In vitro performance of degenerated disc cells

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Abstract

Degeneration of the intervertebral disc (IVD) is a common condition in dogs. Dogs can be divided into two groups with regard to a breed predisposition in the development of IVD degeneration: chondrodystrophic (CD) and Non-chondrodystrophic (NCD) dog breeds.

To research a potential regenerative therapy with the growth factors TGF-β1 and BMP-2, this study was set up to investigate the effects of these growth factors on the chondrogenic and/or fibrotic differentiation in canine Nucleus Pulposus Cells (NPCs), with regard to expression of various genes, and the differences between NCD and CD breeds regarding these.

For this research, cells from the NP of CD and NCD breeds were used. These cells were put in a pellet culture, where 4 different conditions were applied: a control, TGF-β1, in a concentration of 10 ng/mL, BMP-2 in a concentration of 100 ng/mL and of 250 ng/mL. With qPCR, expression levels of various genes involved in, among others, chondrogenesis and fibrosis, and fold changes of these genes were calculated.

The results here reported show a better chondrogenic effect of BMP-2 on the NP cells of CD dogs than TGF-β1 had in these cells. However, in these same CD dog derived cells, the samples treated with BMP-2 also showed a greater fibrotic effect compared to those treated with TGF-β1.

For NCD dogs, both TGF-β1 and BMP-2 showed comparable results in increasing expression of chondrogenic genes. However, as addition of BMP-2 possibly is associated with a higher turn-over of Collagen type II for cells derived from the NP of NCD dogs, a slight preference for BMP could be made based on the results of this results for these cells.

Introduction

Preface

The main function of the intervertebral disc (IVD) is to enable movement of the vertebrae, while maintaining the stability of the spine in total. (1) This function may be at risk if degeneration of the IVD occurs. This degeneration can lead to various diseases of the spine, so-called IVD-related diseases, such as degenerative lumbar stenosis and herniation of the IVD. Bergknut et al. (2) estimated the life time prevalence, i.e. the proportion of dogs that will develop disease, for these IVD-related diseases, to be 3,5%. However, this prevalence is much higher in certain breeds such as Miniature Dachshunds, in which the prevalence was estimated to be 20%. (2)

Degeneration of the IVD is a condition in which the cells and extracellular matrix of the IVD will be altered. This happens due to various changes in, for example, the chemical compounds of the IVD. As a result, the IVD loses the characteristics it needs to proper maintain its function of giving stability and flexibility to the spine.(3) This loss of function may lead to structural failure of the IVD, thereby creating a vicious cycle of continuous damage and inadequate repair. (1,3)

Current therapies to treat IVD degeneration focus on alleviating pain. Examples of these therapies are anti-inflammatory or analgesic medications and surgery. However, none of these therapies can repair the degenerated IVD. With regenerative treatments, this could be possible. (1)
An example of a regenerative therapy which could be used to repair a degenerated IVD, would be the use of growth factors to stimulate anabolic processes and/or inhibit catabolic processes. These growth factors can, for example, decrease cell apoptosis and/or enhance chondrogenic matrix production.

Growth factors that may be used for those regenerative therapies are TGF-β1 and BMP-2. The aim of the study reported here is to investigate the effect of these growth factors on the chondrogenic and/or fibrotic differentiation of canine Nucleus Pulposus Cells (NPCs), with regard to gene expression. Also, this study aims to examine the differences in reaction of NPCs derived from non-chondrodystrophic (NCD) and chondrodystrophic (CD) breeds. The hypothesis is that BMP-2 will have a chondrogenic effect on the canine NPCs and less fibrotic effect than TGF-β1.

Anatomy of the healthy IVD

The intervertebral disc is located between the vertebrae of the spine. The spine of the dog consists of 7 cervical, 13 thoracic, 7 lumbar, 3 sacral and a variable number of coccygeal vertebra. (3) The intervertebral discs are positioned between all vertebrae from the second vertebral body of the cervical vertebrae to the first sacral vertebra. (3)The vertebral disc consists of three layers. Its center is formed by the nucleus pulposus (NP), and its outer layer by the annulus fibrosus (AF). The connection between these two layers is formed by the transition zone (TZ) (figure 1). (3) The ventral and caudal sides of the IVD are limited by the cartilaginous endplates (Eps). The dorsal and ventral limits are formed by dorsal and ventral longitudinal ligaments. (4)

As is to be seen in figure 1, the NP of a healthy IVD is a mucoid, translucent, bean-shaped structure. (3) The ground substance of the NP is composed of proteoglycans and collagen type II. The most occurring proteoglycan in the IVD is aggrecan, which is composed of a protein backbone with negatively charged glycosaminoglycan (GAG) side chains, namely chondroitin sulfate and keratin sulfate. (3,5) These GAG's create a strong osmotic gradient, attracting water into the NP. Therefore, the NP consists for over 80% of water.

The most common cell type in the healthy NP is the notochordal cell. These are large cells, characterized by cytoplasmatic vessels. They have few mytochondria and are therefore believed to rely mostly on anaerobic metabolism. Notochordal cells are found in clusters and produce the matrix of the NP.(6,7)

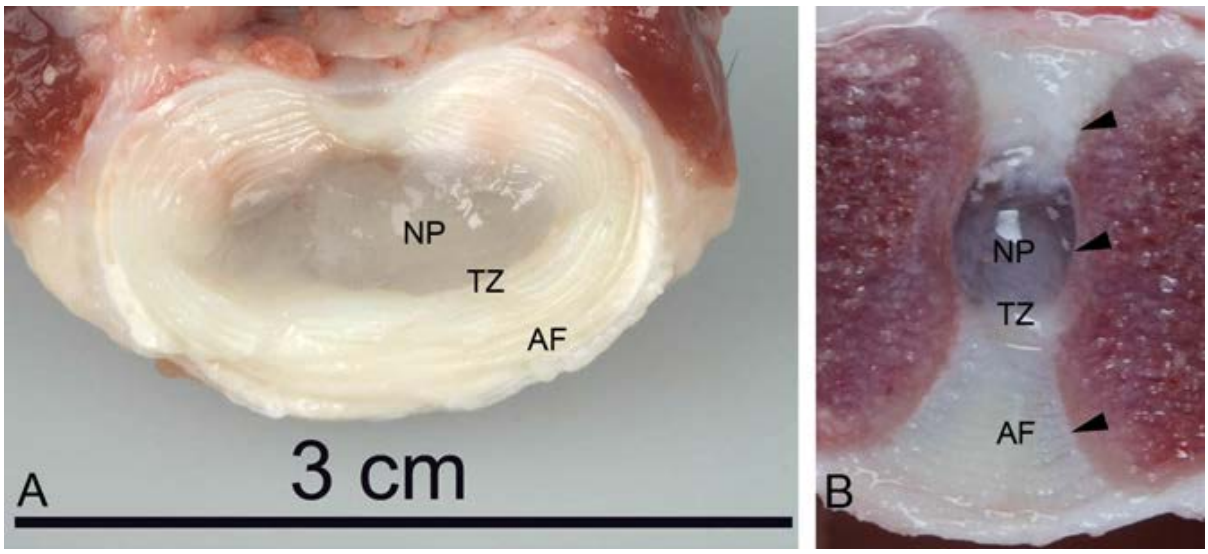

Fig. 1. Transverse (A) and sagittal (B) sections through a L5–L6 intervertebral disc of a mature NCD dog. The arrowheads point out the EP.(3)

The outer layer, the AF, of the healthy vertebral disc is composed of a dense network of multiple, organized, concentric fibrous lamellae. These lamellae consist mainly of collagen type I. It is known that these lamellae have an oblique orientation that alternates in direction for layers of successive lamellae in humans, and probably also in dogs. (5,8) The cells in the outer layer of the AF are fibrocyte-like cells, whereas a mixed population of fibrocytes and chondrocyte-like cells appears in the more inner layers. Towards the center of the IVD, the AF becomes more cartilaginous and less fibrous. This zone forms the interconnection between the AF and the NP and is called the transition zone. (3,8)

The EP's are positioned between each vertebral body and the NP and AF of the IVD (fig. 1 B). They consist of hyaline cartilage, with adjacent blood vessels. The EPs have an important function in supplying nutrients to the IVD. As only the outer layers of the AF have a, limited, blood supply, the deeper parts of the IVD are depending on diffusion and osmosis from capillary buts through the EPs. Larger molecules are transported by a pumping mechanism created by physiological loading of the IVD. (5,6)

Function of the IVD

The biomechanical function of the IVD is 'to transmit compressive forces between vertebral bodies and to provide mobility as well as stability to the spinal segment'. (6) This function is achieved in that an IVD can act like a water bed: the inner NP behaves like a fluid and the outer AF as an elastic layer that can restrain the NP. (5)

The IVD can be subjected to five loading conditions: axial compression, shear, tension, bending and torsion. During axial compression, a compressive force is applied through the centre of the NP, causing the disc to decrease in height and to become wider. The NP will absorb most of this compressive load, thereby providing a counter pressure in all directions. The AF protects the NP against shearing due to the applied load and the internal swelling pressure by absorbing this pressure, thereby resisting collapse of the IVD (3,9).

