), whereas in dogs still little is known. In order to improve treatment strategies based on COX-2 inhibitors, it would be helpful to obtain clinical data about the level of COX-2 expression in dogs with clinical signs and different stages of IVD degeneration. COX-2 inhibition will reduce the process of inflammation and degeneration, and as such would be a good therapeutic strategy for pain management and perhaps make regeneration of the IVD possible (Samad *et al.,* 2001; Willems *et al.*, 2015b). Since dogs show similar pathophysiological aspects in IVD degeneration as humans, these therapeutic and regeneration strategies could potentially be generalized to human medicine (Bergknut *et al.,* 2012).

Increased COX-2 protein expression in the NP at late stages of IVD degeneration

This study has shown significant higher expression of the COX-2 protein in the NP when dogs with an IVD Pfirrmann-score grade IV+V were compared to dogs with grade II of IVD degeneration. This indicates that in early stages of degeneration, COX-2 expression in the NP is significantly lower than in the end stages of IVD degeneration. After correction for location of the hernia, there was also a significant difference between Pfirrmann-scores grade III and grade II of IVD degeneration. These results are in line with previous research on dogs without clinical signs, where significant more COX-2 expression was found in dogs with Pfirrmann-score grade IV+V compared to dogs with grade I and grade II (Willems *et al.*, 2015b). Differently, in the present study a significant difference in COX-2 expression levels between dogs with grade III compared to dogs with grade II was also demonstrated. These findings are in agreement with observation in another cohort of clinical samples where PGE_2 content of the NP is increased in grade II-V compared to grade I (Willems *et al.*, 2015b). It would be interesting to compare the COX-2 expression level in dogs with and without clinical signs, which are in the same stage of degeneration according to the Pfirrmann-score. In this way, it will be possible to conclude whether COX-2 has influence, as expected, in pain generation or mainly plays a role in the process of IVD degeneration.

COX-2 expression in the NP is not significant different for the various stages of degeneration based on the histological Boos-score. Since Pfirrmann-score and Boos-score are found to correlate (Kranenburg *et al.*, 2013; Seiler *et al.,* 2003), a significant higher level of COX-2 expression was expected for increasing levels of the Boos-score. This discrepancy could be due to the small sample-size (n=44),

whereby outliers have a high impact. Another, maybe even more likely, explanation is that most of the dogs in this study had a Boos-score between 8-12 (70,5%). This means that not all scores were equally represented in the study population. This could be resolved by including additional clinical samples in the present study representing high (12-20) and low (0-8) Boos-scores. High Boos-scores, for instance, include canine patients with new bone formation or bone irregularities (see Appendix III, parameters of the Boos-score) and are scored as a Pfirrmann-score grade V (Kranenburg *et al.*, 2013; Seiler *et al.,* 2003). A low Boos-score could be more difficult to obtain. These dogs may have fewer structural irregularities in their IVD, therefore may have less severe clinical symptoms and not be nominated for surgery.

When COX-2 expression in the NP was compared with the clinical neurological score, no significant difference was found. This indicates that clinical neurological scores did not affect COX-2 expression in the NP. An explanation could be that the clinical scoring system is more focused on functional changes, which probably can occur at all levels of inflammation, and is not focused on levels of pain. Indeed PGE_2, whereby COX-2 is involved in the synthesis, is supposed to be a major factor in the genesis of a painful disc (Burke *et al.*, 2001; Kawabata, 2011). Hence, the type of herniation and location of herniation probably have more influence on the clinical neurological score rather than the level of COX-2 expression in the NP on the clinical neurological score. From a clinical point of view, it is not possible to correlate findings from clinical examination with the COX-2 expression in the NP.

Higher COX-2 protein expression in the NP compared to the AF

There was no significant difference between COX-2 expression in the AF and degeneration grade, according to Boos-score and Pfirrmann-score. This could be explained by that NP cells respond differently than AF cells to an inflammatory stimuli and mechanical stress, and therefore produce different levels of COX-2 (Miyamoto *et al.,* 2006). It could also indicate that the production of inflammatory mediators is more pronounced at NP level than at AF level, which is also suggested by others (Willems *et al.,* 2015b). This assumption is confirmed by the fact that in this study the total COX-2 expression was significantly higher in the NP than in the AF.

