Utrecht University

The anti-inflammatory effect of caveolin-1 on Canine Nucleus Pulposus cells

A.M.C. van der Hoeven, Utrecht University, Faculty of Veterinary Medicine, Department of companion animal sciences, Orthopedics

Supervised by: dr. M.A. Tryfonidou, A.R. Tellegen MSc

INDEX

Abstract

Intervertebral disc degeneration (IVDD) is a common disease in dogs and humans, characterized by lower back or neck pain and/or neurologic deficits. Inflammation and matrix destruction are components of degeneration. Besides trauma and mechanical stress, genetic predisposition is known to be involved in the development of IVDD. Caveolin-1 is one of the genes known to be involved in IVDD. It is already known to have an anti-inflammatory function in macrophages, however its precise function in the intervertebral disc is yet unknown. Therefore, this research internship focused on investigating the anti-inflammatory effect of caveolin-1 on nucleus pulposus (NP) cells of the chondrodystrophoid (CD) dog *in vitro*. This was determined in a 7 day culture experiment of pellets of CD dog NP cells. The GAG and DNA content, gene expression and histology of Safranin-O/Fast-Green and collagen type I and II staining were used to study the effects of caveolin-1. Caveolin-1 supplementation increased the GAG content of NP pellets cultured in the inflammatory conditions and induced an increase of gene expression of matrix components while it reduced the gene expression of catabolic genes. However none of these changes were significant. Safranin-O/Fast Green staining indicated that caveolin-1 supplementation counteracted the catabolic TNF-α effects. In conclusion, caveolin-1 may have an anti-inflammatory effect on NP cells of CD dogs *in vitro*.

Keywords: intervertebral disc degeneration, canine, genetic predisposition, regenerative medicine, caveolin-1, anti-inflammatory

Introduction

Intervertebral disc

The intervertebral disc is situated between the vertebral bodies. Through the intervertebral foramina, nerves step out to innervate the surrounding tissues. The physiological function of the intervertebral disc is to transmit forces between the vertebral bodies and to enable movement without loss of the stability.(1,2) The intervertebral disc functions as a cushion of the spine. In figure 1, a schematic image is shown of a lumbar spine in cross-section.(3)

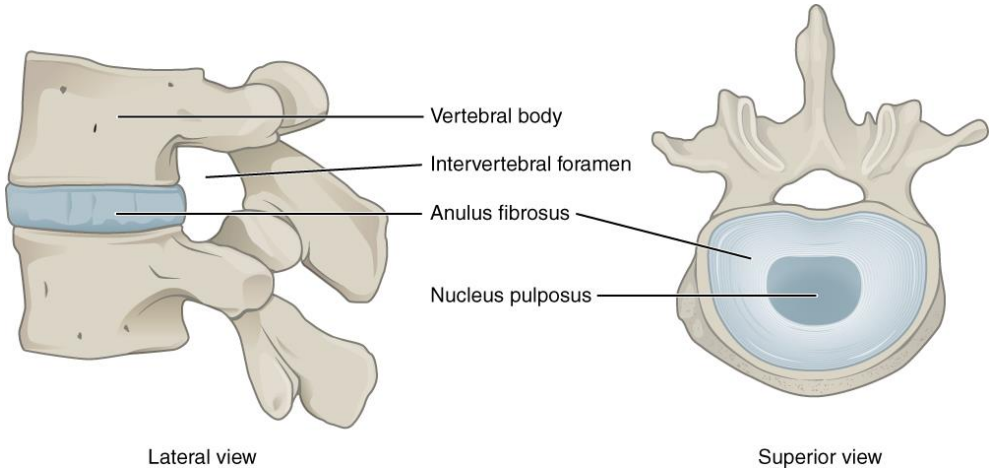

Figure 1 Image of a lateral and superior view of the human spine. The vertebral bodies are separated and united by an intervertebral disc. This provides padding and allows movements between the vertebral bodies. The intervertebral disc is composted by a fibrous outer layer (annulus fibrosus) and a mucoid center (nucleus pulposus). The intervertebral foramen provides exit of a spinal nerve.
Reprinted from 'General structure of a vertebra' from the Anatomy & Physiology: The Vertebral Column 2013 (3)

A healthy intervertebral disc is composed of a nucleus pulposus, a transition zone, an annulus fibrosus and endplates. The **nucleus pulposus (NP)** is a mucoid, translucent, bean-shaped structure, mainly composed of water.(1) The NP is made up of aggrecan, collagen type II and lower amounts of collagen type I, VI, IX and XI. The proteoglycans which are present in the NP are hydrated and incorporated into the collagen type II network. The NP is composed of chondrocyte like cells (CLC's) and notochordal cells (NC's).(1) The NC's enable storage of glycogen and disappear with increasing age, with loss of NC's the chondrocytes will predominate.(1,4) Besides the glycogen storage, it is thought that the big vesicles which the NC's are recognizable for, might have a function in osmoregulation of the NP. (1) The **annulus fibrosus (AF)** is composed by collagen type I, III, V and VI.(5) The outer AF has a lamellar appearance of the collagen and a fibroblastic cell population.(1,2) The inner annulus has a lower amount of collagen, but a higher amount of proteoglycan aggregates, this causes a more cartilaginous and less fibrous appearance.(1,2) This inner annulus is also called **the transition zone (TZ)**. (1,4,6) The **endplate (EP)** is the cartilaginous border between an intervertebral disc and the intervertebral bodies cranial and caudal to it.(1)

Intervertebral disc degeneration

Intervertebral disc degeneration (IVDD) is a cause of back pain in both humans and dogs.(1) It is known there are shifts in expression of proteoglycans and collagen when degeneration develops. The amount of glycosaminoglycans (GAGs) and collagen type II decreases whereas the amount of collagen type I increases.(1,7) This causes the intervertebral disc to become more rigid, which causes dehydration and shrinkage of the NP.(1) Thereby, it is mentioned the innermost regions of the disc, the nucleus pulposus, relies on diffusion for receiving its nutrients. Even in a healthy disc, the

permeability is relatively poor and the distance over which the diffusion must occur is large, this limits the delivery of nutrients to the innermost region. The ongoing degeneration causes a further limitation in nutrient supply and a vicious circle occurs.(5,8)

The NC's in the NP are replaced by CLC's. However, the age at which this replacement occurs, is different for the chondrodystrophoid (CD) and the non-chondrodystrophoid (NCD) dog breeds. In CD-dogs this replacement takes place before one year of age, which is also the reason why IVDD is more common in these breeds when compared with NCD-breeds.(9) In NCD-dogs however the replacement of the NCs occurs at 7-8 years of age.(10) Examples of CD-breeds are the Dachshund, the French bulldog and the Pekinese. Their short legs are a result of a disturbed endochondral ossification.(1)