Bending, torsion and shear loads are considered likely to result in traumatic disruption of the IVD, and are therefore resisted by the activity of other stabilizing elements of the vertebral column, such as ligaments of the spine and articular facets (9). During bending of the IVD, the hydrostatic pressure in it will increase, causing the fibres of the AF to change orientation. However, the capacity of the IVD to resist bending is directly related to the volume of the NP. (3)

The IVD is rarely exposed to pure tensile loads, because the trunk muscles are constantly compressing the IVD. (3)

Degeneration of the IVD

Degeneration of the IVD can be described as an 'aberrant, cell-mediated response to progressive structural failure'. (5) It is a multifactorial process, characterized by changes in the cells and matrix of the NP, TZ, AF and EPs. Degeneration of the IVD is a result of a combination of pathological and age-related changes. During regeneration, a decrease in GAG content and an increase in collagen content will occur, resulting in loss of water and a more rigid structure of the IVD. (3,8) Because of the loss of water, the IVD will no longer be able to fulfil its role as a hydraulic cushion. (3,8)

With IVD degeneration, the ability of the IVD to withstand normal stress levels will decrease. Normal stress levels can therefore cause damage in a degenerated IVD. As the IVD has a low regeneration capacity due to it having little blood supply and a small number of cells, and as the matrix of the degenerated IVD is of inferior quality, the obtained damage will be inadequately repaired. Therefore, the IVD will get even more easily damaged, and so on. This way, a vicious cycle of damage and inadequate repair will arise, resulting in more degeneration.(3) An example of this cycle is given in figure 2.

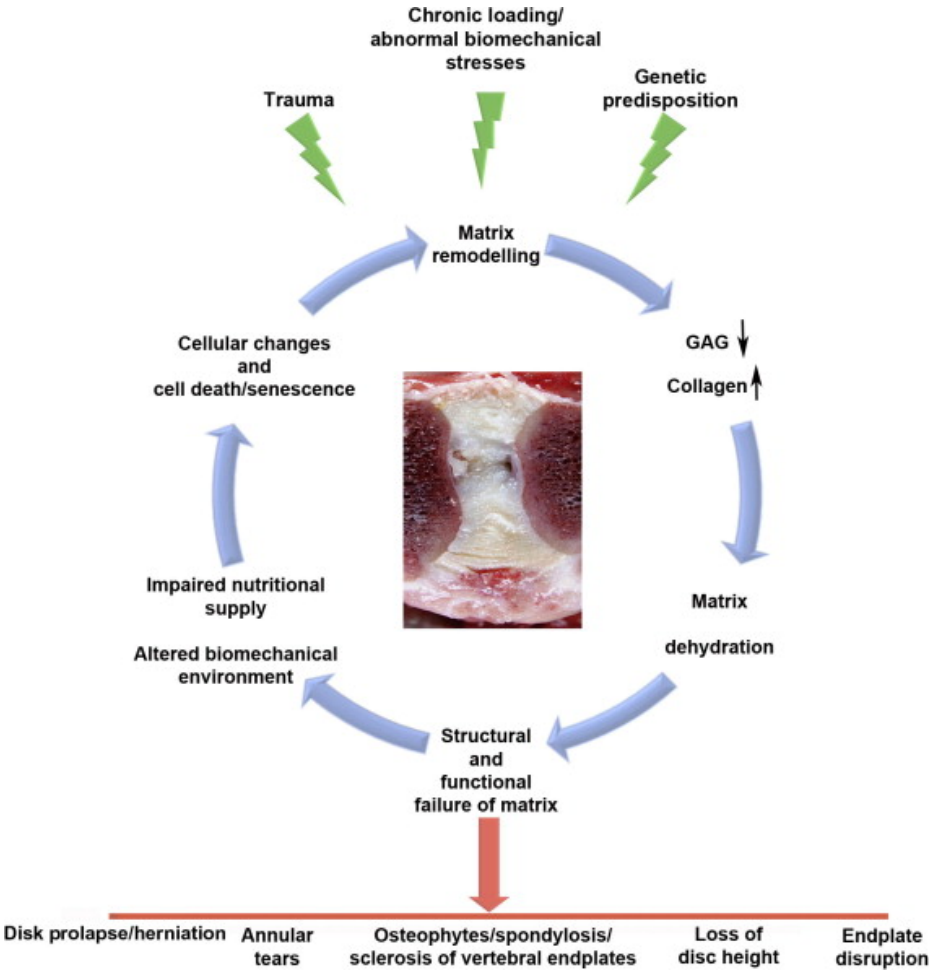

Figure 2. The vicious cycle of IVD degeneration, showing the factors and consequences of the process of degeneration. (3)

Histologically, early degeneration of the IVD is characterized by changes in the cells of the NP: the large clusters of notochordal cells which are normally seen in the NP will disappear and they will be replaced by smaller clusters of notochordal cells or chondrocyte-like cells. Also, the matrix will change to a more hyaline cartilage-like matrix with disorganized collagen fibers. This process is called chondrification. (3) In the AF the cross-links between the annular fibers will increase in number, thereby preventing the movement of the lamellae. This way the AF will be unable to perform its

normal function. The EPs will become thicker in early stages of IVD degeneration and more irregular in later stages, and may then disrupt.(3)

The biomechanical function of the IVD will be affected by the degeneration. Due to the reduced proteoglycan content of the NP, dehydration and loss of NP size. The AF will take over the load-bearing function of the NP. The consequence of this is an increase in size of the AF, thereby causing it to become stiffer and weaker. As a result, the AF will not be able to resist tensile forces, causing the IVD to bulge when exposed to physiological loading. Also, annular defects or tears can occur, through which NP material can extrude. Due to the dysfunction of the IVD other spinal components, such as ligaments, facet joints and vertebral bodies, will be affected. Possible consequences of this are osteoarthritic changes, sclerosis and spondylosis. (3)

The ethology of degeneration of the IVD is still not entirely clear. It is a multifactorial disease, and is associated, as partly explained above, with chronic physicomechanical overload, trauma, inadequate metabolite and nutrient transport, cell senescence and death, altered levels of enzyme activity, changes in matrix macromolecules, changes in water content, age and genetic predisposition. (3,5)

Breed predisposition

Based on genetic predisposition for IVD degeneration, dogs can be divided into two groups: chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds. CD breeds are characterized as dogs with disproportionally short limbs. These short limbs are due to a disturbed endochondral ossification (10). Examples of such breeds are the dachshund, the French and English bulldog and the Basset Hound. A study to the incidence of IVD-degeneration related diseases (2), such as herniation and Degenerative lumbosacral stenosis, found a higher prevalence of these diseases in CD breeds. However, some NCD breeds, such as German Shepherd Dogs and Doberman Pinschers, seem also to be genetic predisposed for diseases associated with IVD degeneration. (2)

Apart from this difference in incidence, CD and NCD dogs differ in mean age of developing the disease. In CD dogs, IVD degenerative disease develops around 3-7 years of age, but degenerative changes can already be found in these CD dogs as young as 3-4 months of age. In contrast, NCD dogs develop disease later in life, often when they are around 6-8 years of age. In CD dogs, the regions most affected by IVD degeneration are the cervical or thoracolumbar spine, but the degeneration process does simultaneously occur in all discs of the vertebral column. This is not the case for NCD dogs, where the degeneration often occurs in only one disc, and effects of the degeneration are mostly seen in the caudal cervical or lumbosacral spine. (8,10)

One of the reasons for this differences in IVD degeneration in CD and NCD dogs could be a difference in cell type. The normal cell type in the juvenile, healthy NP is the notochordal cell. However, with ageing and early degeneration of the IVD, these cell type will be replaced by chondrocyte-like cells. In NCD dogs the notochordal cell will remain the most common type until middle or old age. In CD dogs however, this replacement will occur before 1 year of age. (11,12)

Growth factors

The two growth factors used in this research, Transforming Growth Factor-β1 (TGF-β1) and Bone Morphogenetic Protein 2 (BMP-2), both have an up-regulating effect on the synthesis of the proteoglycan aggrecan and GAG synthesis. (13) As loss of proteoglycans, especially aggrecan, is important to sustain the high water content of the NP(5), these growth factors are of high interest in research to regenerative treatment of IVD degeneration.

Research has also shown that BMP-2 and TGF-β1 upregulate mRNA levels for type I and type II collagen in human IVD cells. (13) However, another research to the effect of BMP-2 on matrix production in rat IVD cells showed an upregulation of collagen II, but not of Collagen I.(14) Both collagen I and II are fibrillar molecules. (13,14) But, whereas collagen II is a major component of the healthy IVD, collagen I synthesis is increased during degeneration, and this contributes to fibrosis of the IVD.(15)

BMPs, such as BMP-2, are members of the TGF-β superfamily, which also includes, among others, TGFβs (13,16). BMPs have a wide range of biologic activities, because they regulate growth, differentiation, chemotaxis and apoptosis in various cell types and organs.(16)

BMP-2 is known as an osteogenic protein, and its pathway does result in bone and cartilage formation. BMP-2 can act on cells involved in the ossification of bones via the Smad-1/5/8 pathway (17). This Smad pathway is activated when the BMP binds to its receptor (BMPR). This receptor is composed of 2 proteins, known as type I and type II receptors (14,16,18), and these types can each be divided into three subtypes, all of which can be bound by BMPs. BMP-2 uses two type I receptors, named ALK 3 and 6, and 2 types of type II receptor.(17) When BMP-2 binds to its receptors, it activates an intracellular protein called 'receptor regulated Smad' (R-Smad), which is made up of Smad 1, 5 and 8. Eventually, this pathway will activate the transcription of osteoblast-specific genes.(17)

Apart from its functions as an osteogenic protein, BMP-2 is also known as a differentiation factor that turns mesenchymal cells into cartilage- or bone-forming cells. It can stimulate the differentiation of some cell types, for example fat and muscle cells, toward cells of the osteoblastic lineage. (16) It can also maintain articulate chondrocyte phenotype in long-term cultures (14).