Differences in COX-2 protein expression in the AF for various neurological scores

A significant difference was found in COX-2 expression in the AF between dogs with a neurological-score of Grade I and Grade II. Dogs with symptoms as paraparesis/tetraparesis (but still ambulatory) expressed significant more COX-2 in the AF than dogs with only pain symptoms. This is contrary to the findings in the NP; no significant difference was found in COX-2 expression in the NP in different stages of the neurological-scores. However, one should bear in mind that dogs with grade II of clinical neurological score already can have high levels of COX-2 expression in the AF. From a clinical perspective, nerve root and spinal cord compression cause pain and neurological symptoms (Sharp and

Wheeler, 2005). Hence, inflammation in the AF in early stages of degeneration may have more influence on clinical neurological symptoms based on the findings of the present study. Nerve endings and blood vessels have only been found in the outer lamellae of the AF in healthy IVD (Bergknut *et al.*, 2013b; Urban and Roberts, 2003; Willenegger *et al.*, 2005). If these neurons get compressed due to malformations caused by inflammation, with high levels of COX-2, neurological signs will occur. This is confirmed by Willenegger *et al.,* who described that even limited pathological processes in the outer layers of the IVD are prone to give neurological signs (Willenegger *et al.,* 2005).

Study limitations

Although the research did fulfill its aim in regard to COX-2 protein expression, some of its limitations should be noted. The percentage of COX-2 positive cells was determined by manual counting of one observer. This method is subjective and could be improved by scoring with more than one observer. In this study, none of the dog's drug history is taken into account. It is presumable that the dogs were treated with anti-inflammatory drugs prior to surgery, or during surgery for a well-balanced anesthesia. Non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids could have been used. Both of the drugs are working in the arachidonate cascade and could have had influence in the results of the COX-2 expression level.

For this study surgically obtained samples were used. Despite all the advantages of this kind of samples, the disadvantage was that not all sections contained NP and AF parts of the IVD.

The sample size limitation had a few consequences. First only one confounder could be submitted to the model to retain enough power, since for every 10 cases one variable can be submitted to the model (Maxwell, 2000). When more samples could have been included in the study, a better statistical model could be created. Furthermore due to sample limitations, large confidence intervals were estimated. Last, some categories contained less than three dogs and as a result these categories could not be incorporated into the statistical analysis.

Conclusion, clinical implication and future plan

In this study it is shown that dogs with clinical signs of IVD degeneration showed higher levels of the COX-2 protein in the NP with increasing levels of degeneration, according to the Pfirrmann-score. The production of COX-2 seems to be more pronounced in the degenerated NP than in the AF. With this knowledge, intradiscal application with a controlled release COX-2 inhibitor should have the most analgesic effect in dogs with a Pfirrmann-score grade III, IV or V. To obtain regenerative effects, grade III is the most ideal since no irreversible structural changes have developed yet. Future studies are needed to investigate if COX-2 inhibition in the 'optimum stage' of IVD degeneration will stop the process of degeneration and make regeneration possible and if it provides effective analgesia.

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Appendix I

Optimization of the immunohistochemistry protocol of CCL5

The optimization for the staining of the chemokine CCL5 was started by using the most simplistic immunohistochemistry protocol. This protocol was the same as described for COX-2, except that CCL5 primary antibody was introduced instead of the COX-2 primary antibody. For the antibody dilution the choice was made to start with a 1:50 and a 1:200 dilution. Because the CCL5 primary antibody was a rabbit-derived antibody, a rabbit secondary antibody (Envision anti-rabbit K4003, Dako) had to be used. The outcome of this first staining round was that the 1:50 dilution had stained the test-sample highly intensely and the 1:200 dilution had stained the sample a little too lightly.