The NC's disappear with ageing, but this can be enhanced when risk factors are present. Examples of these risk factors are noted by Bergknut *et al* (2012): 'IVDD is described as an aberrant, cell-mediated response to progressive structural failure of the IVD and is associated with genetic predisposition, chronic physicomechanical overload and trauma, inadequate metabolite and nutrient transport to and from the cells with the IVD matrix, cell senescence and death, altered levels of enzyme activity, changes in matrix macromolecules and changes in water content'.(1) These factors are shown in figure 2.(1)

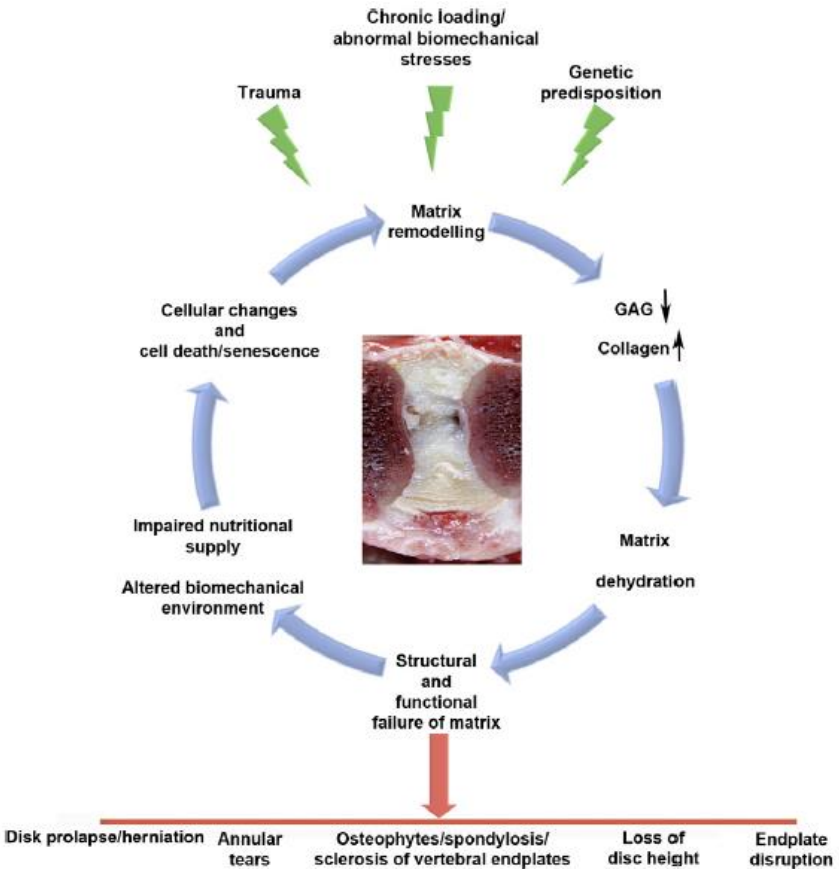

Figure 2 Schematic presentation of the pathophysiology of intervertebral disc degeneration.
Reprinted from 'intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration,' by N. Bergknut *et al*, 2012, The Veterinary Journal 195 (2013), p 284 (1)

The ingrowth of nerves and blood vessels in the EP during degeneration is the cause of pain in the disc.(11) Normally intervertebral discs are avascular and aneural. By mechanical stimulation and degeneration of the IVD, angiogenic and neurogenic signalling molecules cause synthesis of blood vessels and nerve growth.(2)

Inflammatory influences

The cellular switch from NC's to CLC's is mediated by cytokines.(12) Some pro-inflammatory cytokines initiate development of degeneration, while others cause further degeneration of the intervertebral disc.(12) Examples of inflammatory cytokines are Tissue Necrose Factor (TNF)-α, Interleukin (IL)-1β , IL6, IL8, IL-10 and IGF-1.(1,12-14)

Le Maître *et al* (2007) found in human IVDs an increase in matrix degenerative enzymes, regulated by the pro-inflammatory influences, which caused connective tissue degradation.(2) Thereby the cytokines might play a role in nerve- and vessel ingrowth and macrophage accumulation, these factors are all of interest in IVDD.(2) It is found pro-inflammatory cytokines are produced inside the IVD itself, this implicates the cells inside the IVD are responsible for production of the cytokines and thus for the occurring inflammation.(11)

IL-1β and TNF-α both have pro-inflammatory effects, some of their effects are overlapping. These effects are caused by activating common signalling cascades and thereby induce similar target genes. Both cytokines are demonstrated to stimulate the production of IL-6, which also gives a pro-inflammatory response. *In vitro* IL-1β and TNF-α caused a suppression in proteoglycan production and proteoglycan depletion in the disc cell is initiated by aggrecanse.(15)

Both TNF- α and IL-1β are thus associated with IVDD, however opinions differ concerning the impact of the discussed inflammatory components.

TNF-α

A study of Weiler *et al* (2004) showed synthesis of TNF-α takes place in both the NP and the AF and expression of TNF-α is associated with the increase of degeneration.(12) TNF-α is a very potent pro-inflammatory cytokine (12,16) which stimulates synthesis of IL-1, IL-6(17), IL-8 and PGE_2. Furthermore, TNF-α causes an upregulation of matrix metalloproteinases (MMP) and decreases collagen- and proteoglycan synthesis. TNF-α is involved in nerve root injury since it is known to be neurotoxic and increases vascular permeability.(12)

Given the fact TNF-α is present in adults of more advanced age, TNF-α is not likely to be involved in developing IVDD, but it causes more extensive degeneration.(12) Le Maître *et* al (2004) claimed TNF-α does not contribute to disc degeneration.(18) In humans it is found, IL-1 is more of impact in decreasing matrix synthesis which is important in the IVDD.(13,19) However, Grang *et al* (2001) suggested TNF-α most probably is directly involved in both the process of degeneration and in genesis of pain.(16) Walter *et al* (2015) demonstrated it is possible the healthy bovine IVD gets infiltrated by inflammatory cytokines like TNF-α and this induces changes in IVD biomechanics.(20)

IL-1β

Le Maître *et al* (2007) states that IL-1β is the main component of the inflammation in IVDD in humans.(13) Both IL-1α and IL-1β are involved in cartilage homeostasis because of its ability to switch the CLC's from anabolism to catabolism.(2) CLC's express and produce the active receptor of IL-1, and produce both isoforms of IL-1 (IL-1α and IL-1β) both in degenerated and non-degenerated discs. However, in the degenerated IVD an increase of the IL-1 receptor is found, which points towards an increased responsiveness of the CLC's to IL-1.(18) IL-1α and –β both increase

proteoglycan release and degradative enzyme production. IL-1β also increases MMP production and pain mediators as PGE2.(2) Grang *et al* (2001) suggested IL-1β is produced in response to mechanical stimuli and IL-1 promotes the production of factors involved in disc degradation and of proteinases.(16)

