CANINE NOTOCHORDAL CELLS IN TREATING INTERVERTEBRAL DISC DEGENERATION; CELL MARKERS AND REGENERATIVE CAPACITY OF EXCRETORY PRODUCTS

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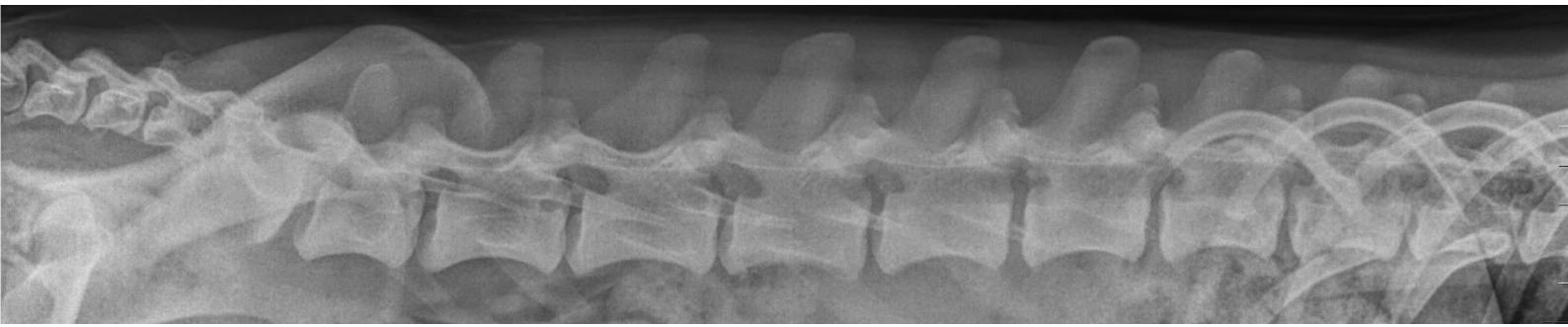

General introduction

Chronic lower back pain causes substantial economic and humanistic damage worldwide [1]. One of the major causes of lower back pain is the degeneration of the intervertebral disc (IVD) [2]. The IVD is located between most vertebrae [3]. It consists of three components: a gel like nucleus pulposus (NP) on the inside, an annular fibrosus (AF) surrounding the NP and cartilaginous endplates that connect the IVD to the vertebrae [3]. IVD degeneration often starts in the NP through a switch in cell type [4]. A healthy NP has clusters of large vacuolated cells, called notochordal cells [4]. These cells produce extracellular matrix and they instruct other cells in the NP to produce extracellular matrix as well. This specific NP extracellular matrix contains, among other things, collagen (mainly type II) and proteoglycans such as aggrecan in a specific ratio [5, 6]. These cell products give the NP its biomechanical properties to withstand compressive pressure between the vertebrae and to enable mobility while maintaining stability of the spine. In a degenerated NP, the large vacuolated cells are being replaced by smaller non-vacuolated cells [4]. These cells do not give the same instructions to surrounding cells as the notochordal cells, resulting in the production of less collagen type II and less proteoglycans. The biomechanical properties of the NP change, and the NP is no longer capable of withstanding physiological pressures. This results in a vicious cycle through which more and more structures of the IVD degenerate.

In humans, the shift from notochordal cells to small non-vacuolated cells is viewed as a process of maturation since earlier research has determined that in humans, around the age of 10, all notochordal cells have disappeared [4, 7]. Chondrodystrophic (CD) dog breeds, such as the beagle, the dachshund and the Lhasa apso, also show this cell shift in the IVD during maturation [8]. These dogs usually develop IVD disease in the cervical or thoracolumbar spine around 3-7 years of age. The remarkable similarities between IVD degeneration in CD dogs and humans make the CD dog a very useable disease model in the search of a therapy for both species [9]. In non-chondrodystrophic (NCD) dog breeds notochordal cells remain the predominant cell type present in de NP. However, these dogs can also develop IVD degeneration but do so at a later age (around 6-8 years of age) and typically in a different region of the spine (caudal cervical or lumbosacral spine) [8].

There is no curative therapy available for IVD degeneration at the moment. Patients with IVD degeneration are currently treated conservatively through painkillers, physical therapy and sometimes through surgery [10]. None of these treatments result in a functional IVD and most of the time the therapy is not successful in alleviating the patient's pain. In search of a curative therapy, regenerative medicine might offer part of the solution.

Notochordal cells (NCs) seem to be a perfect candidate for a cell based therapy that might even cure IVD degeneration [11]. The depleted NC population of the degenerated IVD could be replenished by functional NCs. These large, vacuolated functional NCs then produce the excretory products necessary to instruct the other cells in the NP to produce the right extracellular matrix, thereby having a regenerative effect on the degenerated disc. However, this approach still has some big hurdles to overcome.

Humans and CD dogs only have NCs early in their lifetime and each disc has a limited amount of NCs [4,7,8]. It is hard to harvest these cells since they bear quite unspecific markers which can also be found on non-notochordal cells that reside in the NP. When NCs are harvested and cultured, they dedifferentiate and lose their functional capacity [12, 13]. These circumstances make normal tissue

donation a nonviable option. This has led to an approach where stem cells are differentiated into notochordal-like cells[14]. However, to be able to evaluate this differentiation, it is necessary to know what phenotypic markers are to be found on functional NCs.

Furthermore, research by Bach *et al.* (2014-2018) has determined that the excretory products of NCs have a regenerative effect on other cells in the NP by stimulating these cells to produce collagen type II and proteoglycans in several different species, including canines and humans [15-19]. Therefore, notochordal cell conditioned medium (NCCM) and notochordal cell derived matrix (NCM) have also been proposed as a possible treatment strategy for IVD degeneration. However, the effect of NCM on the phenotype of the degenerated NP cells *in vivo* has not been investigated yet.

This report consists of two sections each with a separate aim being:

1. Identifying specific NC markers in canine NPs;
2. Investigating the effect of NCM on the NP cell phenotype and extracellular matrix production in canine IVDs.

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Delineating markers to identify notochordal cells in the canine nucleus pulposus.

Abstract

Background: Intervertebral disc (IVD) degeneration in humans and chondrodystrophic (CD) dogs is a naturally occurring process. It often starts with a shift in cell type from large vacuolated notochordal cells to small non-vacuolated cells. When developing a therapy to either retain or restore the notochordal cell (NC) population, readout parameters are needed to determine the specific phenotype of NCs. A specific marker set to identify these cells in the (canine) nucleus pulposus (NP) is currently lacking.
Objective: Investigating the expression of several NP/progenitor markers in the canine NP to identify the specific phenotype of NCs.
Methods: Immunohistochemical stainings for several markers were performed on canine NPs of different ages and breeds (stillborn pups, mature non-chondrodystrophic and chondrodystrophic dogs). The markers that were tested included proposed NC makers brachyury, TIE2, CD24, CK8 and 19 and mesenchymal progenitor marker CD73.
Results: CK19 was present in both the puppy and the non-chondrodystrophic (NCD) dog NPs, but more abundant in the puppy. There was a high expression of CD24, brachyury, TIE2 and CD73 in the NP of the puppy, whereas the NCD and CD NP only showed some intranuclear staining of TIE2.
Conclusion: CD24, CK19, Brachyury and CD73 seem to be useful canine NC markers. Further research is necessary to confirm the findings in this study, to be able to perform statistical analysis and to determine which markers select for functional NCs.

Introduction

Intervertebral disc (IVD) degeneration in humans and chondrodystrophic (CD) dogs is a naturally occurring process. It often starts with a shift in cell type from large vacuolated notochordal cells (NCs) to small non-vacuolated cells. When developing a therapy to either retain or restore the NC population, readout parameters are needed to determine whether the NCs are indeed present. However, NCs bear mostly unspecific markers[1]. Some of these markers are elaborated on below.