TGF-β1 is also a member of the TGF –β superfamily. It regulates the transcription of genes using the Smad-pathway, like BMP-2. However, unlike BMP-2, it can next to the Smad 1/5/8-pathway activate the Smad 2/3 pathway. According to Blaney Davidson et al (19), who researched the role of TGF- β1 in osteoarthrosis, the Smad 2/3 pathway is the most common used pathway in young mice. This pathway is activated by binding of TGF- β1 to its type II receptor, which in turn recruits ALK-5. This complex phosphorylates Smad 2/3. This pathway is known to be important in maintenance and protection of healthy cartilage. However, TGF- β1 can also signal via ALK-1, thereby activating the Smad 1/5/8-pathway. This pathway stimulates Collagen II degradation via MMP-13. It is proposed by Blaney Davidson et al. that, with higher age, the ALK1/ALK5 ratio increases, causing Smad 1/5/8 to become dominant relative to Smad 2/3.

Materials and methods

Experimental design

For this research, cells from the NP of CD and NCD breeds were used. From both CD and NCD breeds, cells from 5 donor dogs were used. These cells were obtained from a biobank. These cells were put in a pellet culture. Four different conditions were applied. These conditions were: a Control, TGF-β1 (R&D systems, Minneapolis, MN, USA), in a concentration of 10 ng/mL, BMP-2 (TETEC, Reutlingen, Germany), in a concentration of 100 ng/mL and BMP-2 (TETEC, Reutlingen, Germany) in a concentration of 250 ng/mL. These conditions were each applied to NP cells of both CD and NCD dogs. Each condition was used on 7 pellets of CD dog derived NP cells, and 7 pellets of NCD dog derived NP cells. As there were 4 conditions, this makes a total of used pellets of 56 pellets used in this research. The samples used in this research were collected at the 7th day of the experiment.

Crushing, RNA isolation and cDNA synthesis

The pellets were crushed using plastic 'pellet pestles' (Qiagen, Austin, TX, USA). The pellets were thrown into liquid nitrogen, when they froze, they were crushed using the pestles until they fell apart. Then, 350 µl RLT+β-mercaptoethanol was added and the sample was put on dry ice before they were stored at -70˚C. After the pellets were crushed, the RNA was isolated using RNeasy Micro kit (Qiagen, Austin, TX, USA). And for the synthesis of cDNA, the iScript^{TM} cDNA Synthesis Kit (Bio-rad Laboratories, Hercules, CA, USA) was used according to manufacturer's instructions.

qPCR

Glyceraldehyde phosphate dehydrogenese (GAPDH), hypoxanthine phosphoribosyl transferase (HPRT), Ribosomal protein S19 (RPS 19) and succinate dehydrogenase (SDHA) were used as housekeeping genes and used to normalize results. The genes measured in this research can be divided into 6 categories: chondrogenic markers (*SOX 9, Aggrecan (ACAN), Collagen IIA1* (*Col2A1*), and *a disintegrin metalloproteinase with thrombospondin motifs-5* (*Adamts-5)*), a fibrotic marker (*Collagen type I* (*Co I)*), proliferation markers (*Cyclin D1*), markers for activation of the SMAD pathway (*Activin receptor-like kinase-1* and *-5* (*ALK-1 and ALK-5), plasminogen activator inhibitor 1* (*PAI-1), DNA-binding protein inhibitor domain 1 (ID-1)*), markers for the hypertrophy and maturation of chondrocytes(*Axis inhibition protein* (*AXIN2*), *Collagen type X* (*Coll X*), *matrix metallopeptidase 13* (*MMP-13*), and *tissue inhibitor of metallopeptidases* (*TIMP-1*)) and markers for apoptosis (*Bcl-2-associated X protein* (*BAX*), *B-cell lymphoma 2* (*Bcl 2*) and *caspase 3* (*CASP3*)). For all genes, forty cycles of denaturation, annealing and elongation were done.

Analysis

The results of the qPCR were analysed using Bio-RAD CFX manager to exclude dimers and obtain an efficiency of 100% for the standard line. When a sample did not show a value for multiple genes, it was left out of the analysis.The ΔCt values for the different genes were standardized using the mean value of the housekeeping genes. Then they were normalised relative to the Control group of the CD cells. The positive N-fold change was calculated by calculating (10^(-1/slope) to the power of the ΔCT values of the corresponding sample. For making the graphs, the mean values of these N-fold changes and its standard deviations were used. For the statistical analysis, first a tests of normality was done to measure whether the data was normal or non-parametric distributed. If the data were normally distributed, a 1-way-ANOVA with Tukey as a post-hoc test was done, and if they were non-parametric distributed a Mann-Whitney U test was conducted.

Results

The genes used in this research project can be divided into 6 general groups: chondrogenic markers, fibrotic markers, proliferation markers, markers for activation of the SMAD pathway, markers for the hypertrophy and maturation of chondrocytes and markers for apoptosis. The results are given in figures 3,4,5,6 and 7 and are presented as a fold change relative to the mean of the CD control sample, which was used to normalize the ΔCt values.

Chondrogenic markers:

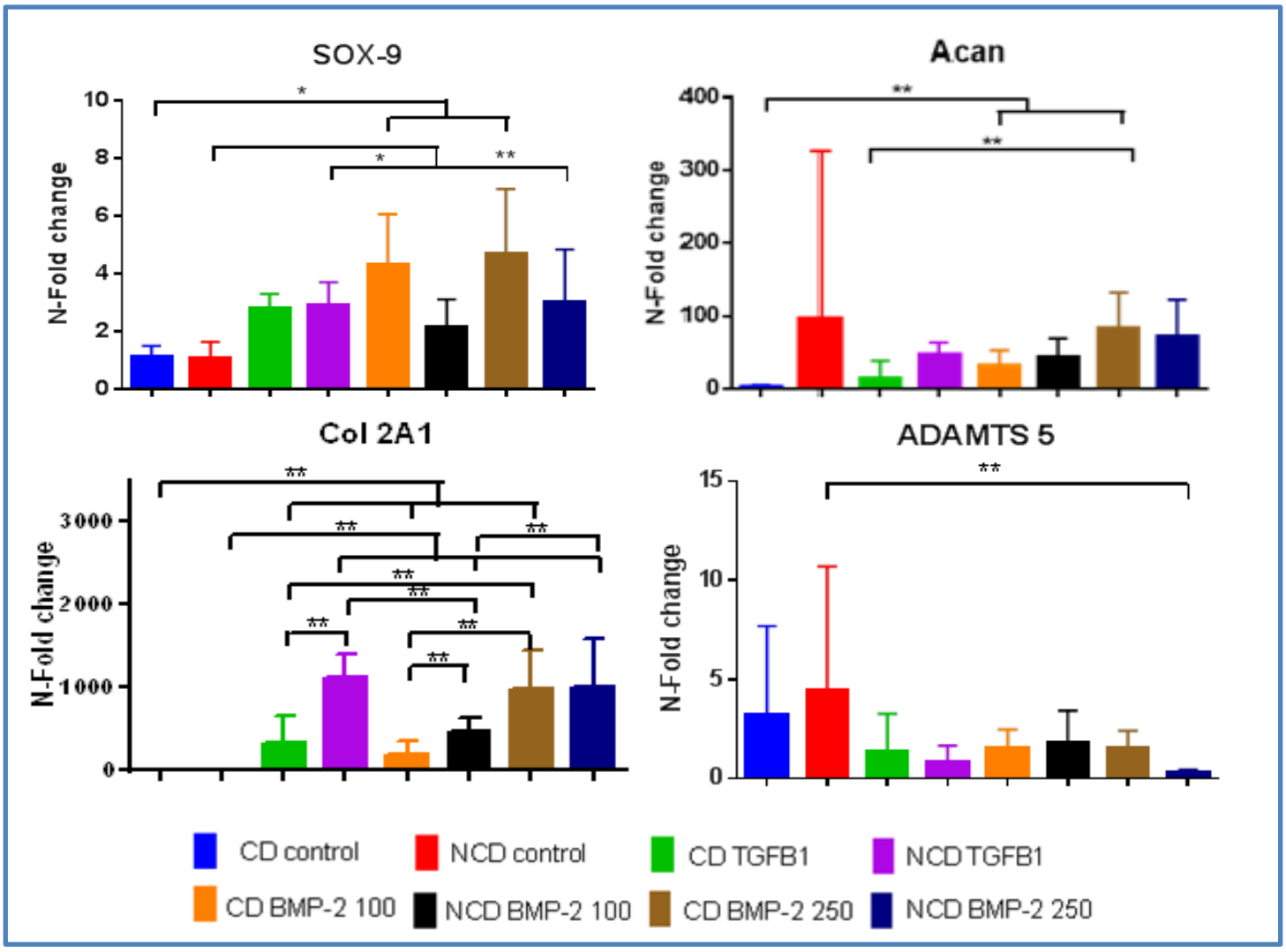

Figure 3: mean mRNA expression of chondrogenic genes: *SOX-9, Acan, Col 2A1* and *ADAMTS 5*. The bars represent the significant differences between conditions. (*p<0.01, **p<0.05)

Genes with a role in the chondrogenesis of the IVD, used in this research, are: *SOX 9, Aggrecan* (*ACAN*), *Collagen IIA1* (*Col2A1*), and *Adamts-5*. The mean mRNA expression levels of these genes are shown in figure 3 in fold changes.