Senescence

Senescence is thought to worsen the degeneration. Cellular senescence is an appellative for shortening of the telomeres. This causes a limit with respect to mitosis, cells cannot divide anymore. There are two kinds of senescence: replicative senescence (RS) and stress-induced premature senescence (SIPS). The RS is the intrinsic senescence and is telomere dependent.(19,21) In ageing cells the length of the telomeres reduce, so multiplication through mitosis cannot occur anymore. The SIPS is the extrinsic senescence pathway and is telomere-independent. The cell is in a standby mode, it remains viable but cannot replicate.(21) However, senescence can also occur when the cells are exposed to stress. The stressor can be radiation or UV light but also oxidative stress can cause DNA damage. Stress can also be induced by exposure to inflammatory cytokines such as TNF-α or IL-1.(19,21) In case of IVDD, where inflammatory cytokines indeed play a role, the SIPS is suggested to worsen the process of degeneration. As explained earlier, angiogenic factors will cause synthesis of blood vessels and thereby increase the oxygen level in the IVDD. This can act synergistically with the senescence induced by oxygen.(22) Le Maître *et al* (2007) found data which implicate that the senescent phenotype is linked to the increased production of degradative enzymes which may be brought about by the catabolic cytokine IL-1, known to be increased in disc degeneration.(22)

Genetic predisposition

The degeneration finds its origin in the replacement of NC's by CLC's, the difference between the dog types is the age at which the replacement occurs. Since in the CD-dog breeds this replacement occurs before one year of age, this was thought to be genetic predisposed. (9,10,23) In a previous review, several genes are shown to play a role in the development of IVDD in those breeds. Most of these genes are either affected by genetic polymorphisms or are increased in replication in the individual. One of the genes associated with the development of IVDD is caveolin-1 (CAV1).(10,24,25)

Caveolin-1

Caveolin-1 (cav-1) is associated with numerous biological functions. It is suggested that cav-1 might be a protective modulator in animal sepsis and suppresses tumours(26,27), thereby it is thought to play a protective role in the inflammatory response.(28) However, there are also experiments which show cav-1 might induce inflammation.(29,30) Thereby, it is also known to induce growth of tumors in different organs.(31) Caveolin-1 is a regulator of Wnt/β-catenin signalling and this is a crucial factor in the maintenance of NC health and physiology. Unpublished data show that caveolin-1 acts as an inducer of matrix synthesis by acting as a growth factor. Disturbances in the function of caveolin-1 can initiate IVDD.(24)

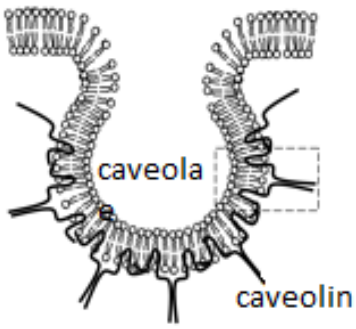

Figure 3 Schematic drawing of caveolin inside the cellular membrane.
Reprinted from 'Transcytosis: Crossing Cellular Barriers' by P.L. Tuma *et al*, 2003, Physiological Reviews, 83 (3), p 897 (29)

Caveolin is mainly located in the cell membranes of the notochordal cells and functions within caveolae membranes.(21,32-34) These caveolae are located at or near the cell membrane and are attached to the cell membrane via a short caveolar neck or they reside intracellularly as illustrated in figure 3.(33)

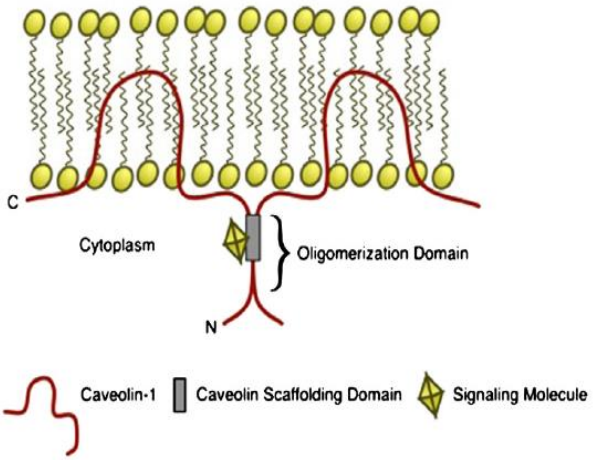

Figure 4 The caveolin scaffolding domain (CSD) interacts with signaling molecules. Reprinted from 'Role of caveolin-1 in fibrotic diseases' by Gvaramia D *et al*, 2013, Matrix Biology, 32, p 309 (30)

Caveolin-1 consists of the caveolin scaffolding domain (CSD) (figure 4), the critical functional domain of the protein.(26) This domain can both interact with and inhibit signalling molecules that possess for example the caveolin-binding sequence motif, such as the TGF-β receptor, the epidermal growth factor receptor (EGFR) and the TLR4.(35-37) Caveolin-1 is thought to play a role in the organisation and concentration of specific lipid classes and lipid-modified signalling molecules.(19,33,38) Caveolin-1 can interact with these signalling molecules in different ways. Namely, via a direct interaction with a molecule, compartmentalization of signalling platforms or by modulating signalling protein activation by caveolar endocytosis.(35) Caveolae have several functions, but the best-known is incorporated in endocytosis, thereby it is thought there might be a role in both cellular transport processes and signal transduction. (33) New studies indicate that cav-1 confers long-lasting stability to the caveolar vesicles that interact with endosomes.(39,40) Caveolin-1 is suggested to correlate with the formation of caveolae membranes.(33)

Caveolin-1 and IVDD

In IVDD there is a downregulation of this caveolin-1, which will result in a loss of NC's. Caveolin-1 knock-out (KO) mice showed to have relatively few healthy NC clusters in the NP. The cells which were present showed signs of apoptosis and the NP contained abundant intracellular chondroid matrix, similar to the CLC-rich NP.(10,24,41) The NP of the caveolin-1 KO mice contained rounded cells, with a smaller amount of cytoplasm lacking the typical vacuolar appearance.(24)

As caveolin-1 is a regulator of Wnt/β-catenin, down-regulation of caveolin-1 causes a down-regulation of the caniconal Wnt signalling. This signalling pathway regulates the renewal and apoptosis of the NCs and NP stem cells. Down-regulation may result in an increased apoptosis and decreased self-

renewal of these cells. Wnt signalling is likely to play a role in the transition towards the aged or degenerated CLC-rich NP.(24)

Caveolin-1 also turns out to regulate the interaction of caveolin with the actin filaments of the cytoskeleton. Smolders *et al* (2013) found: 'Caveolin-1 is crucial for NC maintenance and IVD health, and this protein may be regarded an exciting target for developing ways to regenerate the IVD'.(24)