Potential canine notochordal cell markers

Cluster of differentiation 24 (CD24), cytokeratin 19 (CK19), cytokeratin 8 (CK8), Brachyury and TIE2 are considered NC markers. CD24 has been identified as a NC marker by Weiler *et al.* in 2010 [2]. In the nucleus pulposus (NP), the amount of CD24 positive cells decreases with the increase of disc degeneration and transplantation of CD24 positive NP cells promotes recovery of the degenerated disc [3]. CD24 is located on the cell surface and the protein acts as a cell adhesion molecule by binding with other cells or with the extracellular matrix. Cytokeratin 19 and cytokeratin 8 are both part of the cytoskeleton and therefore reside in the cytosol of the cell. Cytokeratin 19 is already expressed in the notochord, the embryological precursor of the NP [4]. Several studies in rats, bovines and humans have determined that CK19 is significantly higher expressed in NPs of healthy IVDs compared to degenerated IVDs [5-7]. Cytokeratin 8 can be found in foetal and adult human discs [8]. A double labelling experiment in human discs of several ages has also shown that CK19 and CK8 are often co-expressed by cells in the NP in cells with the typical notochordal appearance [2]. However, a study completed in 2010 found CK8 to be expressed in the mature bovine disc, even though bovine NCs disappear before birth [9]. Brachyury is considered a young NC marker and is already expressed in the developing notochord and the notochord is considered a precursor of the NP [9]. There is conflicting literature regarding the expression of brachyury in the adult NP. There are studies that have not found any immunopositivity for brachyury in the adult human NP [10], but others have found brachyury in the adult NP and even the degenerated NP, though they did see decreases with age and degeneration [11, 12]. Unfortunately, no canine specific research can be found regarding the expression of brachyury in the healthy adult NP. TIE2 was identified as a NC marker by Sakai *et al.* in 2012 using a colony forming assay (CFA) in mice and humans where TIE2 positive NP cells were capable of forming a colony, and TIE2 negative NP cells were not [13]. Immunostaining against TIE2 showed expression of this protein in canine NPs and TIE2 positivity of the NP decreases with age and degeneration in humans [13]. In 2018, Sakai *et al*. successfully labelled and isolated human, canine, bovine and murine TIE2 positive NP cells using flow cytometry [14].

In addition, we hypothesize that CD73 might also be present in NCs. CD73 is recognized as a mesenchymal stem cell (MSC) marker [15]. CD73 expression has also been found in cells of the NP[16]. Since CD73 is an epitope of MSCs, this marker might be present in progenitor cells such as the NC, which is a progenitor cell that also progresses from the mesodermal lineage [17].

This study aimed at identifying specific NC markers in canine NPs.

Materials and methods

Donors and NP tissue collection

NPs were collected from several dogs of different age and breeds. After fixation in 4% neutral buffered formaldehyde the NPs were dehydrated through graded alcohol steps. After dehydration, samples were embedded in paraffin and 5 µm sections were mounted on Microscope KP+ slides (KP-3056, Klinipath B.V., Duiven, The Netherlands). Details about the donors that were used are indicated in **table 1.1**.

*Table 1.1 Breed, age and cause of death of the donor dogs. *these dogs are not related.*

Donor	Breed	Age	Cause of death
Puppy 1	Labrador retriever	0 days	Stillborn
NCD dog 1*	Mongrel	3 years	Euthanized
NCD dog 2*	Mongrel	3 years	Euthanized
NCD dog 3*	Mongrel	3 years	Euthanized
CD dog 1	Beagle	5 years	Euthanized

Immunohistochemical staining of the nucleus pulposus.

Information about the antibodies used for the staining and the type of antigen retrieval used can be found in **table 1.2**. The appropriate canine NP slides were deparaffinized using consecutive baths of Xylene (2 times 5 minutes), 100% ethanol (2 times 3 minutes), 96% ethanol (2 times 1 minute) and 70% ethanol (2 times 1 minute). Slides were washed with PBS (2 times 5 minutes) and incubated with DAKO Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark) for 10 minutes to suppress endogenous alkaline phosphatase and peroxidase. Slides used for CD24, CK8 and brachyury staining were pre-treated with a 10 mM sodium citrate buffer with a pH of 6 at 70 degrees Celsius for 30 to 60 minutes, depending on the specific protocol. Slides used for CK19, CD73 and TIE2 staining did not require antigen retrieval. After another washing step with PBS-T 0.1% (2 times 5 minutes), sections were treated for 30 minutes at room temperature with PBS/BSA 5% to saturate excess protein-binding sites. The slides were incubated with the primary antibodies and stored overnight at 4 degrees Celsius. After a washing step with PBS-T 0.1% (2 times 5 minutes), the slides were incubated for 1 hour with the appropriate secondary antibody, which was a mouse (VWRKDPVM110HRP, Immunologic, Duiven, The Netherlands), rabbit (VWRKSDPVR110HRP, Immunologic, Duiven, The Netherlands) or goat secondary antibody (SC2053, Santa Cruz Biotechnology, Dallas, Texas, United States of America) depending on which primary antibody was used (see **table 1.2**). Another washing step with PBS (2 times 5 minutes) was performed and the sections were incubated in Bright DAB substrate kit (VWRKBS04-110, VWR, Radnor, Pennsylvania, United States of America) for 2 minutes. Slides were briefly rinsed in demi water and counterstained with Mayers hematoxylin (Merck 1.09249.0500, Darmstadt, Germany) for 1 minute. Slides were then rinsed in running tap water for 15 minutes and dehydrated through consecutive baths of 70% ethanol (2 times 1 minute), 96% ethanol (2 times 1 minute), 100% ethanol (2 times 3 minutes) and Xylene (2 times 5 minutes). Finally, a coverslip was glued on the slides using Pertex (VWRKAM-0801, VWR, Radnor, Pennsylvania, United States of America) and the slides were dried overnight.

Table 1.2 Source, concentration and antigen retrievel of the primary antibodies

Antibody	**Source**	**Antibody concentration**	**Antigen retrieval**
CD24	LSbiosciences, LS-C87657 mouse monoclonal antibody	1 µg/mL	60 minute 10 mM sodium citrate buffer (pH 6) at 70 degrees Celsius
CK19	Abcam, ab9221 mouse monoclonal antibody	40 µg/mL	none
CK8	Thermofischer, MA1-19037 mouse monoclonal antibody	10 µg/mL	60 minute 10M sodium citrate buffer (pH 6) at 70 degrees Celsius
CD73	IHCPlus, LS-B8284 rabbit monoclonal antibody	0.5 µg/mL	None
Brachyury	R&D systems, AF2085 goat polyclonal antibody	2.5 µg/mL	30 minute 10 mM sodium citrate buffer (pH 6) at 70 degrees Celsius
TIE2	Santa crus, sc-234 rabbit polyclonal antibody	4 µg/mL	None

Obtaining microscopic images of the nucleus pulposus and data evaluation.
The Olympus BX43 light microscope was used to image the NP slides. Images with magnifications of 4x, 10x and 20x were taken from multiple locations of each slide. Subsequently, the immunopositivity of the images was evaluated.

Results

The microscopic images of all immunohistochemical marker stainings can be found in **figure 1.1**.The NP of the stillborn puppy stained positive for CD24. The positive staining is visible in the membrane and cytoplasm of some cells. Two NCD dogs and the CD dog stained negative for CD24. In one NCD dog there were a couple of NP cells that showed some cytoplasmic staining. The puppy NP showed abundant protein expression of CK19 in the cytoplasm. In the NPs of the NCD dogs there was also some CK19 protein detected, while this was absent in the NP of the CD dog. The pup NP showed nuclear protein expression of brachyury and nuclear and cytoplasmic expression of TIE2. Brachyury and CD73 were not present in the NCD and CD dog NP, but TIE2 staining appeared to be present in the nucleus of cells in both the NCD dogs and CD dog. Since all samples, including the positive control tissue, stained negative for CK8 (results not shown) it is likely that the protocol did not work properly.