The expression level of *Sox-9* was significantly increased compared to the CD control for both CD BMP-2 conditions. Compared to the NCD control, expression in the NCD conditions of TGF-β1 and BMP-2 (250) was higher.

For the cells derived from CD dogs, a significant increase was shown in the expression of *ACAN* in cells which were treated with BMP-2 (100) and BMP-2 (250). The increase of *ACAN* expression was also significant for CD BMP-2 (250) in comparison to CD TGF-β1.

For *collagen II*, an increase in expression for all CD conditions was found compared to the CD control condition. The expression levels of all NCD conditions were also increased relative to the NCD control. The expression of *Collagen II* was significant lower in CD TGF-β1 compared to NCD TGF- β1 and CD BMP-2 (250). NCD TGF-β1 was significantly increased relative to NCD BMP-2 (100). The expression of *collagen II* was lower for CD BMP (100) than for NCD BMP-2(100) and CD BMP-2 (250). Lastly, a decrease of NCD BMP-2 (100) relative to NCD BMP-2 (250) was found.

No differences in expression levels of *Adamts-5* were found except for a decrease in expression in NCD BMP-2 (250) cells in comparison to NCD control.

Fibrotic marker

The expression of the fibrotic marker *Collagen type I* is shown in figure 4. The expression of this gene was increased in the CD condition for BMP-2 (250) compared to the control. Expression in the NCD control was also higher than in the CD control. In CD TGF-β1, expression was lower than expression in CD BMP-2 (250) and NCD TGF-β1. In the NCD TGF-β1 condition, there was a significantly higher expression of *Col I* compared to NCD BMP-2 (250).

Proliferation marker

Cyclin D1 expression is shown in figure 4. This gene is a marker for cell proliferation. Its expression was significantly higher compared to the CD group in the NCD control and both CD BMP-2 conditions. For CD TGF-β1, the expression of Cyclin D1 was significantly higher in NCD TGF-β1, CD BMP-2 (100) and CD BMP-2 (250).

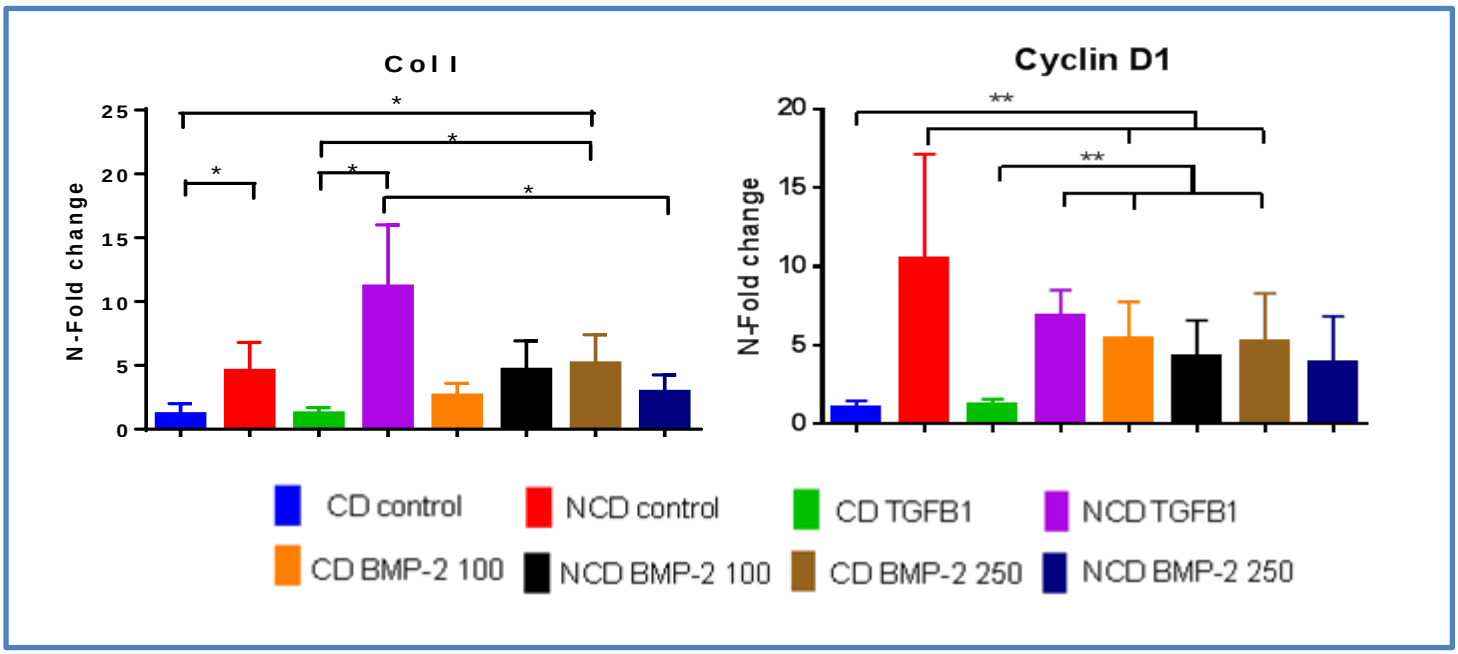

Figure 4: expression of the fibrosis marker *Col I*. And the proliferation marker *Cyclin D1*. The bars represent the significant differences between conditions. (*p<0.01, **p<0.05)

Markers for activation of the SMAD-pathway

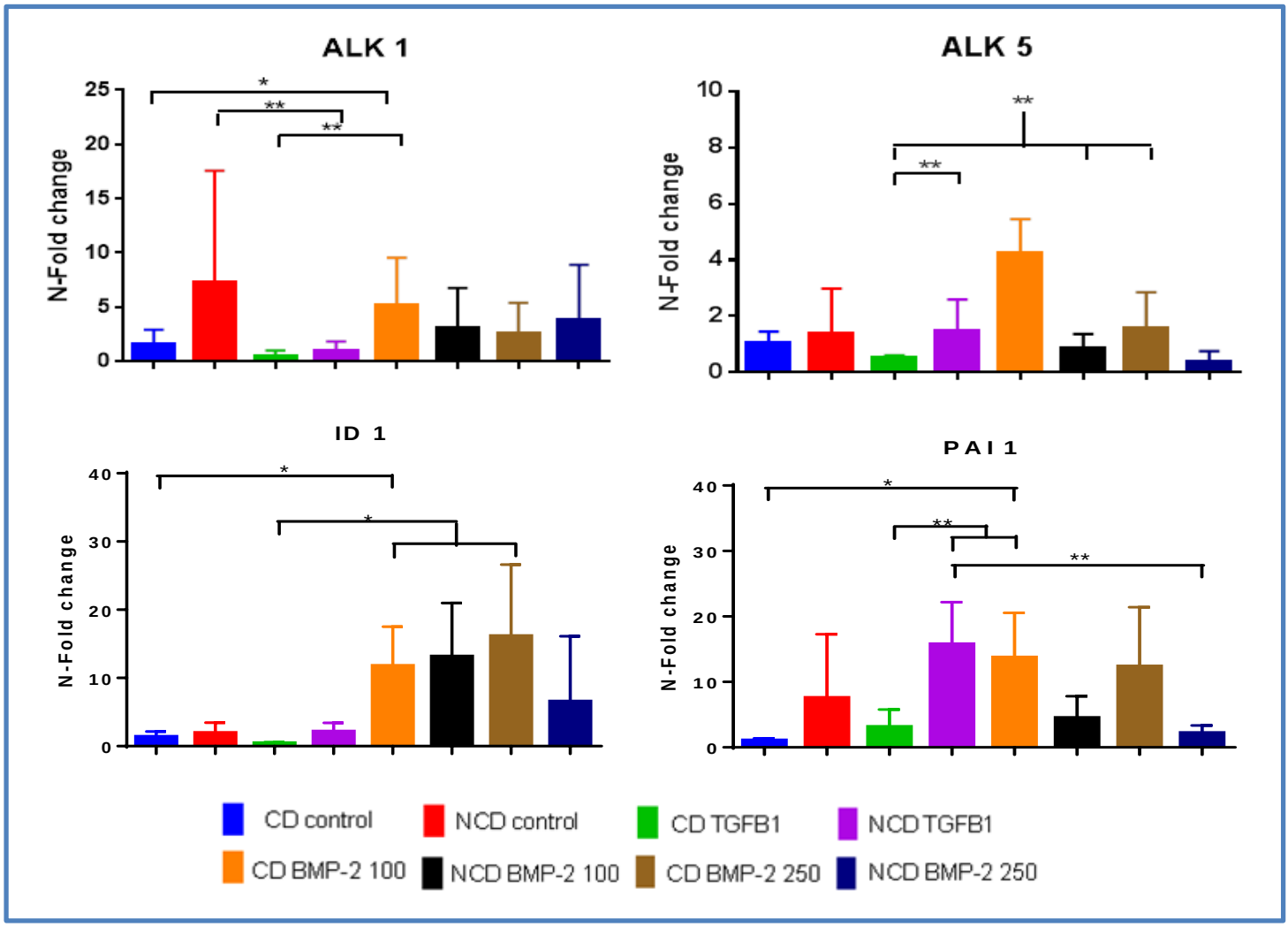

Figure 5: mRNA expression of genes that indicate an activation of the SMAD pathway: *ALK-1, ALK-5, ID 1* and *PAI 1*. The bars represent the significant differences between conditions. (*p<0.01, **p<0.05)