In the next round of optimization antigen retrieval was added to the protocol to look if this would result in a more proper staining with the low antibody concentration (1:200). Formaldehyde fixation and the rest of the paraffin embedding procedure may alter the conformation of the antigen. This eventually could result in a decrease of staining quality. The antigen retrieval was introduced after the deparaffinization and rehydration step. The slides were placed in a citrate buffer at room temperature whereafter the temperature was raised to 70° Celsius. The slides were kept at 70° Celsius for 30 minutes. After that, the slides had to cool down for 1 hour. Next to this cooling down step, the protocol was similar as described for the COX-2 staining with nonspecific endogenous peroxidase activity blocking as the next step. The result of this extra step was hopeful, the staining was clearer for both the 1:50 and the 1:200 CCL5 antibody dilutions. However the 1:200 primary antibody dilution was still too light to use in the experiment.

To investigate whether there was a possibility to use fewer antibodies than in a 1:50 antibody dilution, a round of staining was carried out with an antigen retrieval time of 15 and 45 minutes. The reason for this is that 30 minutes could be too short to repair the antigen conformation or could be too long, so that boiling in the citrate have damaged the antigen. The conclusion of this round of staining was that both boiling times, the shortened as well as the prolonged, didn't make the staining more clear.

Therefore it was decided to do a round of staining with an antibody dilution of 1:100 next to the 1:50 dilution. In addition, the 30 minutes antigen retrieval was reintroduced into the protocol. It was observed that this boiling time had the most proper staining. The result of this fourth round of staining was a better staining with the 1:50 primary antibody dilution relative to the 1:100 dilution.

In the next round of staining, a negative-control (Dako X0903) was performed next to a positive control with both the same antibody concentration (for the CCL-5 a 1:50 dilution) and a 30 minutes antigen-retrieval time. Unfortunately, the negative control had positive staining. This could be due to a-specific binding of the negative-control primary antibody, or by binding of the secondary antibody directly to the tissue sections. To exclude the first possibility, a round of staining was administered with a negative-control primary antibody from another company (Vector I-1000). At the same time a staining protocol was performed with omission of a primary antibody, just the 5% TBS-BSA was

added overnight. This was done to rule out the second option of positivity with the first introduced negative control. In figure 8 a schematic overview is presented of the three immunohistochemistry reactions from this round.

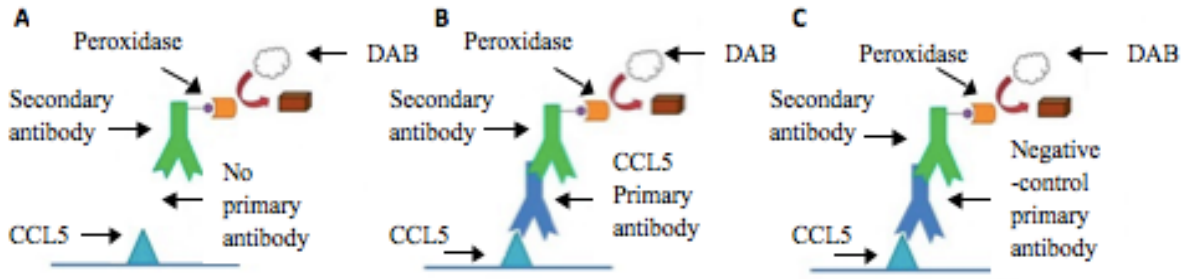

Figure 8. (A) The immunohistochemistry reaction without a primary antibody, (B) the immunohistochemistry reaction with a CCL5 primary antibody and (C) the immunohistochemistry reaction with introducing a negative-control primary antibody.

The result of this staining was still positive with the new introduced negative-control primary antibody and a negative outcome on the tissue sample where no primary antibody was introduced. Moreover a negative control where no negative-control primary antibody is introduced is not acceptable to suppose that an immunohistochemistry protocol is optimized.

Figure 9 shows a summarization of all the different protocols that have been used for the optimization.