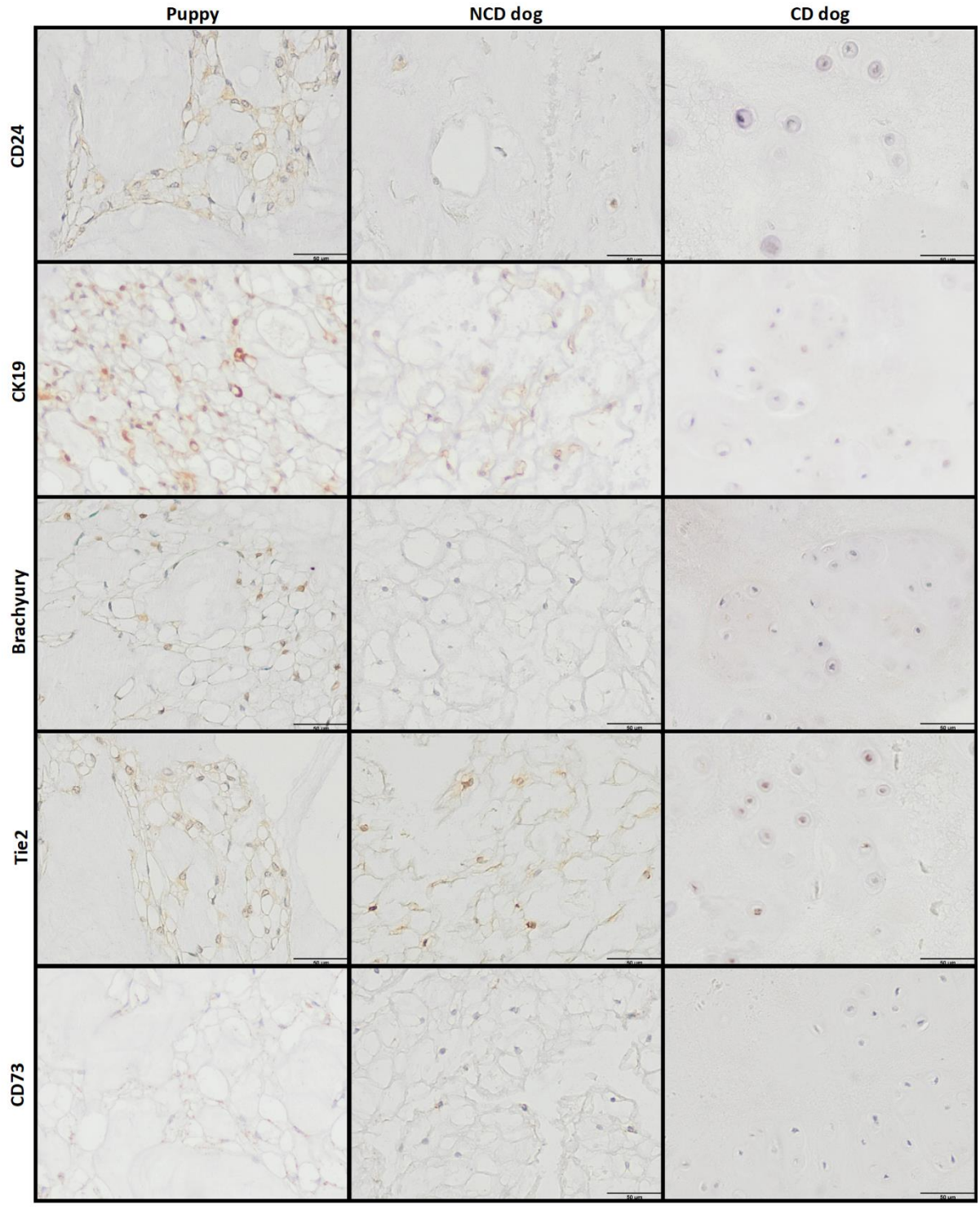

Figure 1.1 20x microscopic images of a puppy, NCD and CD dog nucleus pulposus immunohistochemically stained for CD24, CK19, Brachyury, TIE2 and CD73.

Discussion

In order to delineate markers to identify NCs in the canine NP, immunohistochemical staining of six proposed mature NC markers (CD24, CK19, CK8, Brachyury, TIE2 and CD73) were performed on puppy, NCD and CD NPs. CD24, CK19, Brachyury, TIE2 and CD73 were all present in the NP of the puppy, whereas the NP of the NCD dogs only showed expression of CK19 and intracellular expression of TIE2. The beagle NP did not show protein expression of any of the tested markers, except for TIE2, which was visible in the nucleus of the NP cells.

Unfortunately, the CK8 staining was not successful. There are several possible causes for the failure of this staining including unsuccessful antigen retrieval, incorrect dilution of the antibody for this species or incompatibility of this antibody with canine NP tissue. Optimization of the immunohistochemical CK8 staining on canine NP tissue is needed to further investigate this.

The fact that CD24 stained positive in the puppy NP is an affirmation that the NP of healthy canines contain CD24, something that was already researched in humans by Weiler *et al.* (2010) and Liu *et al.* (2018) [2,3]. However, we expected that the NCD dog NPs would also be positive for CD24. There was one NCD dog where some positive CD24 staining was seen in the cytoplasm of cells. Given the minimal amount of staining and the cytoplasmic location, it is quite unlikely that this is specific staining for CD24. To confirm this, it would be wise to repeat the CD24 staining on more adult NCD dogs.

CK19 stained positive in the puppy NP as well as in the NCD dog NPs. However, it is present in more abundance in the puppy NP. In 2013, Rodriques-Pinto *et al.* reviewed the interspecies variation of novel NP markers [20]. CK19 is listed there as a murine, bovine and human NP marker, but this review did not include CK19 as a canine NP marker. The fact that CK19 has been identified as a NP marker in these three other species makes it probable that this might also be true for canines. The results of the stainings in this study support this hypothesis, but no definite conclusions can be drawn due to the low sample size.

Brachyury is a very well-known NC marker and the results in this study show exactly what we expected; the puppy NP stains positive for Brachyury, but the NCD dog and the CD dog stain negative for Brachyury. These results are in compliance with the existing literature regarding the expression of Brachyury. It would be interesting to investigate the expression of Brachyury in NCD dogs of several ages to determine when exactly the expression is lost.

TIE2 has recently been discovered as a NS marker in canines and humans by Sakai *et al.* (2012, 2018) [14,15]. In our study, the puppy and NCD dog NP clearly stain positive for TIE2 in the nucleus as well as on the plasma membrane. In contrast, in the NP of the CD dog there is only nuclear TIE2 expression detected. Based upon the research of Sakai *et al*., we expected TIE2 expression mainly in the puppy but possibly also in the NCD dog NP, located on the cell membranes.This is not completely in line with the results obtained in this study. When diving further into the research regarding TIE2, it turns out that this marker can sometimes be found in the nucleus after it binds and forms a complex with the ligand caveolin-1[19]. However, further research is needed to determine the function of these TIE2/caveolin-1 complexes in the nucleus of NP cells. Based upon these results, it can be concluded that TIE2 expression on the cell membrane can be used to identify (young) NCs. Testing more NPs of dogs of different ages could clarify when and why the translocation of TIE2 to the nucleus appears.

The results of the CD73 staining are quite interesting. The staining is positive in the NP of the puppy and negative in all NCD dogs and the CD dog. This supports our hypothesis; CD73 seems to be present in the embryonic canine NP but not in the adult NCD or CD canine NP. This is also supported by the human study performed by Wu *et al*. in 2018 where resident NP cells in IVD tissue of donors aged <20 years showed a high expression of mesenchymal stem cell surface markers, including CD73, and resident NP cells in IVD tissue of donors aged >20 years showed a low expression of these mesenchymal stem cell surface markers [16]. Since only one puppy was used in this study, confirmation is needed through the staining of more puppy NPs. This positive CD73 staining does not necessarily mean that it is a NC marker since the protein could be present in other cells of the embryonic NP. However, it does mean that CD73 is present in the embryonic NP and disappears over time. Further research is necessary to elucidate the origin of the CD73 protein in the NP.