Genes that are involved in the mechanism of action of SMAD-pathways, important signalling pathways in the mechanism of action of TGF-β1 and BMP-2, are *Activin receptor-like kinase-1* and *-5* (*ALK-1 and ALK-5*), these are genes coding for receptors of TGF-β1. Genes used as read outs for the two SMAD-pathways relevant for this research were *DNA-binding protein inhibitor domain 1* (*ID 1*) and *plasminogen activator inhibitor 1* (*PAI-1*). The mean fold change of the samples for these genes is shown in figure 5.

An increase in the expression of *ALK-1* was found for CD BMP-2 (100) condition compared to the CD control group. A decrease was found for NCD TGF-β1 relative to the NCD control. Also the expression of *ALK-1* in CD BMP-2 (250) was higher than in CD TGF-β1.

For the expression levels of *ALK-5* no changes relative to the control groups were to be found. However, increases of expression in CD BMP-2 (100) compared to CD TGF-β1, CD BMP-2 (250) and NCD BMP-2 (100) were found. A significant higher expression was also found in NCD TGF-β1 compared to CD TGF-β1.

In *ID-2* a higher expression relative to CD control was found for CD BMP-2 (100). Expression of CD TGF- β1 was lower than the expression levels in CD BMP-2 (100) and CD BMP-2 (250).

For *PAI-1* expression, expression in CD BMP-2 (100) was higher than the expression in the control. The expression in BMP-2 (100) was increased compared to expression in CD TGF-β1, just like the expression in TGF-β1. Expression in CD BMP-2 (250) was significantly higher than in NCD BMP-2 (250).

Markers for hypertrophy and maturation of chondrocytes

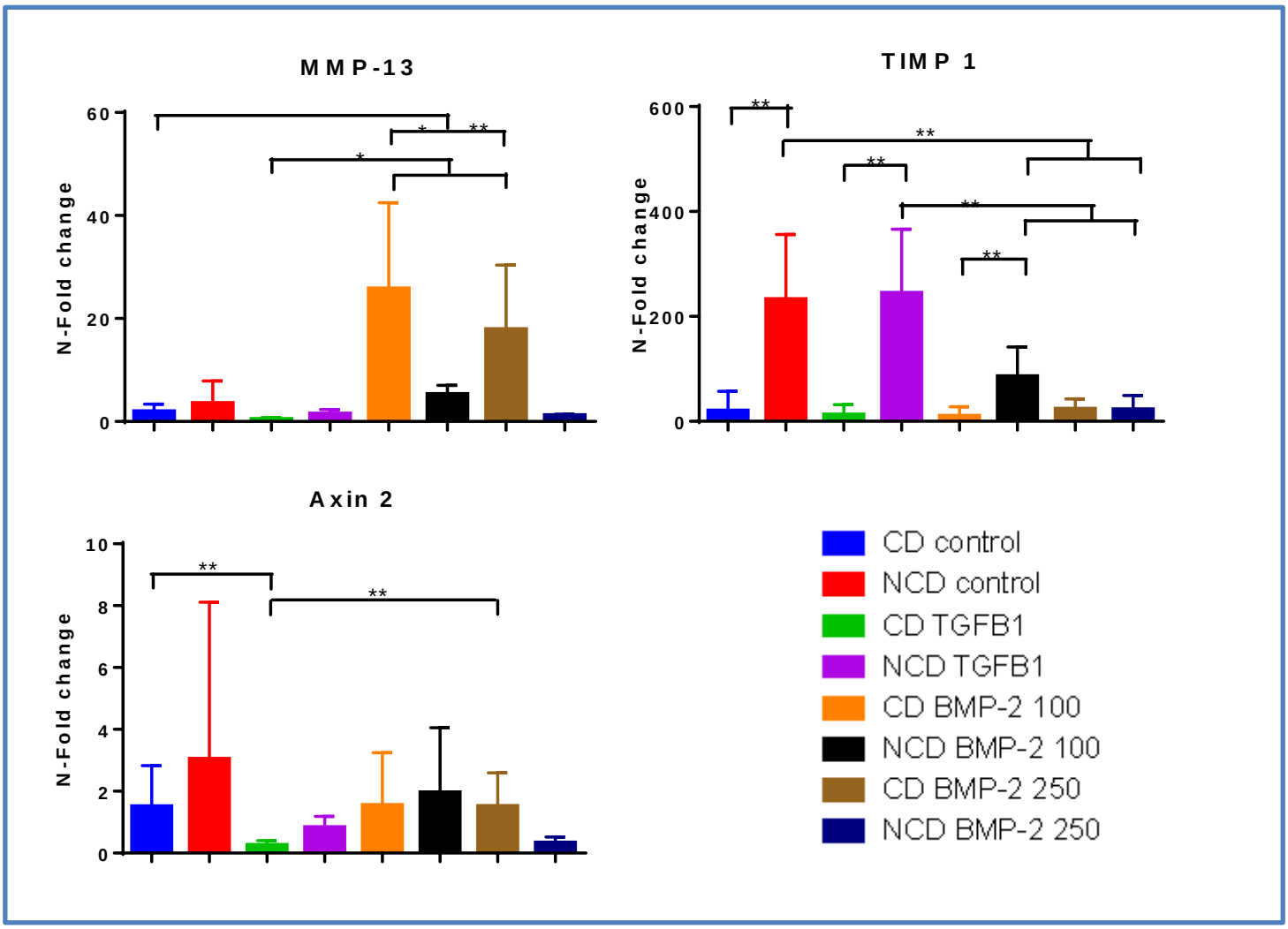

Figure 6: mRNA expression of genes for hypertrophy and maturation of chondrocytes: *MMP-13, TIMP* and *Axin 2*. The bars represent the significant differences between conditions. (*p<0.01, **p<0.05)

Axis inhibition protein (AXIN2), Collagen type X (Coll X), matrix metallopeptidase 13 (MMP-13), and tissue inhibitor of metallopeptidases (TIMP-1) are genes involved in the hypertrophy and maturation of chondrocytes.

The expression of *MMP-13* was significantly increased in comparison to the CD control group for CD BMP (100) and CD BMP (250). These two conditions did also have a higher expression compared to CD TGF-β1.

None of the expression levels of *TIMP 1* from CD-dog derived NP cells were significantly higher or lower compared to the CD control. However, the expression in the NCD control condition was increased compared to CD control. The NCD control had a significantly higher expression of *TIMP-1* compared to NCD BMP-2 (100) and NCD BMP-2 (250). Expression in NCD TGF-β1 was significantly higher than the expression in CD TGF-β1, NCD BMP-2 (100) and NCD BMP-2 (250). CD BMP-2 (100) had a significantly lower expression than NCD BMP-2 (100).

For AXIN2, significant decreases in gene expression in the CD TGF-β1 condition when compared to the CD control, and to the BMP-2 (250) condition, were found.
No expression levels were to be found for Collagen X.