Caveolin-1 and senescence
As explained earlier, senescence is of influence in IVDD, more particular, SIPS.(22) It is assumed caveolin-1 plays a role in this process. Caveolin-1 is suggested to play an important role in the development of SIPS in response to the stress conditions such as IL-1.(19,21) It is stated caveolin-1 causes IVDD when downregulated. (10,24,41)

Caveolin-1 and anti-inflammatory function
Besides the matrix related functions of caveolin-1, it also functions as an anti-inflammatory component in macrophages. Caveolin-1 can interact with membrane receptor signalling via the earlier mentioned ways and interaction with downstream signal molecules.(36) Because it is already known that caveolin-1 can interact with TLR4 it is possible that this receptor may regulate downstream effector functions.(36,42) The conclusion of Wang *et al* (2006) was: 'Caveolin-1 has a protective role for inflammation by suppression of pro-inflammatory cytokine (TNF-α and IL-6) production and augmentation of anti-inflammatory cytokine (IL-10) production in the arthritis model.'(36) Weiss *et* al (2015) observed a higher TNF output when there is a decrease in cav-1 levels(28), which might be due to a loss of TLR4 regulation. Tang *et al* showed that TLR4 activation is required for IL-17 induced tissue inflammation.(43) A decrease in cav-1 may cause an increase of IL-17.(43) Hu *et al* (2008) presents that the expression of cav-1 in polymorphonuclear neutrophils (PMNs) is of importance for the activation of the PMNs.(44)

However, all these outcomes were found in experiments focussed on caveolin-1 found in macrophages.(36) The discoveries mentioned before are suggestive it may also have this effect in the nucleus pulposus cells (NP cells).

Aim of the study
The current treatments of IVDD focus on reducing the pain, but these treatments do not repair or help the recovery.(45,46) Since in IVDD both degeneration of the matrix and inflammation occur, these two mechanism should be tackled. This might be achievable by regenerative medicine.

Several studies support the anti-inflammatory function of cav-1 in macrophages.(28,36,43,47) Given the fact caveolin-1 is known to play a role in IVDD development, we hypothesize that caveolin-1 may also modulate the inflammatory processes occurring during IVDD. In order to investigate this, the main aim of this project was to set up an *in vitro* system where NP cells receive a pro-inflammatory stimulus (TNF-α and IL-1β) and investigate the anti-inflammatory properties of caveolin-1. In this study the matrix anabolism and catabolism is observed on gene expression level, biochemical and histological, the inflammation at gene expression level. The different studies are illustrated in figure 5.

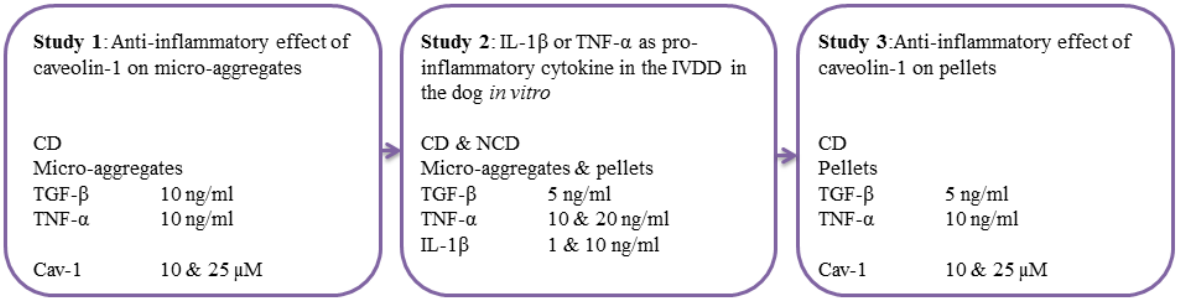

Figure 5 Illustration of different performed studies

Materials and methods

Study design
Nucleus pulposus (NP) cells from CD and NCD dogs were readily available through the Biobank of the orthopaedics group. Prior to the experiment, the cells were expanded by adding standard expanding medium (hg-DMEM,GlutaMAX™, pyruvate (Invitrogen, 31966), 10% FBS

(16000-044, high performance, Gibco), 1% penicillin/streptomycin (p/s) (PAA laboratories, P11-010), 20mM ascorbate-2-phosphate (ASAP) (A8960, Sigma-Aldrich, Saint Louis, MO, USA), 0.02 mM dexamethasone (D1756, Sigma-Aldrich), 1ng/mL basic fibroblast growth factor(bFBF) (PHP105, AbD Serotec, Oxfored, UK), and 0.5% Fungizone (15290-018, Invitrogen)). After expanding the cells, those were loosened with help from Hanks' balanced salt solution (HBSS), trypsine (12604-013, TrypLE™ Express, Gibco, Denmark) and TGF-β (240-B, R&D, Systems Inc Minneapolis, USA). The cells were cultured for 7 days in 5% CO_2, 20% O_2. Microaggregates of 35.000 cells or pellets of 200.000 cells were used and the culture medium was refreshed two times a week. The chondrogenic medium contained hg-DMEM+GlutaMAX (Invitrogen) with 1% insulin-transferrin-selenium (ITS) + premix (354352 Corning, Tewksbury, MA, USA), 20 mg/mL L-Proline (P5607 Sigma-Aldrich), 1% p/s (PAA laboratories, P11-010), 0.5% Fungizone (15290-018, Invitrogen), 0.02 mM dexamethasone (D1756, Sigma-Aldrich) and 20 mM ASAP (A8960, Sigma-Aldrich, Saint Louis, MO, USA), 10µL/mL Bovine Serum Albumin (A3059-1006, Sigma-Aldrich, St Louis, USA) and TGF-β(240-B, R&D, Systems Inc Minneapolis, USA)

When these pellets were formed the culture conditions were adjusted to the conditions preferred for the experiment. These conditions are specified in the corresponding chapters of the pilots. Different donors were used (table 1, 2), in the corresponding pilot, the used donors will be specified. The caveolin-1(Caveolin-1 scaffolding domain peptide (Enzo Life Sciences, ALX-153-064) which was added to the cultured cells is the active protein, the caveolin scaffolding domain(CSD), the intracellular part is added.

Table 1 Donors of which CD NP cells were pooled used in caveolin-1 experiment and IL-1β experiment.