The markers CD24, CD73, CK19, Brachyury and TIE2 (when located on the cell membrane) all seem specific for canine NCs since all of these markers cannot be found in the NP of the CD dog. However, the expression of several of these markers seem to disappear with age; they are seen in the NP of the puppy, but not in the NP of the adult NCD dogs. The question remains whether the function of the cell is influenced by the loss of these markers. If this is true, the NCs of the puppy should, for example, produce better extracellular matrix and stimulate other cells better than the NCs of NCD dog NPs. Future research should focus on linking these phenotypical markers to the performance of the NCs. The findings in this study should be verified through a more extensive study with more donor dogs to validate these results and to gather enough power to perform statistical analysis.

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The effect of NCM on the expression of cytokeratin 19 and aggrecan in degenerated canine intervertebral discs

Abstract

Background: Intervertebral disc (IVD) degeneration in dogs often starts with the disappearance of notochordal cells (NC) from the nucleus pulposus (NP). NC secreted substances are known to have a regenerative effect on the NP. Without them and their matrix, the jelly-like NP converts into a more rigid structure uncapable of fulfilling its biomechanical function. NC conditioned medium (NCCM) and NC derived matrix (NCM) have been proposed as a possible therapeutic strategy for IVD degeneration.
Objective: The aim of this study is to determine whether intradiscal treatment with NCM stimulates the production of aggrecan and upregulates the expression of cytokeratin 19 (CK19) in mildly and moderately degenerated canine IVDs.
Methods: Six beagles were used in this study. Three IVDs of each beagle were subjected to partial removal of the NP to induce moderate IVD degeneration (NX-IVD). Three other mildly degenerated IVDs of each beagle were also included (NoNX-IVD). Each mildly and moderately degenerated IVD of each beagle received a different treatment: no treatment, a onetime injection with 50 µL 10 mg/mL porcine NCM or two injections with 50 µL 10 mg/mL three months apart. The beagles were euthanized 6 months after the initial injection and an aggrecan and a CK19 immunohistochemical staining was done on each IVD.
Results: In the noNX-IVDs as well as the NX-IVDs, a lot of donor variation was seen with regard to the aggrecan content. However, the noNX-IVDs all show more aggrecan content in the NCM treated IVDs in comparison with the not treated IVDs. No CK19 immunopositivity is seen in the noNX-IVDs, apart from 1 IVD that was not treated with NCM. Although a visible response to the NCM treatment is seen in the NX-IVDs regarding the CK19 expression, these differences were not statistically significant.
Conclusion: *In vivo* treatment of canine mildly and moderately degenerated IVDs with porcine NCM results in more aggrecan in the noNX and NX-IVDs and more CK19 expression in the NX-IVDs. More research is necessary to confirm the therapeutic potential of NCM.

Introduction

The disappearance of notochordal cells (NC) from the nucleus pulposus (NP) often coincides with the onset of intervertebral disc (IVD) degeneration [1]. NCs secrete substances that enhance the regenerative potential of the NP and instruct cells to produce certain cell products. Without these substances secreted by NCs, communication to produce the right ratio of collagen type II and proteoglycans such as aggrecan seems to be lost [2]. The jelly-like NP converts into a more rigid structure uncapable of fulfilling its biomechanical function.

NC secreted substances have been proposed as a possible therapeutic strategy to slow down, halt or even reverse canine IVD degeneration [3]. Either NC conditioned medium (NCCM) or NC derived matrix (NCM) is used to develop therapies. Multiple studies have been done to confirm the regenerative effect of NCM and NCCM on the NP [4-10]. De Vries *et al*. (2016) confirmed a stimulatory effect of NCCM on the matrix production of NP cells in culture [8]. Mehrkens *et al*. (2013) demonstrated a reduction of cytotoxic stress induced apoptosis in human NP cells after treatment with NCCM and Mehrkens *et al.* (2017) demonstrated that canine NCCM can protect human and murine NPCs from caspase-9 and caspase-3/7 induced apoptosis [9, 10]. Bach *et al.* (2014-2018) have done very extensive research regarding the composition and effects of NCCM [4, 5, 7, 11]. A cross-species effect of NCCM was found when non-human and human NCCM was used to stimulate human chondrocyte like cells (CLCs) derived from degenerated human IVDs [4]. In 2016, Bach *et al.* demonstrated that the soluble factors of porcine and canine NCCM, containing peptides and proteins, exerts more anabolic effects than the pelletable factors of porcine and canine NCCM, containing protein aggregates and extracellular vesicles [11]. In 2017, the effect of porcine NCCM-derived extracellular vesicles versus the effect of porcine NCCM-derived proteins and unfractionated NCCM on canine and human NP cells were compared and although the fractionated NCCM did have an anabolic effect, the unfractionated NCCM was more potent in human CLCs [5].

This study builds on the latest work of Bach *et al*. (2018) where porcine NCM was injected *in vivo* into mildly and moderately (induced) canine degenerated IVDs [7]. Bach *et al*. (2018) concluded that treatment with NCM in moderately canine degenerated IVDs resulted in beneficial effects such as improved Thompson and Pfirrmann scores, increased disc height and induction of collagen type II production [7]. Here, we will examine the effect of *in vivo* treatment with porcine NCM on the expression of cytokeratin 19 (CK19) and aggrecan in mildly and moderately degenerated canine IVDs using immunohistochemical staining. Aggrecan is produced in the healthy NP and therefore might serve as a good parameter to determine the condition of the NP after treatment with NCM [12]. CK19 was selected through several reasons: the outcome of the first part of this study has shown that CK19 might be a possible canine NC marker. Furthermore, a previously conducted study showed that NCM treatment increased the protein expression of CK19 of canine NP cells *in vitro* (unpublished data). In this study we aim to delineate whether this increase is also seen *in vivo*, which would indicate that NCM could have a beneficial effect on the NP cell phenotype.

Materials and methods

Overall study design

The objective of this study is to determine what effect treatment with porcine NCM has on the expression of CK19 and aggrecan in the IVD of canines with mild and moderate IVD degeneration. Procedures were approved and conducted in accordance with Animal Experiments Committee guidelines (project number: AVD108002015285) and performed by Bach *et al.* in 2018 [7]. Five otherwise healthy adult beagles (N=5) were included in this study. Per dog, 6 IVDs were used. Since the beagle is a chondrodystrophic (CD) dog, IVD degeneration naturally sets in during maturation. No further degeneration was induced in IVDs T11-T12, L2-L3 and L6-L7. These IVDs were considered to be mildly degenerated. Further degeneration was induced 6 weeks prior to the experiment in IVDs L1-L2, L3-L4 and L7-S1 by performing a partial NP removal on the left side of the spine. After the partial removal of the NP, these IVDs were considered to be moderately degenerated. T11-T12 and L1-L2 were not treated and served as controls. L2-L3 and L3-L4 were treated with one injection containing 50 µL of 10 mg/mL NCM. L6-L7 and L7-S1 were treated with two injections containing 50 µL of 10 mg/mL NCM, the second injection following 3 months after the first injection. Six months after the first injection, the dogs were euthanized and the IVDs were collected. See **figure 2.1** for a visual representation of the study design and see Bach *et al.* (2018) for a more detailed study design and for the tissue preparation protocol [7].