Markers for cell apoptosis

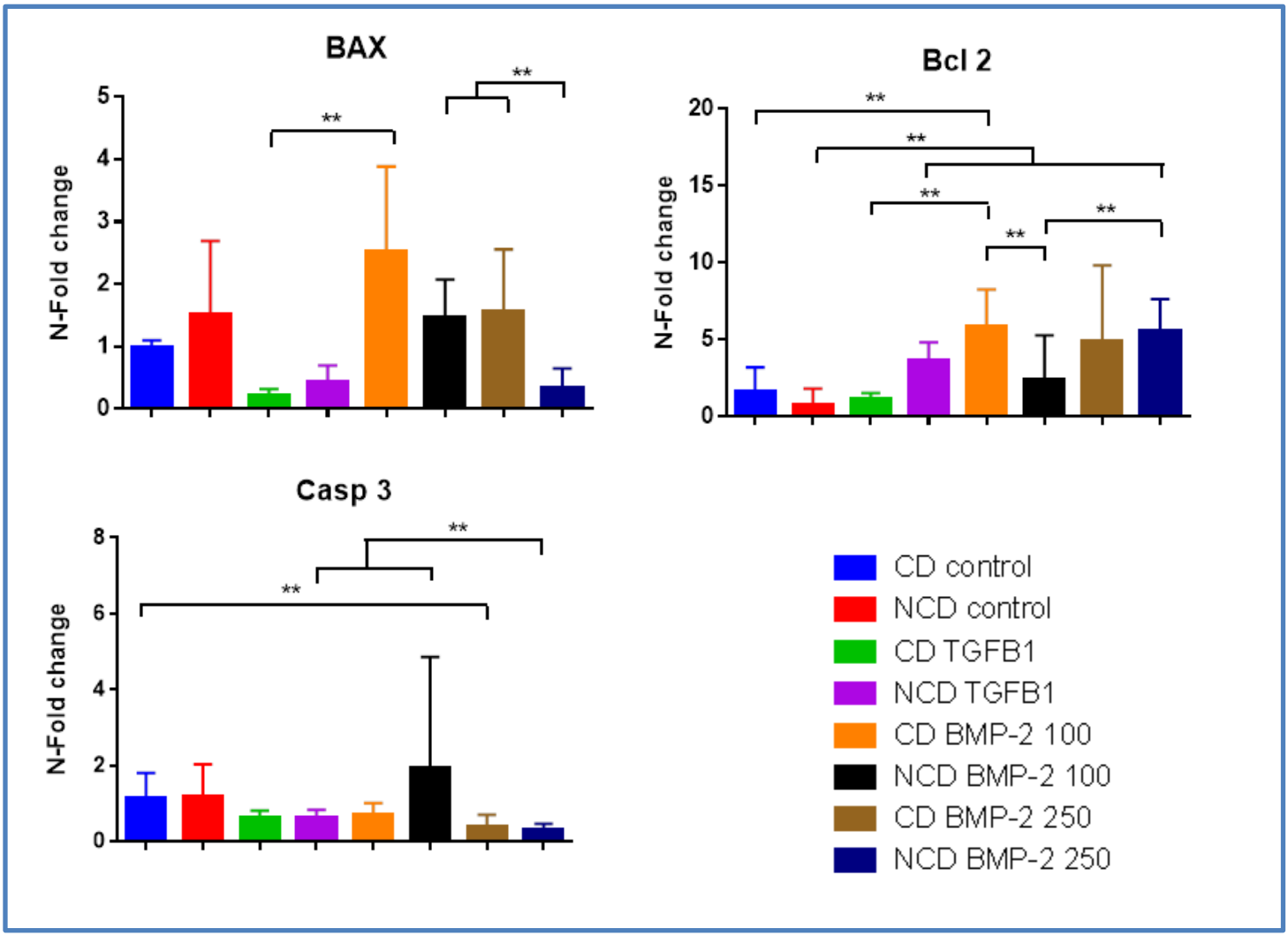

Figure 7: mRNA expression of the markers for apoptosis: *BAX, Bcl 2* and *Casp 3*. The bars represent the significant differences between conditions. (*p<0.01, **p<0.05)

Bcl-2-associated X protein (*Bax*), *B-cell lymphoma 2* (*Bcl 2*) and *caspase 3* (*CASP3*) are regulators of cell apoptosis and where therefore measured in this research. Their N-fold changes are shown in figure .

Bax expression showed no significant changes relative to both control conditions. However, NCD BMP-2 (250) showed a significant lower expression compared to both NCD BMP-2 (100) and CD BMP-2 (250). Also, CD TGF-β1 showed a lower expression than CD BMP-2 (100).

The only higher expression of *Bcl-2* compared to the CD control condition was found for CD BMP-2 (100). Compared to NCD control expression in NCD TGF-β1 and in NCD BMP-2 (250) was increased. Expression in CD TGF-β1 was lower relative to CD BMP-2 (100). NCD BMP-2 (100) had a lower expression compared to CD BMP-2 (100) and compared NCD BMP-2 (250).

For *CASP 3*, the expression in CD BMP-2 (250) expression was lower than the expression in the CD control sample. Expression levels in NCD TGF-β1 and NCD BMP-2 (100) were higher than the expression level in NCD BMP-2 (250).

Discussion

Chondrogenic effects of TGF-β1 and BMP-2

To measure the response of the NP cells on TGF-β1 and BMP-2 regarding chondrogenesis, we estimated the expression of *Sox-9*, *Aggrecan*, and *Collagen type II.* These genes are related to the production of aggrecan and Collagen type II, both important components of the normal NP(3), whose synthesis decreases in the process of degeneration. (13,15) Also, expression of *Adamts 5* was measured, a gene encoding for an aggrecanase.

Sox-9 was measured in this study because it is a gene that has been shown to mediate both Aggrecan and Collagen IIAI production in human NP cells (13), so one would expect the same effect in expression of both these genes for the NP cells from CD and NCD dogs used in this study. In CD dogs, the increased expression of *Sox-9* matched indeed largely with the increase in *Aggrecan* expression for both BMP groups compared to the control group, while *Collagen type II* expression increased by both TGF-β1 and BMP-2 treatment.

In the NCD conditions *Sox-9* expression was increased for TGF-β1 and BMP-2 (250), whereas for *Aggrecan* expression, no differences in fold changes where found. This could be due to the high standard deviation in the control group for expression of *Aggrecan*. A possible explanation for this high standard deviation could be that expression of *Aggrecan* already occurs in NCD NP cells that are not treated with growth factors. Possibly, this is due to the fact that notochordal cells can be found in the NP of NCD dogs (20), and these cells have shown to be able to maintain the expression of A*ggrecan* in chondrocytes.(21)

Nonetheless, *Collagen type II* expression was increased in all NCD conditions treated with growth factors compared to the control group. Whereas for expression of Sox 9, BMP-2 (100) did not increase its expression. This indicates that in expression of *collagen type II*, other genes or factors , along with Sox 9, may also play a role in the regulation of its expression. An example of such a factor could be an increase in expression of BMP-7, which is known to also increase collagen type II and aggrecan production and which expression has shown to be increased by BMP-2. (14)

On the overall*, Collagen type II* expression was increased in all conditions compared to both control groups, indicating a good chondrogenic effect of both TGF-β1 and BMP-2 on both CD and NCD NP cells. However, this effect seems to be greater for NCD TGF- β1 and BMP-2 (100) than for CD TGF-β1 and BMP-2 (100). This could indicate that TGF-β1 in NCD dogs is more effective in signaling by means of the ALK-5 receptor than in CD dogs. However, one should then also expect a similar result for *Aggrecan* expression (19). Also, for NCD dogs, TGF- β1 seemed more potent in increasing the expression of *Collagen type II*, than BMP-2 (100) as the fold change for this condition was higher.

Furthermore, *Adamts 5,* a gene that encodes for an aggrecanase, was decreased only for NCD BMP-2 (250), suggesting that in this condition less breakdown of the Aggrecan occurred resulting in an overall beneficial effect at the biochemical level.

In summary, our results indicated that in up-regulating genes for chondrogenesis, BMP-2 was the most potent growth factor for CD breeds, as it upregulated both *Aggrecan* and *Collagen type II*. In contrast, TGF-β1 and both BMP-2 (250) were most effective in NCD breeds, as they upregulated *Collagen type II*. Furthermore, BMP-2 (250) did also decrease the expression of *Adamts 5,* thereby probably reducing the breakdown rate of Aggrecan. However, to determine whether this result would also be found in the composition of the extracellular matrix, and not only in gene expression, future research, for example a GAG-analysis, would be needed.

Fibrotic effects of TGF-β1 and BMP-2

Collagen type I is a marker for fibrosis in the intervertebral disc. In humans, it's production increases with ageing of the intervertebral disc, also, collagen type I fibres replace type II fibres, causing the disc to become coarser. (5,15)

Our results showed an increase in expression of *Collagen type I* in NP cells from CD dogs treated with BMP-2 (250), whereas NCD dogs TGF-β1 seemed to increase its expression. This increase could indicate more fibrosis in these conditions, but also an increase in a more fibrocyte-like cell type rather than the chondrocyte-like NP cells (22). However, one would expect a simultaneous decrease of expression of *Collagen type II* and *Aggrecan.* Our results did not show such a decrease, as CD BMP-2 (250) and NCD TGF-β1 were among the main conditions that caused increased expression of *Collagen type II*. A possible explanation could be that that a change from the chondrocyte-like NP cell to a fibrocyte-type cell is occurring in these samples,but that both cell types are present at the moment. However, to examine this, and the analysis of the samples at a later time in the experiment could be useful, as the samples used in this research were taken on the seventh day.

The expression of *Collagen type I* was higher in the NCD control group compared to the CD control. This indicates that there is already more fibrosis in these samples. Especially as neither *Aggrecan* nor *Collagen type II* expression was increased in these condition. This result is contrary to what one would expect, as CD dogs have a NP with a higher collagen type I content than the NP of NCD breeds. (10)

So with regard to fibrosis, BMP-2 (250) would probably lead to an increase in the production of collagen type I in CD dogs, contrary to NCD dogs, where TGF-β1 increased the expression compared to BMP-2 (250).

Effects on proliferation of TGF-β1 and BMP-2

Cyclin D1 is a cell cycle regulator (23) and its expression can therefore be used as an indicator for the cell proliferation. It is induced by Wnt/β-catenin activation, an important signalling pathway in, among others, promoting the differentiation of stem cells.(23) It is suggested that activation of this pathway supports the proliferation of cells within the degenerating intervertebral disc. So an increase in *Cyclin D1* expression can be reflecting a regenerative response(23).