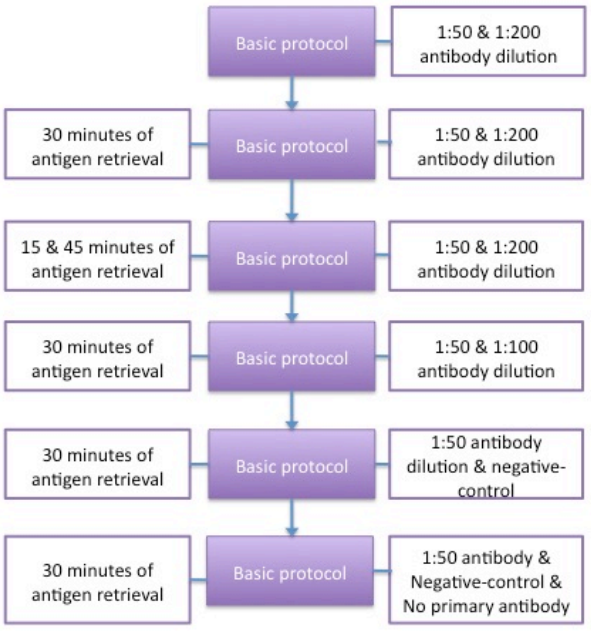

Figure 9. Overview of the CCL5 staining optimization. Basic protocol refers to figure 6, the overview of the COX-2 staining protocol.

Appendix II

Description of the five categories of the MRI-based grading scheme according to Pfirrmann et al. used to classify IVD degeneration in dogs (Pfirrmann *et al.*, 2001).

Pfirrmann grade	Structure	Distinction between NP and AF	Signal intensity	Height of IVD
I	Homogeneous and bright white	Clear	Hyperintense and isointense to CSF	Normal
II	Nonhomogeneous with or without bands	Clear	Hyperintense and isointense to CSF	Normal
III	Nonhomogeneous and gray	Unclear	Intermediate	Normal to slightly decreased
IV	Nonhomogeneous and gray to black	Lost	Intermediate to hypointense	Normal to moderately decreased
V	Nonhomogeneous and black	Lost	Hypointense	Collapsed disk space

NP= Nucleus pulposus, AF= Annulus fibrosus, CSF= Cerebrospinal fluid, IVD= Intervertebral disc

Appendix III

Histological parameters of the modified Boos et al. grading system for the canine intervertebral disc (Boos *et al.,* 2002; Bergknut *et al.,* 2013a).
Morphology of the AF
0 Well-organized, half ring-shaped, collagen lamellae
1 Mild disorganized; some lose half ring-shaped structure, most lamellar layer, still distinguishable (<25%)
2 Moderately disorganized; partly ruptured AF loss of half-ring shaped structure (25-75%)
3 Completely ruptured AF; no or few distinguishable half-shaped collagen lamellae (>75%)
Chondrocyte metaplasia of AF
0 No chondrocyte morphology, just spindle-shaped fibroblasts
1 Mild chondrocyte proliferation (i.e. limited to inner most AF layers)
2 Moderate chondrocyte proliferation (i.e. chondroid cells up to outer layers of the AF)
3 Marked chondrocyte proliferation (i.e. chondroid cells up to outer layers of the AF)
Tears and cleft formation
0 Absent
1 Rarely present
2 Present in intermediate amounts
3 Abundantly present
4 Scar/tissue defects
Chondrocyte proliferation of NP
0 No proliferation
1 Increased chondrocyte-like cell density
2 Connection of two chondrocytes
3 Small size clones (i.e. several chondrocytes group together, i.e. 2-7 cells)
4 Moderate size clones (i.e. >8 cells
5 Huge clones (i.e. >15 cells)
6 Scar/tissue defects
Presence of notochordal cells in NP
0 Abundantly present (>50%)
1 Present (1-50%)
2 Absent
Matrix staining of the nucleus pulposus with Alcian blue/Picrosirius red staining
0 Blue stain dominates
1 Mixture of blue and red staining
2 Red stain dominants
Endplate morphology
0 Regular tickness; homogeneous structure
1 Slightly irregular tickness
2 Moderately irregular tickness
3 Severely irregular thickness with interruption of the endplate

New bone formation
0 Absent
1 Minor new bone formation
2 Moderate amounts of new bone formation
3 Abundant new bone formation; tendency towards bridging/complete bridging
Subchondral bone sclerosis
0 No sclerosis (<2 x the thickness of the dorsal ventral cortex)
1 Mild sclerosis (2-4 x the thickness of the dorsal ventral cortex)
2 Moderate sclerosis (>4 x the thickness of the dorsal ventral cortex
3 Severe subchondral bone irregularities
AF= Annulus fibrosus, NP= Nucleus pulposus