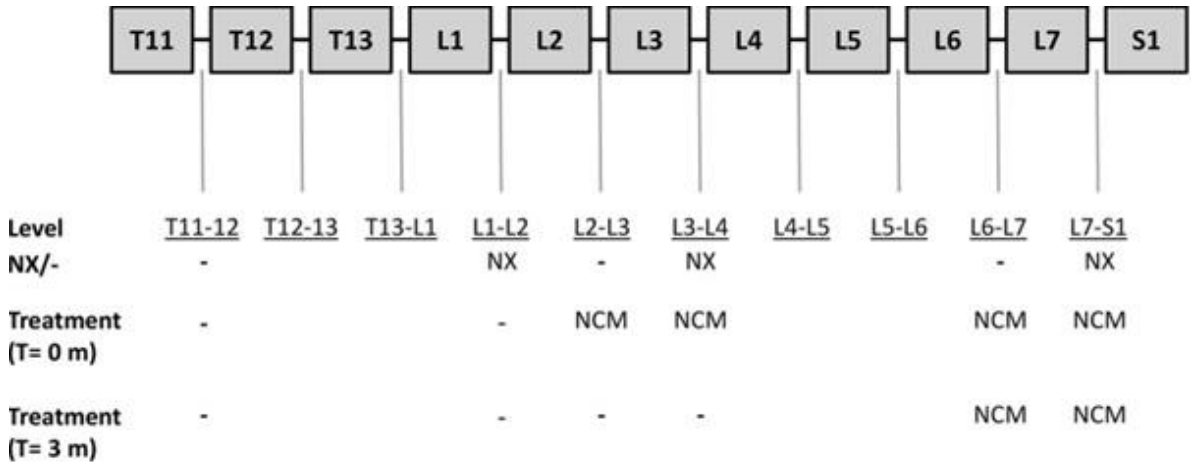

Figure 2.1 - Visual representation of the study design. The level indicates between which vertebrae the intervertebral disc (IVD) in question is situated. IVDs without any description in the treatment area were not used in this study. The row with NX/- indicates whether a partial nucleotomy (NX) was performed or not (-). The treatment at T=0 indicates whether an intradiscal injection with porcine notochordal cell derived matrix (NCM) was performed at 0 months. The treatment at T=3 indicates whether a second intradiscal injection with porcine NCM was performed at 3 months.

Immunohistochemical staining for aggrecan and cytokeratin 19 of the intervertebral disc

Information about the antibodies used for the staining and the type of antigen retrieval can be found in **table 2.1**. The canine IVD slides were deparaffinized using consecutive baths of Xylene (2 times 5 minutes), 100% ethanol (2 times 3 minutes), 96% ethanol (2 times 1 minute) and 70% ethanol (2 times 1 minute). Slides were washed with PBS (2 times 5 minutes) and incubated with DAKO Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark) for 10 minutes to suppress endogenous alkaline phosphatase and peroxidase. The aggrecan staining protocol includes an antigen retrieval, described in the subsection below. Slides were washed with PBS-T 0.1% (2 times 5 minutes) and treated with PBS/BSA 5% to saturate excess protein-binding sites. The slides were incubated with the primary antibodies (see **table 2.1** for more information) and stored over night at 4 degrees Celsius. After a washing step with PBS-T 0.1% (2 times 5 minutes), the slides were incubated for 1 hour with

the mouse secondary antibody (VWEKDPVM110HRP, Immunologic Duiven, The Netherlands), conjugated with HRP. Another washing step with PBS (2 times 5 minutes) was performed and the slides were incubated in Bright DAB substrate kit (VWRKBS04-110, VWR, Radnor, Pennsylvania, United States of America) for 2 minutes. Slides were briefly rinsed in demi water and counterstained with Mayers hematoxylin (Merck 1.09249.0500, Darmstadt, Germany) for 1 to 2 minutes. Slides were then rinsed in running tap water for 15 minutes and dehydrated through consecutive baths of 70% ethanol (2 times 1 minute), 96% ethanol (2 times 1 minute), 100% ethanol (2 times 3 minutes) and Xylene (2 times 5 minutes). Finally, a coverslip was glued on the slides using Pertex (VWRKAM-0801, VWR, Radnor, Pennsylvania, United States of America) and the slides were dried overnight.

Antigen retrieval aggrecan

Slides destined for aggrecan staining needed to be pre-treated with Pronase and Hyaluronidase. After de DAKO Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark) described above, the slides were washed with PBS-T 0.1% (2 times 5 minutes) and incubated with Pronase 1 mg/ml for 60 minutes at 37 degrees Celsius. The slides were washed with PBS-T 0.1% (2 times 5 minutes) again and incubated with Hyaluronidase 10 mg/ml for 60 minutes at 37 degrees Celsius. The specific pre-treatment for aggrecan staining was then completed and the slides were further treated equally to the CK19 slides, as described above.

Table 2.1 Source, concentration and antigen retrieval of the primary antibodies

Antibody	**Source**	**Antibody concentration**	**Antigen retrieval**
Aggrecan	Abcam, ab3778 mouse monoclonal antibody	32 µg/mL	60 minute Pronase 1 mg/ml at 37 degrees Celsius 60 minute Hyaluronidase 10 mg/ml at 37 degrees Celsius
CK19	Abcam, ab9221 mouse monoclonal antibody	40 µg/mL	none

Obtaining microscopic images of the intervertebral discs and data evaluation

The Olympus BX43 light microscope was used to image the IVD slides. All pictures were taken from the NP of the IVDs Three random images with a 10x and 20x magnification were taken from each aggrecan stained slide and two random images with a 20x magnification were taken from each CK19 stained slide. Immunopositivity for CK19 was quantified. For the statistical analysis, IBM SPSS statistics 22 was used. To check whether the data were normally distributed, a Shapiro Wilks test was performed. Since the data were not normally distributed a Kruskal Wallis and Mann-Whitney U test were performed. P-values < 0.05 were considered significant.

Results

Aggrecan in NCM treated NPs that were not subjected to partial nucleotomy

Figure 2.2 shows the 10x magnified microscopic images of the NPs that were not subjected to a partial nucleotomy. It is hard to find a trend between the control NPs and the 1x and 2x NCM treated NPs. Dog 2 and dog 5 seem to have the expected progression of produced aggrecan; the least aggrecan is visible in the control NPs, a bit more is seen in the 1x NCM treated NPs and the 2x NCM treated NPs seem to have produced the most aggrecan. In dog 1, the cells in the control NP seem to produce more aggrecan than the cells in the 1x NCM treated NP and the 2x NCM treated NP seems to produce a similar amount of aggrecan as the control NP. Dog 3 shows no difference between the amount of aggrecan produced in the control NP, the 1x NCM NP and the 2x NCM NP. In dog 4, the 1x NCM treated NP produced the largest amount of aggrecan, followed by the 2x NCM treated NP.

Aggrecan in NCM treated NPs that were subjected to partial nucleotomy

Figure 2.3 shows the 10x magnified microscopic images of the NPs that were subjected to a partial nucleotomy. The control NX NPs of all dogs except dog 5 produced less aggrecan then the control NPs that did not have a partial nucleotomy. This is in line with what was expected; mildly degenerated NPs produce more aggrecan then moderately degenerated NPs. Only dog 3 shows the expected trend regarding aggrecan production and treatment with the least aggrecan produced by the NX control NP and the most aggrecan produced by the 2x NCM treated NX NP. Dogs 1, 2 and 4 have the largest production of aggrecan in the 1x NCM treated NX NP.