Expression of *Cyclin D1* was increased in the NCD control group compared to the control condition of CD. This could suggest that the proliferation rate in NCD breeds is already high, even without the addition of TGF-β1 and BMP-2. However, this result seems to be opposed to the findings of Smolders et al (23), who found a higher expression of *Cyclin D1* in early intervertebral disc degeneration in cells derived from the NP of CD dogs than in those of NCD dogs. They interpreted that this result could be explained by a difference in cell type between those two groups. Namely, in NCD dogs, notochordal cells remain the predominant cell type in the NP until late in life, whereas in CD dogs, these cells are replaced by chondrocyte-like cells before one year of age.

In the CD groups, both BMP-2 conditions were increased compared to the control. This result suggests that for CD breeds BMP-2 has a positive effect on the cell proliferation, contrary to the result in NCD breeds, where none of the growth factors showed any differences in expression compared to the control.

In summary, with regard to the proliferation marker *Cyclin D1*, both TGF-β1 and BMP-2 seem to increase expression for CD breeds, reflecting a possible regenerative effect of these 2 growth factors for CD breeds. In NCD breeds, no effects of TGF-β1 and BMP-2 were found on expression of *Cyclin D1*.

Activation of the SMAD-pathway of TGF-β1 and BMP-2

TGF-β1 can signal by means of 2 pathways. The first pathway is activated by binding of TGF-β1 to its receptor ALK5. Activation of this receptor is linked with an increase in expression of *Aggrecan* and *Collagen type II.* After binding with TGF-β1 ALK-5 activates the SMAD 2/3 pathway, which leads to an increased expression of the downstream marker *PAI*. (19)

Another pathway by which TGF-β1 functions, is the SMAD 1/5/8 pathway. After binding of TGF-β1 with another receptor, ALK1, this pathway is activated. Activation of the SMAD 1/5/8 pathway is associated with an increase in *MMP-13* expression and chondrocyte terminal differentiation. Activation of this pathway can be shown by an increased expression of its downstream marker, ID-1. (19)

BMP-2 can also activate SMAD 1/5/8, and thus lead to an increased expression of ID-1, but it does so not by binding with the receptor ALK-1, but instead by binding with its receptor ALK3/6. (17) To investigate the effect of TGF-β1 and BMP-2 on the expression of the receptors for ALK1 and ALK5 and the difference between CD and NCD breeds regarding these expressions, the expression of *ALK1* and *ALK5* and their downstream markers, were measured.

For *ALK1*, there were no differences in expression between CD and NCD breeds without the addition of a growth factor.

However, in CD breeds, BMP-2 (100) seemed to increase expression of *ALK1*. This could suggest that BMP-2 has a upregulating effect on the expression of *ALK-1,* but no literature to support this statement could be found. As ALK-1 activation leads to chondrocyte terminal differentiation, an important factor in osteoarthritis (19), and to production of MMP-13, a type-II collagen degrading protein (19), so a high expression of this pathway is unwanted. For CD dogs TGF-β1 would thus be more effective with regard to ALK-1 activation.

For NCD dogs, a lower expression of *ALK1* was found in the presence of TGF-β1. This could indicate that in these cells, TGF-β1 leads to a lower expression of *ALK1*, and maybe to lesser activation of this receptor.

To investigate whether or not an increased expression of ALK1 will be accompanied with an increased activation of SMAD 1/5/8, the expression of it downstream marker *ID-1* was measured.

For *ID-1* an increasing effect on expression was found for CD BMP-2 (100). This finding is in line with our findings for ALK-1 receptor expression, suggesting an increased action by means of this receptor in the presence of BMP-2 (100) in CD breeds. It has been shown that in rat intervertebral disc cells, BMP-2 was able to increase the expression of TGF-β1 (14), so the higher expression of *ID-1* is possibly not only due to activation of SMAD 1/5/8 of BMP-2 itself via ALK 3/6 (17) but could also be caused by the higher expression of *ALK-1* and binding of TGF-β1 with this receptor.

In NCD derived cells, no significant changes compared to the control groups were found. However, there was a lower expression found on the level of the receptor ALK1. This lower expression thus does not seem to have a decreasing effect on the regulation in the activation of the SMAD 1/5/8 pathway, possibly because of another factor which can activate this pathway by other means than the ALK1 receptor.

So in NCD dogs neither TGF nor BMP-2 have an increasing or decreasing, effect on the activation of SMAD 1/5/8 pathway. Whereas in CD dogs, BMP-2 (100) increased both *ID-1* and *ALK1* expression, and therefore an higher activation of SMAD 1/5/8.

For *ALK-5*, an increase in expression for CD BMP-2 (100) compared to CD TGF-β1 and CD BMP-2 (250) was found. As activation of the receptor ALK-5 leads to activation of the SMAD 2/3 pathway, one would expect a corresponding increased expression in *Aggrecan* and *Collagen type I* (19). However, although an increased expression for both these genes was found in CD BMP-2 (100),

our results showed also increased expression levels of these genes for CD BMP-2 (250) for *Aggrecan* and for CD TGF-β1 and CD BMP-2 (250) for *Collagen type II.* This could indicate a higher activation rate of the pathway itself, rather than an increase in expression of its gene. To see whether or not this could be the cause, the expression of *PAI-1*, a downstream marker for ALK-5 activation, was measured.

Expression of *PAI-1* was increased for CD BMP-2 (100), indicating that the found increased expression of *ALK-5* indeed also leads to a higher activation of the SMAD-2/3 pathway. However, the expression pattern of *PAI-1* only partly correspondents with the found expressions of *Aggrecan* and *Collagen type II,* so possibly other factors or pathways, are causing upregulation of *Aggrecan* and *Collagen type II*.

Without the addition of a growth factor, there were no differences between CD and NCD breeds in ALK5 and ALK1 receptor expression, or activation of the SMAD-2/3 and SMAD-1/5/8 pathways as shown by expression of *PAI-1* and *ID-1*. So neither NCD nor CD dogs showed a tendency toward either aggrecan and collagen type II production (SMAD-1/5/8) or MMP-13 upregulation (SMAD-2/3, see paragraph 'hypertrophy and maturation of chondrocytes of TGF-β1 and BMP-2) effect to without the addition of the growth factors.

In summary, our results with regard to SMAD 1/5/8 activation, (*ID-1*) suggest that for CD breeds, the addition of BMP-2(100) will lead to the most activation of this pathway, also expression of the receptor *ALK-1* was increased for BMP-2 (100).

For NCD breeds, TGF-β1 seemed to induce less activation of SMAD 1/5/8 and expression of *ALK-1* than did BMP-2. As activation of SMAD 1/5/8 will induce chondrocyte terminal differentiation and production of MMP-13, a type-II collagen degrading protein (19), its activation will have a negative effect on chondrogenesis.

SMAD 2/3 was mainly increased in the CD BMP-2 (100) condition and the NCD TGF-β1 condition. As activation of SMAD 2/3 leads to an increase in expression of *aggrecan* and *Collagen type II*, this will probably lead to a positive chondrogenic effect.

So, like the results for chondrogenic effects of TGF-β1 and BMP-2, for CD breeds BMP-2 seems to have a higher chondregenic effect and activation of SMAD 2/3, whereas in NCD breeds, TGF-β1 was the most potent growth factor in increasing SMAD 2/3 and upregulation of *Aggrecan* and *Collagen type II*.

Hypertrophy and maturation of chondrocytes of TGF-β1 and BMP-2

MMP-13 is a protein that degrades Collagen type II(19), and it has shown to increase during degradation of the human IVD. (15)

Our results showed a high expression for MMP-13 in both CD BMP-2 conditions, suggesting a high degradation rate of Collagen type II in these conditions. However, expression of *Collagen type II* was also increased in these conditions, suggesting a high turn-over rate of collagen type II.

Activation of SMAD 1/5/8 pathway is correlated with *MMP13* expression. (19) The results of *ID-1* expression, a measure for SMAD 1/5/8 activation, did show an increase in expression for CD BMP-2 (100), similar to the increase for *MMP-13* expression.

Our results showed no significant increase in expression of MMP-13 for any of the TGF-β1 conditions. This could suggest that it does not activate SMAD 1/5/8 in both CD and NCD breeds. This observation is supported by the expression of *ID-1*, which did also not show an increase in expression.

TIMP 1 is an inhibitor of MMPs, and can be up-regulated by TGF-β1 through the SMAD 2/3 pathway.(24) Our results showed an increased expression of *TIMP 1* in the NCD control and TGF-β1 conditions compared to the corresponding CD conditions.

In both BMP-2 conditions for NCD breeds, expression of *TIMP1* was decreased compared to the other NCD conditions. So, although no increase in *MMP-13* expression was found for NCD TGF-β1

and the control, the MMP-13 present will decrease Collagen type II and have a beneficial effect on the content of the matrix.

In contrast to the NCD conditions, no differences in *TIMP-1* expression were found between the CD conditions. However, as *MMP-13* expression was increased in both CD BMP-2 conditions, a high degradation of Collagen type II was probably present in these conditions. In combination with the found increased expression of *Collagen type II* also found in both CD BMP-2 conditions, a high turn-over rate will probably be present in these conditions.