Breed	Age in months	Gender
Beagleton	121	Male
Beagle	40	Male
Beagle	58	Male
Beagle	62	Male

Table 2 Donors of which NCD NP cell mix was pooled for IL-1β experiment

Breed	Age	Gender
Mixed	26 months	Male
German Shepherd	10 years	Male
Mixed	14 months	Male
Jack Russell	11 years	Male

DNA and GAG content
After the culture experiment was ended, cells were digested by overnight incubation at 60 °C of Papain digestion solution (pH 6, 200 mM H_2NaPO_4 * 2 H_2O) (21254, Boom B.V. Meppel, the Netherlands) 10 mM EDTA(100944, Merck Millipore, Amsterdam, the Netherlands.), 10mM cysteine

HCL(C7880, Sigma-Aldrich, Saint Louis, USA) and 10 mM papain (P3125, Sigma-Aldrich, Saint Louis, USA). This was performed to digest the cell pellet through which the GAG and DNA content could be measured. The DNA content per mg cell culture was measured using the Qubit ds DNA High Sensitivity Assay Kit (Q32851, Invitrogen , Paiseley, UK). 1x number of samples (n) µL of the Qubit Reagent was added to 199x n µL of the Qubit Buffer, this combined formed the working solution. The high sensitive standards(S_1, S_2) have specific values which cleared the Fluorometer. This was performed by adding 10 µL of the standard to 190 µL of the working solution. The samples which were going to be measured, were present in amount of 5 µL and were added to 195 µL of the working solution. The DNA content was measured in ng/mL. The GAG content was measured with a dimethyl methylene blue (DMMB) assay. The DMMB staining solution should be prepared one day before use. This was done by adding 16 mg DMMB to 5 mL of 100% ethanol, the total solution should be incubated on a roller bench for 2-16 hours. Further, 2.37 g NaCl and 3.04 g Glycine should be added to 1 L distilled water. When the pH is set on 3.0 the DMMB can be added to the solution. The chondroitin sulphate C (0.5 mg/mL) standard was than diluted 1:50, dilution series of 1:4 were made to provide the standard line. 100 µL of the diluted standard line was pipetted in duplo. The samples were diluted in PBS-EDTA, based on the expected amount of GAGs. The PBS-EDTA was prepared by adding 0,04 M Na2HPO4, 0.06 M H2NaPO4 * 2H2O and 0,01 M Na2EDTA * 2 H2O to 1L distilled water. 100 µL of the diluted sample was pipetted in the plate. Directly after adding 200 µL of the DMMB, the absorbance (540/595) was measured using a microplate reader (Tecan).

Gene expression
Deep frozen samples were crashed with the aid of pestles (P9951-901, Argos Technologies, Elgin, USA). Thereafter, 350 µl RLT (151012987, Qiagen, Hilden, Germany) + β-mercaptoethanol was added to achieve cell lysis and samples were stored at -70°C until further analysis.

RNeasy micro spin columns (74004, Qiagen, Valencia, USA) were used to perform the RNA isolation. The samples were defrosted, equal volume 70% ethanol was added and transferred into the micro spin columns. Columns were centrifuged for 30 sec at 13200 rpm, flow-through was discarded and columns were washed with 350 µl RW1 buffer (151013997, Qiagen, Hilden, Germany). The micro spin columns were centrifuged for 30 sec with max speed again and the flow-through was thrown away again. 80 µl DNAse (RNAse-Free DNase Set, 79254, Qiagen, Valencia, USA) was added on the membrane and incubated for 15 min. DNAse is used to assure the present DNA is broken down, so there is no remaining DNA left. Columns were washed again with 350 µl RW1 buffer and centrifuged for 30 sec at maximum speed. 500 µl RPE^+(151014626, Qiagen, Hilden, Germany) was added, followed by centrifuging for 30 sec on maximum speed in a new collection tube. 500 µl 80% ethanol was added, the samples were centrifuged for 2 minutes and again a new 2 mL collecting tube (151032285, Qiagen, Hilden, Germany) was taken. Centrifuge with open cover for 5 minutes. The current 2 mL tube was replaced by a 1,5 mL tube (151030138, Qiagen, Hilden, Germany). 17 µl RNase free water (151022581, Qiagen, Hilden, Germany) was pipetted directly on the membrane, incubated 1 min and centrifuged for 1 min. The flow-through was pipetted directly on the membrane, and centrifuged for 1 minute. The flow-through which is obtained in this last step is the RNA.

cDNA was synthesised from RNA using the iScript cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands). Before starting the synthesis, it should be calculated what the desired amount of RNA would be. The maximum is 15 µL, if less is necessary it is filled up to 15 µL with nuclease-free water. The iScript mix is synthesised by adding 4 µL x n of the iScript reaction mix and 1 µL x n of the iScript reverse transcriptase. These two components should be mixed. To each Eppendorf PCR tube 5 µL of the iScript mix is added. This makes a total amount of 20 µL in each tube. This complete reaction was incubated mix 5 min at 25ºC, 30 min 42ºC, 5 min 85 ºC and hold it at

4 ºC. Primer sequences were obtained from previous work (table 3). Before PCR could be started, cDNA should be diluted 10x and a standard line should be made, this consisted of a pool of cDNA from all the samples. The S_1 is the highest standard, a serial dilution of 4x was made toward S_7. The final master mix consisted of MQ, SYBR green Supermix and the forward and reverse primer. In each Eppendorf PCR tube 6.0 µL of the master mix was added and 4.0 µL of the 50x diluted cDNA.

qPCR was performed using the iQT™ SYBR green Supermix (Bio-Rad, Veenendaal, the Netherlands), which causes fluorescence in the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands).

To normalize the target gene expression, four reference genes were used (table 3).