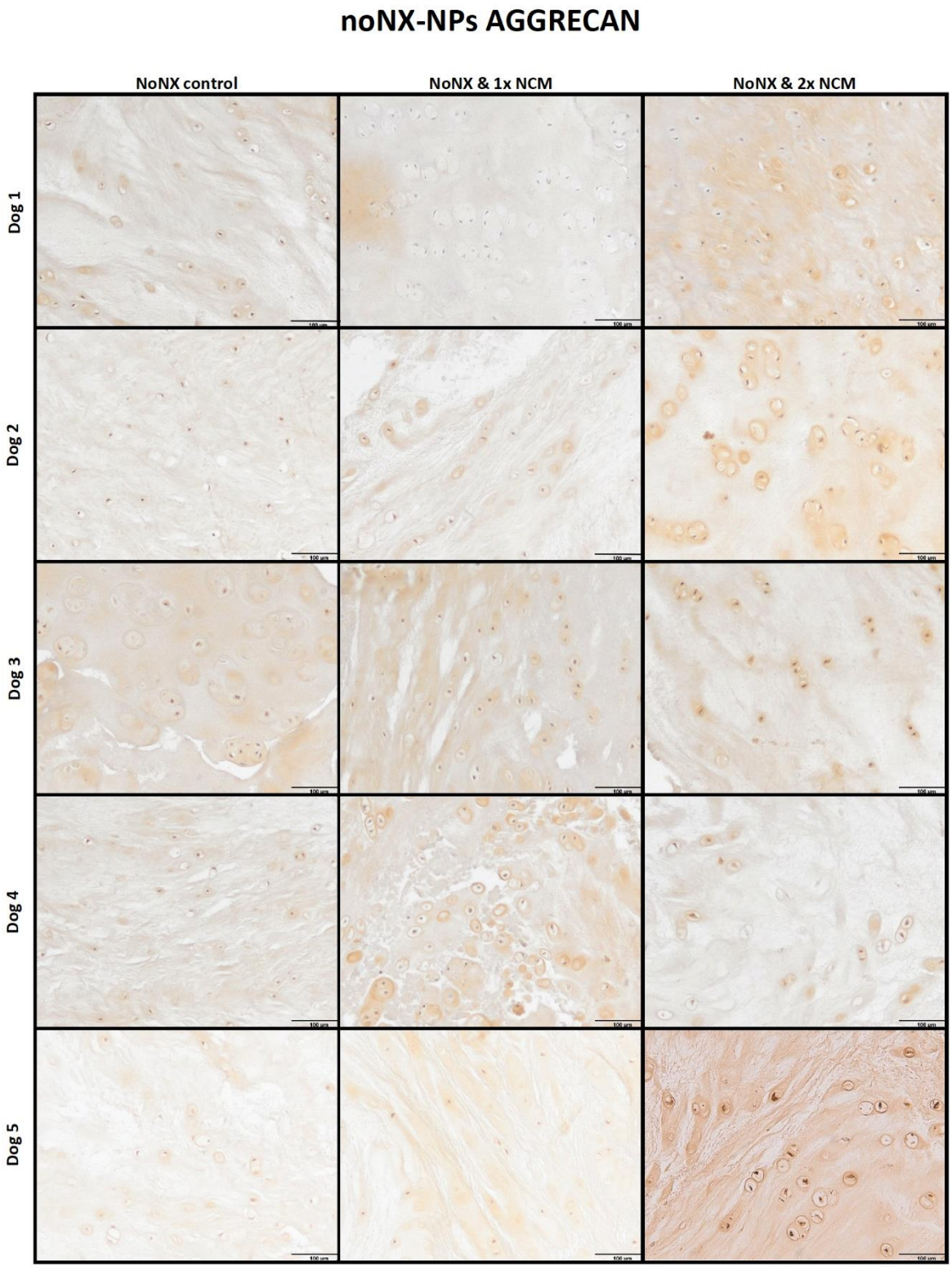

Figure 2.2 - 10x magnified microscopic images of mildly degenerated canine nucleus pulposus (noNX-NPs, n=5) without receiving a treatment (control) or injected ones with notochordal cell derived matrix (1x NCM) or twice (2x NCM) immunohistochemically stained for aggrecan.

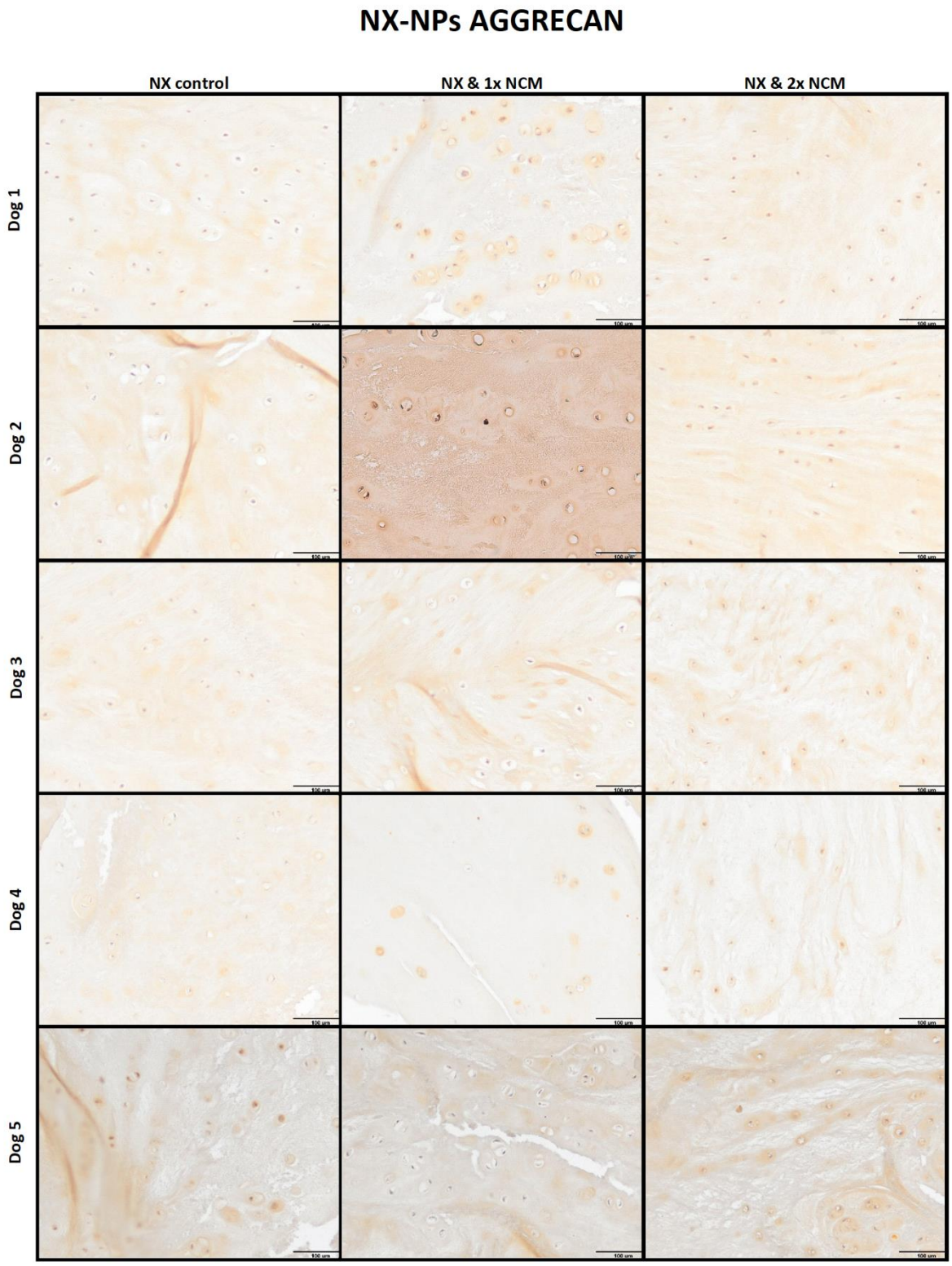

Figure 2.3 - 10x magnified microscopic images of mildly degenerated canine nucleus pulposus (NX-NPs, n=5) without receiving a treatment (control) or injected ones with notochordal cell derived matrix (1x NCM) or twice (2x NCM) immunohistochemically stained for aggrecan.

CK19 in NCM treated NPs that were not subjected to partial nucleotomy.

Figure 2.4 shows the 20x magnified microscopic images of the immunohistochemical staining for CK19 of the NPs that were not subjected to a partial nucleotomy. There appeared to be no CK19 protein expression in any of the NPs, except for some cells in the untreated IVD of dog 5. No significant differences between the CK19 expression in the non-treated, 1x NCM treated and 2x NCM treated NPs were found. In **figure 2.6**, a visual representation of the ratio positive cells in two 20x magnified microscopic images of the NP of each dog can be found.

CK19 in NCM treated NPs that were subjected to partial nucleotomy.

Figure 2.5 shows the 20x magnified microscopic images of the NPs that were subjected to a partial nucleotomy. Dog 2 and 3 stained positive for CK19 in the 2x NCM treated NPs. Dog 1 and 4 stained positive in the 1x NCM treated NP, and in the 2x NCM treated NP. Dog 5 stained positive for CK19 in the control NP and the 1x NCM treated NP. No significant differences were found. No significant differences between the CK19 expression in the non-treated, 1x NCM treated and 2x NCM treated NPs were found. In **figure 2.6**, a visual representation of the ratio positive cells in two 20x magnified microscopic images of the NP of each dog can be found.