Axin 2 is a read out parameter for the activation of canonical Wnt signalling, a pathway involved in the maintenance and differentiation of stem cells (23). This pathway has an important in early IVD degeneration. (11,23)

Our results showed a decrease in *Axin 2* expression in CD breeds for TGF-β. According to Smolders et al. (23) a higher activation of *Axin 2* is associated with an increase in canonical Wnt signalling. In this article, there was a simultaneous higher gene expression of cell cycle regulators, such as *cyclin D1* and therefore they suggested that canonical Wnt signalling supports the proliferation of cells within the degenerating IVD. However, in our results TGF-β showed a decrease in expression of *Axin 2*. This result could suggest a subsequently decrease in canonical Wnt signalling in the NP cells derived from CD breeds in the presence of TGF-β1. As Wnt/β-catenin, probably reflects a regenerating response, (23) TGF-β1 will probably not stimulate regeneration in CD breeds. This is further supported by the found *Cyclin D1* expression in our results, which did not differ with the control.

No difference between the CD and NCD controls was found. This finding is in contrast with the results of Smolders et all. (11,23) who found an increase in expression of *Axin-2* in IVD cells derived from CD dogs. Therefore, it would be interesting to investigate coupes of these dogs with regard to weather the cells mainly consists of CLCs or NCs. However, another research of Smolders et al (11), which divided both CD and NCD groups into CLC-rich, mixed, and NC-rich, did also find an increase in expression when they compared CD and NCD groups, but a decrease in expression when they compared CLS-rich groups with NC-rich and mixed groups. Another explanation for not finding any differences between these groups could be the high standard deviation in the NCD control condition.

Collagen type X expression was not to be found in our results. Type X collagen is a short-chain collagen, that is present in hypertrophic chondrocytes of the growth plate(25). It has shown to be present in degenerated IVD's in humans(26), and in cells of the degenerative NP of CD dogs (25). A possible explanation for not finding any gene expression of *Collagen type X* in our results could be that the cells were obtained too early in the experiment, and that on a later date there will be expression of *Collagen type X*.

Effects of TGF-β1 and BMP-2 on cell apoptosis

The regulators of cell apoptosis measured in this research were *Bax, Bcl 2* and *Casp 3*. Loss of cells is believed to be an important factor in the loss of extracellulair matrix in the process of IVD degeneration(27).

Up-regulation of *Bax* induces cell apoptosis. It increases the permeability of the mitochondrial outer membrane, and Bax is an important factor in inducing apoptosis by the so-called 'mitochondrial pathway', an intrinsic pathway in the process of apoptosis(28). In a normal, human, intervertebral disc expression of Bax is low. However, in the process of intervertebral disc degeneration, expression of Bax will be increased. (28)

In our results, *Bax* was increased for CD BMP-2 (100) compared to CD TGF-β1, suggesting an increased cell apoptosis of CD cells in the presence of BMP-2 compared to TGF-β1. A possible

explanation for this finding could be that TGF-β1 has shown to protect human annulus fibrosus cells from autophagy. It is possible the mitochondrial pathway was involved in this process. (29).

For NCD BMP-2 (250), a lower expression of *Bax* compared to NCD BMP-2 (100) and CD BMP-2 (250) was found, suggesting a decrease of apoptosis for NCD cells in the presence of a higher concentration of BMP-2. Also, this result suggests a greater effect of BMP-2 (250) on NCD cells compared with CD cells with regard to a decrease of *Bax*-mediated apoptosis, but no research was found to explain this difference found between NCD and CD breeds.

Bcl 2 is a protein located on the outer cell wall of mitochondria and, contrary to Bax, it prevents cell apoptosis (30).

In our results, expression of *Bcl 2*, suggested a lower apoptosis rate in CD BMP-2 (100), especially when taken into account that *Bax* expression was not increased for this condition compared to the control.

Bcl 2 expression was higher for CD BMP-2 (100) than for NCD BMP-2 (100), suggesting a greater effect of BMP-2 for CD cells with regard to the prevention of apoptosis of cells by Bcl 2. Remarkably, *Bcl 2* expression in CD BMP-2 (100) was increased compared with CD TGF-β1, whereas the same result was found for *Bax* expression, suggesting a compensating effect of Bcl 2 on the *Bax* mediated apoptosis, as they have an opposing effect.

Another important factor in apoptosis is the expression of *Caspase 3*, which is involved in executing apoptosis.(21,31) Also, it is a down-stream target of Bcl-2.(21)

In our results, *Casp 3* expression was decreased in CD BMP-2 (250), indicating a lower number of cell deaths by apoptosis for this condition. However, the same result was not found in Bcl-2 expression, indicating a possible effect of another pathway on the expression of *Casp 3*.

For NCD, expression of Casp 3 in TGF- β1 and BMP-2 (100) was increased compared with the expression in NCD BMP-2 (250). Indicating a higher apoptosis-rate for those two conditions. As the same result is also not found in Bcl-2 expression, another pathway by which means BMP-2 results in inhibition of the production of *Casp 3* may be possible. As this effect is only seen for BMP-2(250) and not in BMP-2(100), a concentration higher than 100 ng/mL may be needed for this effect.

Conclusion

With regard to genes that have a chondrogenic effect our results indicated that in CD dogs, BMP-2 would be more effective than TGF-β1 in stimulating expression of *Aggrecan, Collagen II* and *Sox-9.* For NCD dogs, the results showed similar increases in expressions for both BMP-2 (250) and TGF-β1 for expression levels of *Collagen II* and *Sox-9*. However, as BMP-2 (250) also decreased the expression of *Adamts*, thus stimulating the breakdown of Aggrecan, TGF-β1 would likely be more potent in chondrogenesis than BMP-2 in NCD dogs.

For production of *Collagen I*, our results indicated a stimulation of expression in cells derived from CD dogs of BMP-2 (250), indicating that this growth factor has a stimulating effect on fibrosis in CD dogs. For NCD dogs, none of the growth factors seemed to have an upregulating effect on *Collagen I* production.

For *Cyclin D1* our results showed an increased expression in NCD dogs compared to CD dogs without addition of a growth factors. This suggests a high proliferation rate in these cells. For CD dogs, an increase in expression was found in both BMP-2 conditions. This could indicate a positive effect on the proliferation of these cells in the presence of BMP-2.

With regard to genes that indicate an activation of the SMAD pathways, our results suggested a higher rate of functioning of SMAD 1/5/8, as indicated by an increase in *ID-1* expression, for BMP (100) in the cells derived from CD dogs, this result corresponded with an increase in *ALK-1* expression. As activation of SMAD 1/5/8 is associated with chondrocyte terminal differentiation, activation of this pathway will result in fibrosis of the IVD and is therefore undesirable in this study. However, BMP-2 (100) also showed to increase *PAI-1* expression in CD breeds, indicating a higher activation of the SMAD 2/3 pathway, which is associated with *Aggrecan* and *Collagen II* expression.

For NCD only a decrease of *ALK-1* expression was found in the TGF-β1, but there were no indications for an associated downregulation of SMAD 1/5/8 functioning. For genes associated with SMAD 2/3 no noteworthy changes in gene expression were found in NCD dogs.

Regarding genes that are involved in hypertrophy and maturation of chondrocytes, an increase in expression of *MMP-13* was found to be increased in CD dogs for both BMP-2 groups, indicating degradation of collagen type II in this sample. Another change in gene expression for CD dogs was found for *Axin-2*, which expression was decreased in the TGF-β1 condition, possibly fitting with a non-regenerative response. For NP cells derived from NCD dogs, there was a higher expression of *TIMP-1* in this control condition compared to the CD control condition, indicating an inhibition of present MMPs. For both NCD BMP-2 groups, however, a decrease in expression of *TIMP-1* was found, indicating less inhibition of MMPs, and therefore probably a higher degeneration of collagen type II.

With regard to genes involved in cell apoptosis, the gene expression level of *Bcl-2* in the presence of BMP-2 (100) was increased for cells derived from CD dogs, indicating a lower cell apoptosis in this condition. BMP-2 (250) also seemed to induce a lower apoptosis rate, as *Casp-3* expression was decreased in the CD BMP-2 (250) sample. For NCD dog derived cells, both TGF-β1 and BMP-2 (250) showed an increase in *Bcl-2* expression, indicating a decrease in cell apoptosis for both these conditions.

In summary, for CD dogs BMP-2 seemed to have a better chondrogenic effect on the NP cells than TGF-β1. It did however, also stimulate more genes, such as Collagen type I and MMP-13, involved in a fibrotic effect in CD dogs, more so than TGF-β1did. But considering that TGF-β1 also did not show much of an upregulating effect in most chondrogenic genes, preference would be given to BMP-2 based on the results of this research for cells derived from the NP of CD dogs.

For NP cells derived of NCD dogs, the effects of both TGF-β1 and BMP-2 with regard to chondrogenic effects were much more alike. However, BMP-2 showed a decrease in *TIMP-1* expression, an inhibitor of MMP-13, and a decrease of TIMP-1 can therefore possibly lead to a higher degradation of Collagen type II, as expression of *Collagen type II* is also increased, there will be a high turn-over rate of Collagen type II. Therefore, a slight preference for BMP-2 would be made based on the results in this research for NP cells derived from NCD dogs.

However, to draw good conclusions with regard to the chondrogenic and fibrotic effects of both BMP-2 and TGF-β1 and the differences between CD and NCD dogs, further research will be appropriate.

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