Table 3 Primer sequences

Gene	*Primer sequence*	*Product Size (bp)*	*PCR temperature (ºC)*
HPRT*	Fwd: 5'-AGCTTGCTGGTGAAAAGGAC-3' Rev: 5'-TTATAGTCAAGGGCATATCC-3'	104	56+58
SDHA*	Fwd: 5'-GCCTTGGATCTCTTGATGGA-3' Rev: 5'-TTCTTGGCTCTTATGCGATG-3'	92	61
RPS19*	Fwd: 5'-CCTTCCTCAAAAAGTCTGGG-3' Rev: 5'-GTTCTCATCGTAGGGAGCAAG-3'	95	61+63
GAPDH*	Fwd: 5'-TGTCCCCACCCCCAATGTATC-3' Rev: 5'-CTCCGATGCCTGCTTCACTACCTT-3'	100	58
Aggrecan	Fwd: 5'-GGACACTCCTTGCAATTTGAG-3' Rev: 5'-GTCATTCCACTCTCCCTTCTC-3'	110	61-62
Type 1 Collagen	Fwd: 5'-GTGTGTACAGAACGGCCTCA-3' Rev: 5'-TCGCAAATCACGTCATCG-3'	109	61
Type 2 Collagen	Fwd: 5'-GCAGCAAGAGCAAGGAC-3' Rev: 5'- TTCTGAGAGCCCTCGGT-3'	150	60.5-65
MMP-13	Fwd: 5'-CTGAGGAAGACTTCCAGCTT-3' Rev: 5'-TTGGACCACTTGAGAGTTCG-3'	250	65
ADAM-TS5	Fwd: 5'-CTACTGCACAGGGAAGAG-3' Rev: 5'-GAACCCATTCCACAAATGTC-3'	148	61
IL-6	Fwd: 5'-GAGCCCACCAGGAACGAAAGAGA-3' Rev: 5'-CCGGGGTAGGGAAAGCAGTAGC-3'	123	65
PTGES2	Fwd: 5'-GCTCTCAAGACCTACCTGG-3' Rev: 5'-AGTCACTTCCTTTCCCTGG-3'	98	60-62
Casp3	Fwd: 5'-CGGACTTCTTGTATGCTTACTC-3' Rev: 5'-CACAAAGTGACTGGATGAACC-3'	89	61
FasL	Fwd: 5'-ATGTTTCAGCTCTTCCATCTACAG-3' Rev: 5'-CAGAAGGTGGATTGGGTTGAC-3'	114	60
Bcl2	Fwd: 5'-GGATGACTGAGTACCTGAACC-3' Rev: 5'-CGTACAGTTCCACAAAGGC-3'	80	61.5-63
Sox9	Fwd: 5'-CGCTCGCAGTACGACTACAC-3' Rev: 5'-GGGGTTCATGTAGGTGAAGG-3'	105	62+63
BAX	Fwd: 5'- CCTTTTGCTTCAGGGTTTCA-3' Rev: 5'- CTCAGCTTCTTGGTGGATGC-3'	108	58-59
Cav1	Fwd: 5'- CGCACACCAAGGAAATCG-3' Rev: 5'- AAATCAATCTTGACCACGTCG-3'	72	60
Pai1	Fwd: 5'- AAACCTGGCGGACTTCTC-3' Rev: 5'- ACTGTGCCACTCTCATTCAC-3'	98	61.5
IL-1β	Fwd: 5'-TGCTGCCAAGACCTGAACCAC-3' Rev: 5'- TCCAAAGCTACAATGACTGACACG -3'	115	68

*: used reference genes

Safranin-O/Fast Green staining
The pellets were fixed in 4% neutral buffered formaldehyde (4286, Klinipath B.V., Duiven, The Netherlands) at room temperature (RT) for 24 h and dehydrated and embedded in paraffin. Sections of 5 µm were obtained on Microscope KP + slides (KP-3056, Klinipath B.V. Duiven, the Netherlands) and deparaffinised with xylene (two times 5 min) and solutions of ethanol (96%, 80%, 70%, 60%, each solution 5 minutes) caused rehydration. The sections were stained in Weigerts's haematoxylin (640505, Klinipath B.V., Duiven, The Netherlands) for 5 min, followed by running tap water and distilled water. Thereafter, sections were stained with Fast Green (F7252, Sigma-Adrich, Saint Louis, USA) for 4 min, these were washed with acetic acid (5 min) to remove the loose fractions and the sections were counter-stained with Safranin-O (58884, Sigma-Aldrich, Saint Louis, USA) for 5 min. The sections were put in ethanol (twice 96%) for 2-5 minutes, 100% ethanol and xylene to dehydrate, then the sections were mounted (Vectamount, H5000, Vector Laboratories, Burlingame, USA).

Collagen type I and II staining
Sections were deparaffinised with xylene and rehydrated with series of ethanol. The samples were blocked with H_2O_2 (51008600.9025, Boom B.V., Meppel, the Netherlands) for 10 min and washed 2x5 min with PBS+0.1% Tween(PBST0.1%). Antigen retrieval was performed with 1mg/mL pronase (30 min, 37°C) (11459643001, Roche Diagnostics, Almere, the Netherlands). Sections were washed again (2x5 min) with PBST0.1%. Further antigen retrieval was performed with 10mg/mL hyaluronidase (H3506, Sigma-Aldrich, Saint Louis, USA) for 30 min at 37°C. All sections were washed two times with PBST0.1% 5 min each, after which the sections were blocked with 5% BSA in PBS for 30 min at room temperature. Circles were placed with an ImmEdge™ pen (Vector Laboratories, Burlingame, USA) around the samples so the liquid would stay with the sample. The sections were incubated overnight with the primary antibody at 4°C. The primary antibody for mice was put on the samples, collagen type I (0.1µg/mL, ab6308, Abcam, Cambridge, UK) and collagen type II (0.4µg/mL, II-II6B3, DSHB, Iowa City, IA). The negative control was normal mouse IgG_1 (0,4 µg/mL; 3877, Santa Cruz Biotechnology, Heidelberg, Germany)

The next day is started with tipping of the sections horizontally, so the antibody of collagen I will not interfere on the samples meant for collagen II. Then again washing with PBST0.1% to remove the loose fractions. The secondary antibody (EnVision + System-HRP goat Anti-Mouse, K4001, Dako, Glostrup, Denmark) was added and was incubated for 60 minutes at room temperature. The sections were washed two times with PBS 5 min each, after which the samples were incubated with DAB peroxidase substrate solution for 1 min (K3468, Dako, Glostrup, Denmark). The DAB reaction is stopped with MQ and the counterstaining with Haematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) can take place. The samples were placed under running tap water for 10-15 minutes. The samples were put in ethanol (70%, 80%, 95%, 95%, 100%, for 5 minutes each) and xylene (2x5 min) for dehydration. Samples were fixated with Vectamount (Vectamount, H5000, Vector Laboratories, Burlingame, USA) so the sections can be stored over a longer period of time.

Statistical analysis
Statistical analysis was performed by using the SPSS Statistics 22. The data were examined for normal distribution using the Shapiro Wilks test ($n<2000$). When non-normally distributed data was found, the Kruskal Wallis was performed followed by a Mann-Whitney U as post-hoc test. In case of normally distributed data, the ANOVA test was used. Post-hoc testing was performed by using Benjamini and Hochberg correction, this corrected for occurrence of false positives. A p-value < 0.05 was considered significant. When SPSS Statistics 22 was not powerful enough to detect significances, a more sufficient program, R, in where a Cox proportional hazard model (Coxph) could be performed was used. Here the Benjamini and Hochberg Post Hoc analysis was used as well.

Study 1: Anti-inflammatory and prophylactic effects of caveolin-1 in micro-aggregates

Aim of the study :

It is desired to determine both the prophylactic, and the anti-inflammatory effect, so this pilot is subdivided in two experiments. In experiment A, in order to determine whether caveolin could reverse the anti-inflammatory effect of TNF-α, caveolin-1 was added after a pro-inflammatory stimulus was applied. In experiment B the caveolin-1 was added at the same time as the pro-inflammatory stimulus to determine whether it could be effective in preventing the pro-inflammatory effect of the TNF-α.