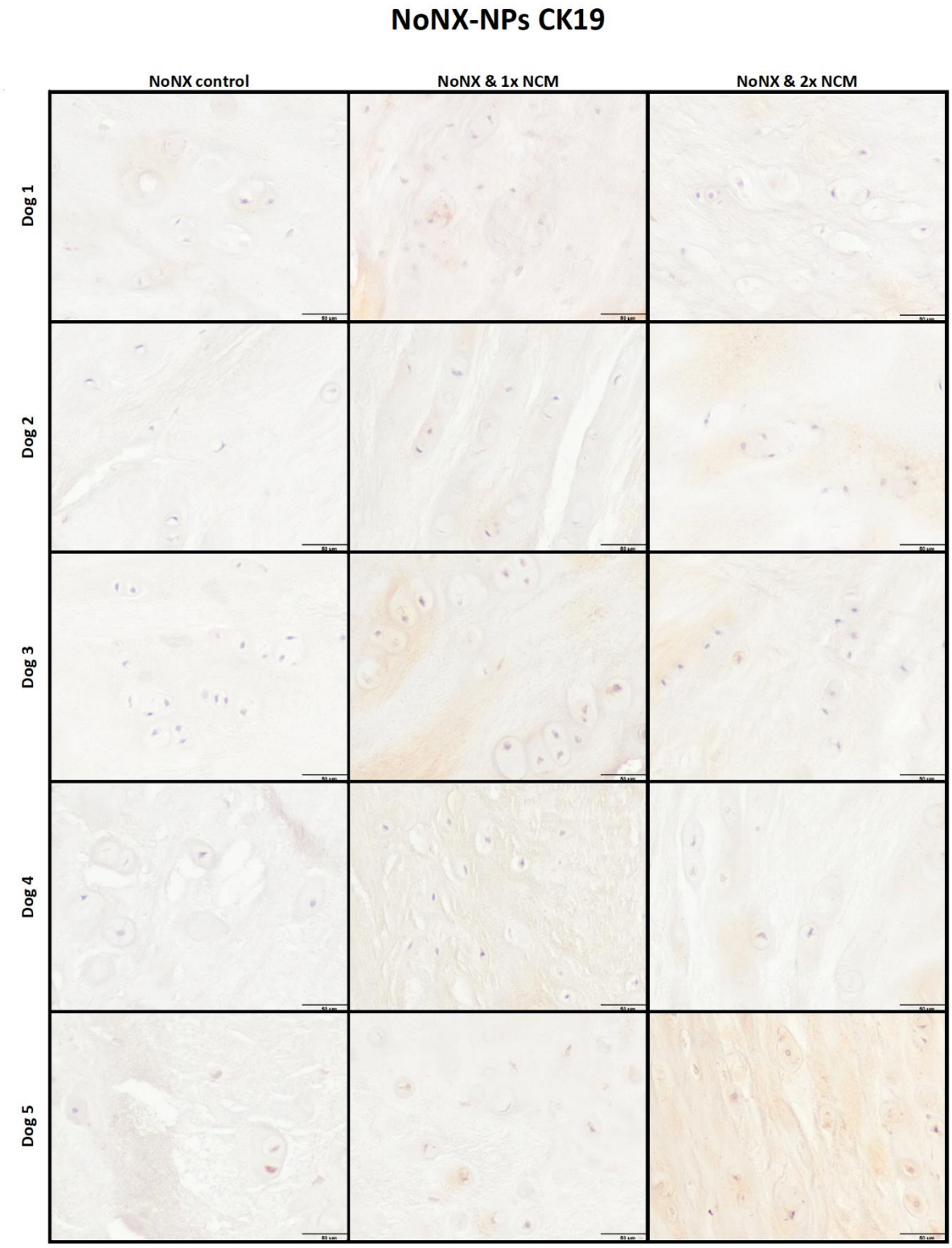

Figure 2.4 - 20x magnified microscopic images of mildly degenerated canine nucleus pulposus (noNX-NPs, n=5) without receiving a treatment (control) or injected ones with notochordal cell derived matrix (1x NCM) or twice (2x NCM) immunohistochemically stained for cytokeratin 19.

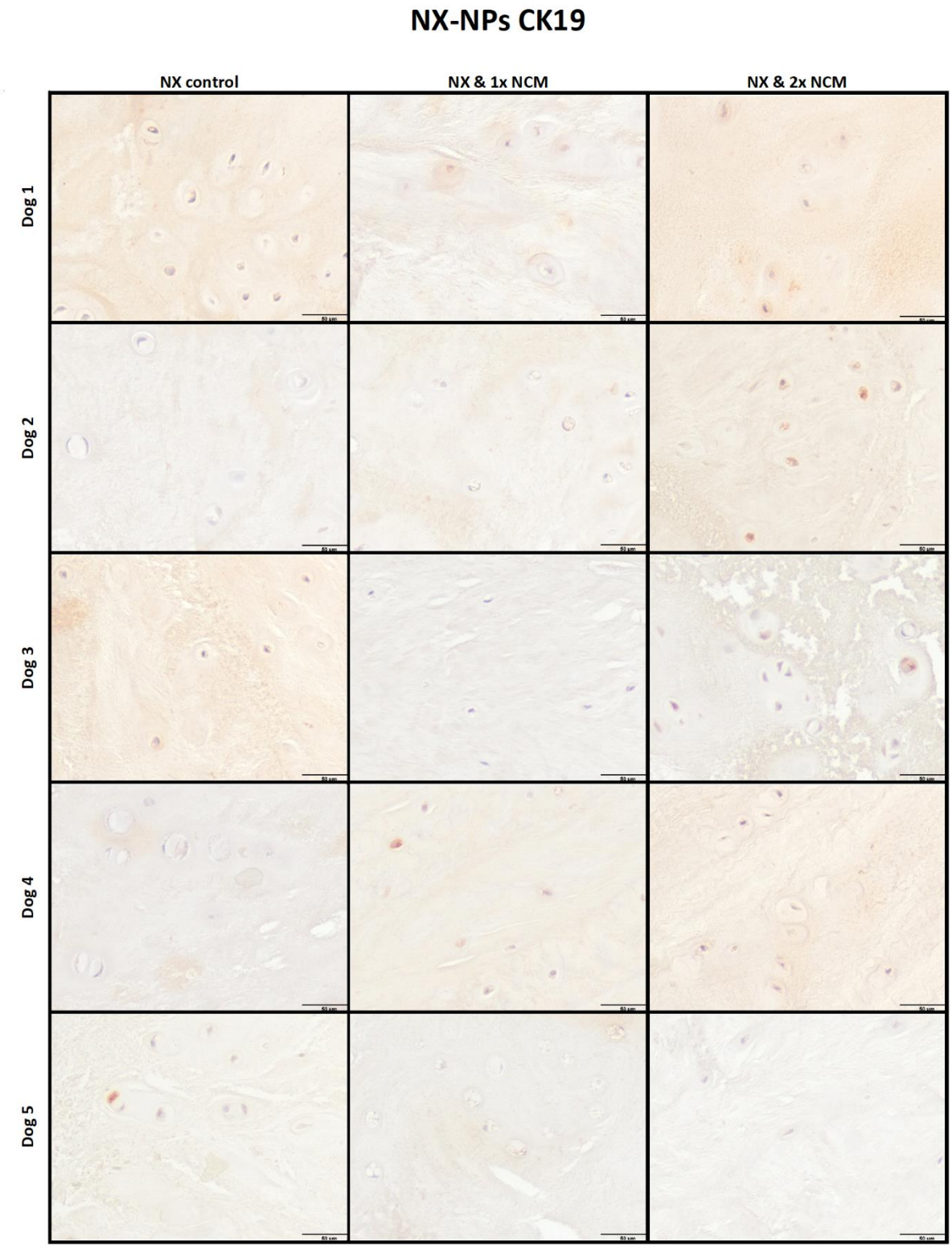

Figure 2.5 – 20x magnified microscopic images of mildly degenerated canine nucleus pulposus (NX-NPs, n=5) without receiving a treatment (control) or injected ones with notochordal cell derived matrix (1x NCM) or twice (2x NCM) immunohistochemically stained for cytokeratin 19.

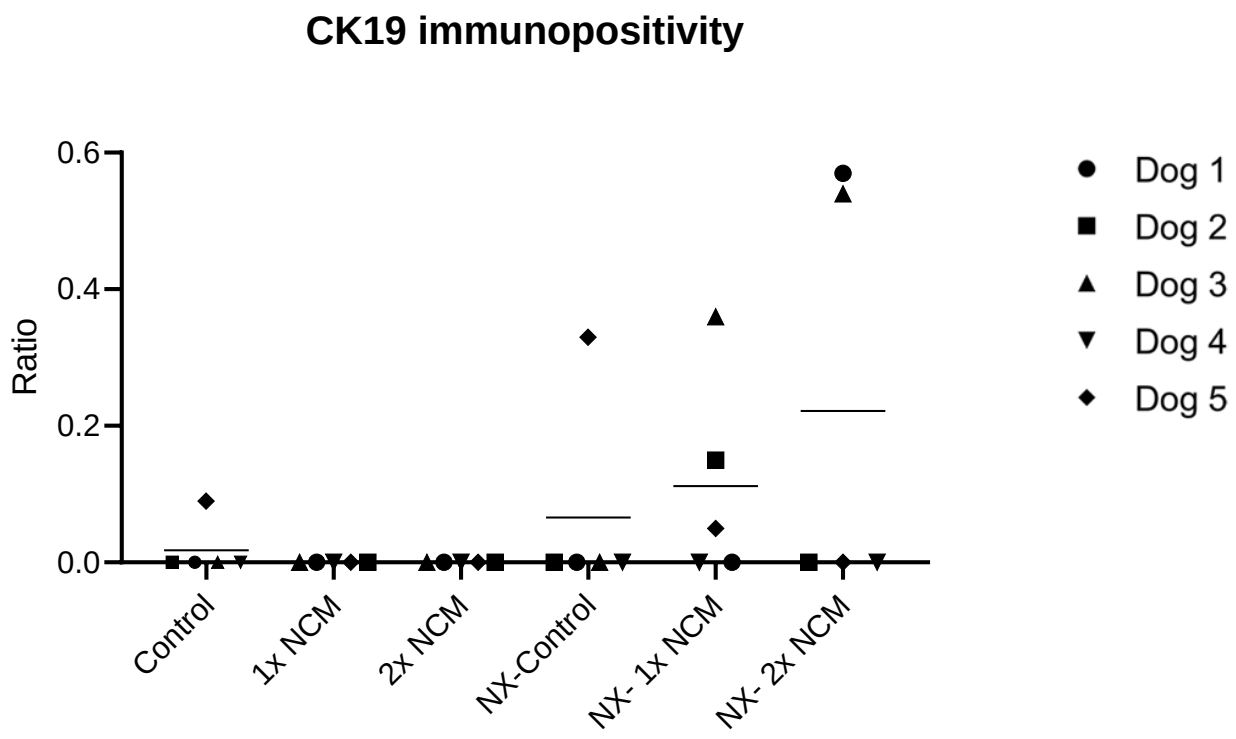

Figure 2.6 – Cytokeratin 19 immunopositivity shown as the ratio positive cells counted in two 20x magnified microscopic images of the nucleus pulposus of each donor (n=5).

Discussion

This study investigated the effect of treatment with NCM on the expression of aggrecan and CK19 in the degenerated canine IVD. It builds on the *in vivo* study Bach *et al.* (2018) in which mildly and moderately degenerated canine IVDs were subjected to no treatment, 1x NCM treatment or 2x NCM treatment [7]. Bach *et al* (2018). had already scored the IVDs using the Boos grading system, determined the DNA content, GAG content and collagen content and investigated the mRNA expression of extracellular matrix-related genes, including *ACAN* – the gene coding for aggrecan. It is interesting to compare some of these results with the results in this study.

When comparing the results of Bach *et al* (2018). regarding the *ACAN* expression and glycosaminoglycan content in the IVDs with our results regarding aggrecan content, we can conclude that in both studies, NCM had a positive effect on the GAG content. This supports the suggestion that NCM stimulates healthy matrix production of degenerated NP cells. However, Bach *et al*. (2018) did not find any difference in NP mRNA expression of *ACAN* between condition. This could indicate that the differences between the treatment groups are not large enough to be found in the gene expression, but it might also be related to the fact that the intradiscally injected porcine NCM contains aggrecan. By injecting this into the IVD, the immunohistochemical images will show an increase in aggrecan even though the canine cells are not producing more aggrecan themselves and the *ACAN* gene is thus not upregulated. Which one of the two theories holds true will have to be sought, for example by labelling the porcine aggrecan in order to delineate how much of the aggrecan visible in the treated IVDs is a direct result of the injection and how much is an indirect result of the injection where factors in the NCM stimulate the canine cells to produce aggrecan.

The obtained CK19 results are very interesting. Although some expression of CK19 is seen in the mildly degenerated discs, there is no trend seen in the CK19 positive ratio regarding the NCM treatment. In contrast, there is a trend in the CK19 positive cell ratio seen in the moderately degenerated IVDs treated with NCM. The low expression of CK19 in the mildly degenerated discs compared with the moderately degenerated discs could indicate that the induction of further degeneration is necessary to increase CK19 expression by inducing a reparative response. Although not significant, in the moderately degenerated IVDs, the CK19 expression was increased when the discs were treated with NCM. In the first part of this thesis, we detected that only NCs express CK19 which could imply that NCM treatment shifts the degenerated NP cells to a more notochordal-like phenotype. All together, these results suggest that it would be very interesting to further dive into CK19 and its relation to the regenerative capacity of nucleus pulposus cells as well as to test the effect of NCM on other phenotypic markers.

When looking at the limitations of this study, it must be stipulated that the three dimensional IVD is studied as a two dimensional slide. Even though it was tried to take a slide from the optical middle of the IVD, this was not measured and differences of the location in the IVD could influence the amount of aggrecan and presence of CK19. It is also important to highlight the fact that in each dog the same IVD segment received the same treatment. It could be that there is some variation between the location of the IVDs when looking at the stage of degeneration and the regenerative capacity.

This study, in combination with the work of Bach *et al* (2018)*.,* shows a possible potency of NCM to be used as regenerative treatment strategy for IVD degeneration. Future research should focus on the working mechanism of NCM by which it induces it positive effects on the degenerated IVD.

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General conclusions

The first study focussed on the delineation of markers to identify notochordal cells (NCs) in the canine nucleus pulposus (NP) by immunohistochemically staining the NP of a puppy, three non-chondrodystrophic dogs and a chondrodystrophic dog for six possible NC markers . The markers CD24, CD73, CK19, Brachyury and TIE2 (when located on the cell membrane) came out as possible useful canine NC markers, but further research is necessary to confirm the findings in this study, since only five dogs were used. Since the NCs disappears with age, and so do some of these markers, it would be interesting to look at the expression of these markers in different canine age groups. The question remains whether the function of the cell is influenced by the loss of these markers. Future research should focus on linking these phenotypical markers to the performance of the cells.

The second part of this study sought to investigate the effect of notochordal cell matrix (NCM) on the expression of CK19 and aggrecan in degenerated canine intervertebral discs. Immunohistochemical staining for aggrecan showed a lot of donor variation, but some positive effect on the aggrecan content is seen in the IVDs treated with NCM. Future research should aim to investigate whether the visible increase in aggrecan content is of canine or porcine origin, to delineate whether the increase in aggrecan is indeed the result of increased production of aggrecan by the canine cells in the NP. The mildly degenerated IVDs showed almost no CK19 immunopositivity, whereasthe moderately degenerated IVDs showed an increased CK19 expression when treated with NCM (non-significant). The fact that more CK19 expression is seen in the moderately degenerated IVDs compared to the mildly degenerated IVDs suggests that further degeneration might be necessary to increase CK19 expression by inducing a reparative response. It would be very interesting to further dive into CK19 and its relation to the regenerative capacity of NP cells as well as test the effect of NCM on other phenotypic markers. This study, in combination with the work of Bach *et al.* (2018) shows a possible potency of NCM to be used as a regenerative treatment strategy for IVD degeneration.