Study design

The used donors of the first pilot are shown in table 1. Micro-aggregates (35.000 cells) were formed and cultured according to the conditions given in figure 6. All pellets received TGF-β to support chondrogenesis of the NP cells. A pro-inflammatory stimulus was provided with TNF-α that was added daily for 5 consecutive days. Caveolin-1 was only added from day 0 (experiment A) to study its anti-inflammatory effects in the presence of inflammation, caveolin-1 was added from day -5 (experiment B) to study its prophylactic effect during induction of inflammation.

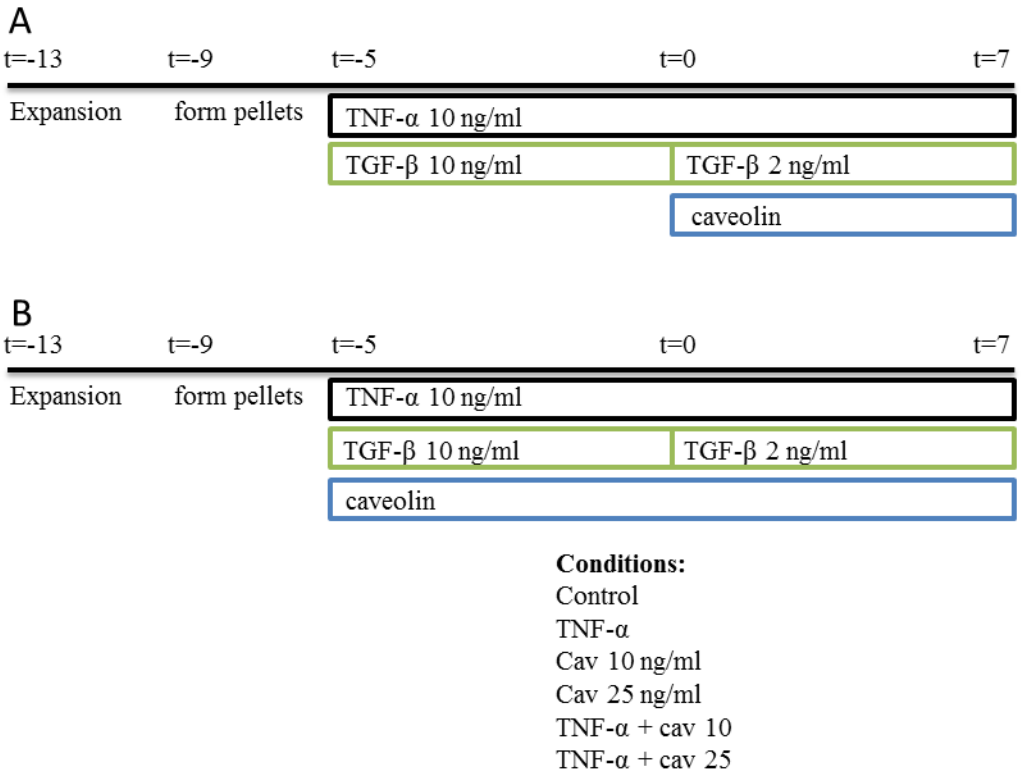

Figure 6 NP cells of degenerated discs were cultured with TNFα (10 ng/ml) and Caveolin-1 (10 and 25 µM).

In the pilot concerning the anti-inflammatory effect of caveolin-1 the qPCR was performed on genes thought to be influenced. For the anabolism, aggrecan (ACAN) and collagen I and II were measured. Aggrecan is catabolised by ADAMTS5. Sox9 is part of the TGF-pathway and therefore also of interest in anabolism. Catabolism is visualised with MMP3 and MMP13 which mainly catabolises collagen II. Caspase 3 (Casp3) is a down-regulator of other caspases, which indicates the expression of Casp3 might reflect the apoptosis incidence.(48) Fas ligand (FasL) promotes apoptosis while Bcl2 decreases apoptosis. IL6 is an inflammatory gene. While COX 1, COX 2, $PTGES_1$ and $PTGES_2$ are part of the PGE_2 pathway. Besides the single measurements, a ratio of BAX/Bcl2 was also obtained, this shows the susceptibility of apoptosis. Bcl2 transmits the survival signal, while BAX transmits the dead signal.(49)

Results

GAG and DNA content

Experiment A: anti-inflammatory effect
In experiment A, where the anti-inflammatory effect is determined, the GAG and DNA of the micro-aggregates were measured both on day 1 and day 7. Although the GAG content increased throughout time (figure 7A), the DNA content decreased in the presence of TNF-α (figure 7B). The GAG/DNA content was comparable over all conditions on day 7 (figure 7C).

The GAG content of the collected medium and the micro-aggregate were added to have a estimation of the total amount of GAGs produced by the cells (figure 7D). The TNF-α, cav10 and cav25 treated micro-aggregates had produced a significantly lower amount of GAGs in comparison with the control group where only TGF-β was present. Furthermore, the TNF-α+cav10 and TNF-α+cav25 treated micro-aggregates produced significantly less GAGs compared to TNF-α alone. There was a dose related effect in the culture groups where caveolin-1 was added: 10 µM caveolin-1 resulted in a higher amount of GAG content when compared with 25 µM caveolin-1, the same pattern was observed in the presence of TNF-α.

The decrease of the GAG release in the medium can be caused by two things. The production of GAGs could be decreased by the cells in the micro-aggregate or the micro-aggregate increased in size since the GAGs were not provided to the medium. Since graph 7D shows a decrease of GAG in the medium, but no increase of the GAG content in the micro-aggregate, it can be stated that the production of GAG by the pellet was lowered.

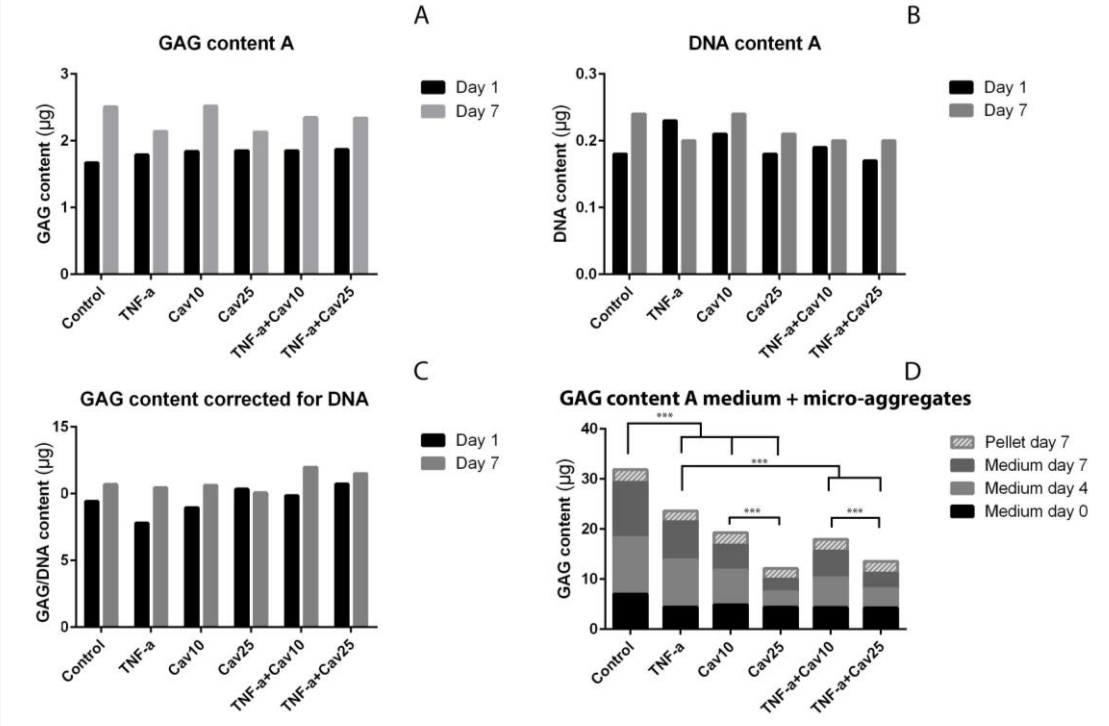

Figure 7 The effect of caveolin-1 on matrix production and DNA content during inflammation in micro-aggregates. **A** GAG content (µg) day 1 and day 7 from pellet. **B** DNA content (µg) day 1 and day 7 from pellet. **C** GAG/DNA ratio day 1 and day 7. **D** Total amount of GAG of medium and pellet combined. Significances are referred to as *: p<0.05; **: p<0.01; ***: p<0.005

Experiment B: prophylactic effect

In experiment B, caveolin-1 was added from day -5 to study the prophylactic effect of caveolin-1 in the presence of a pro-inflammatory stimulus. The sample of day 1 in the condition with TNF-α was lost, so no data could be analysed for this condition in this time frame. The GAG content (figure 8A) of day 7 was increased in all conditions compared to day 1, except for the TNF-α+cav25. Both groups treated with caveolin-1 alone contained more DNA (figure 8B) on day 7 compared with the control group; the same pattern was observed in presence of a pro-inflammatory stimulus, the TNF-α+cav10 and TNF-α+cav25 compared to TNF-α alone. The GAG/DNA of TNF-α+cav10 and TNF-α+cav25 was increased in day 7 compared with day 0 (figure 8C).

As explained earlier, decrease of GAG release in the medium can be caused by increased release of the GAG by the pellet, or by reduced production by the pellet of GAG. The caveolin-1, both 10 µM and 25 µM, provided less GAG than the control group. The TNF-α+cav10 and TNF-α+cav25 produced less GAG when compared with TNF-α (figure 8D). Also, a dose related effect was seen here in the caveolin-1 with doses 10 µM and 25 µM, caveolin-1 in a dose of 25 µM produces less GAGs.

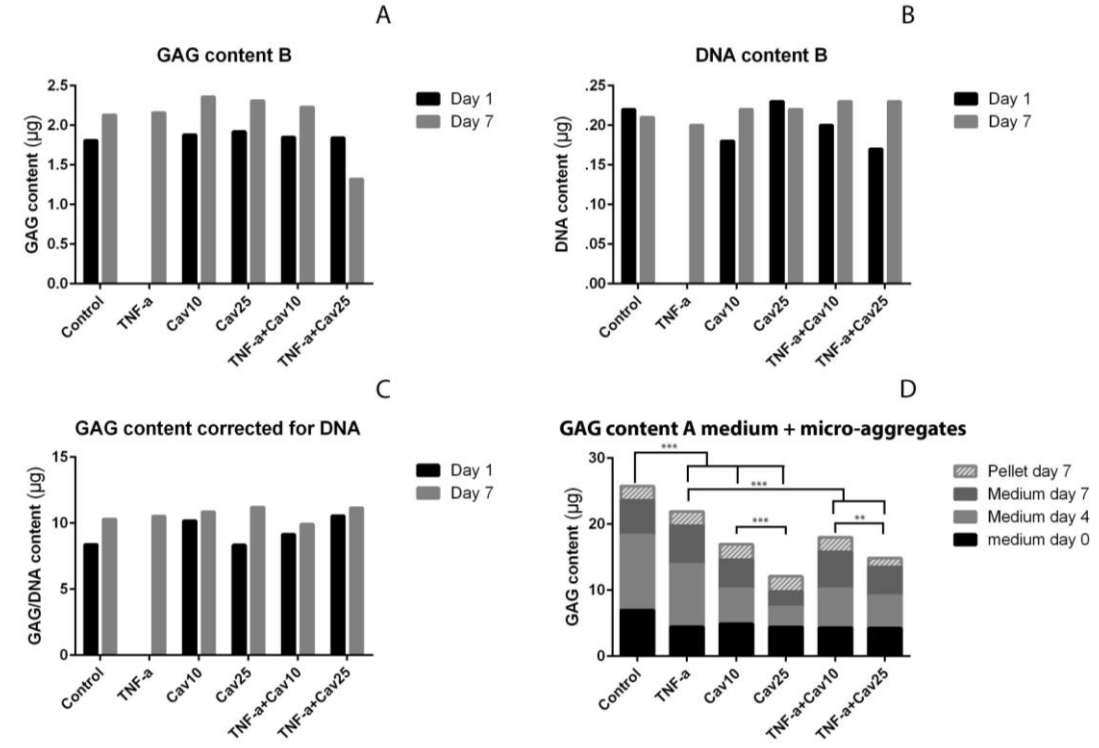

Figure 8 The prophylactic effect of caveolin-1 on matrix production and DNA content in micro-aggregates. **A** GAG content (µg) day 1 and day 7 from pellet. **B** DNA content (µg) day 1 and day 7 from pellet. **C** GAG/DNA ratio day 1 and day 7. **D** Total amount of GAG of medium and pellet combined. Significances are referred to as *: p<0.05; **: p<0.01; ***: p<0.005

Gene expression

Since the number of samples was relatively low, no statistics were performed on these qPCR data. Although all genes mentioned above were sequenced, ADAMTS5, IL-6, PTGES2 and FasL were not sufficient, so data could not be analyzed.

Experiment A: anti-inflammatory effect

In experiment A, ACAN (figure 9A) showed an increase in gene expression in cav25, when compared with TNF-α, the TNF-α+cav25 caused an increase in aggrecan gene expression as well. SOX9 (figure 9B) is involved in the TGF-β pathway and expression was increased when cav25 was added. All conditions showed a decreased expression of the catabolic gene MMP13 (figure 9C) when compared with the control group.

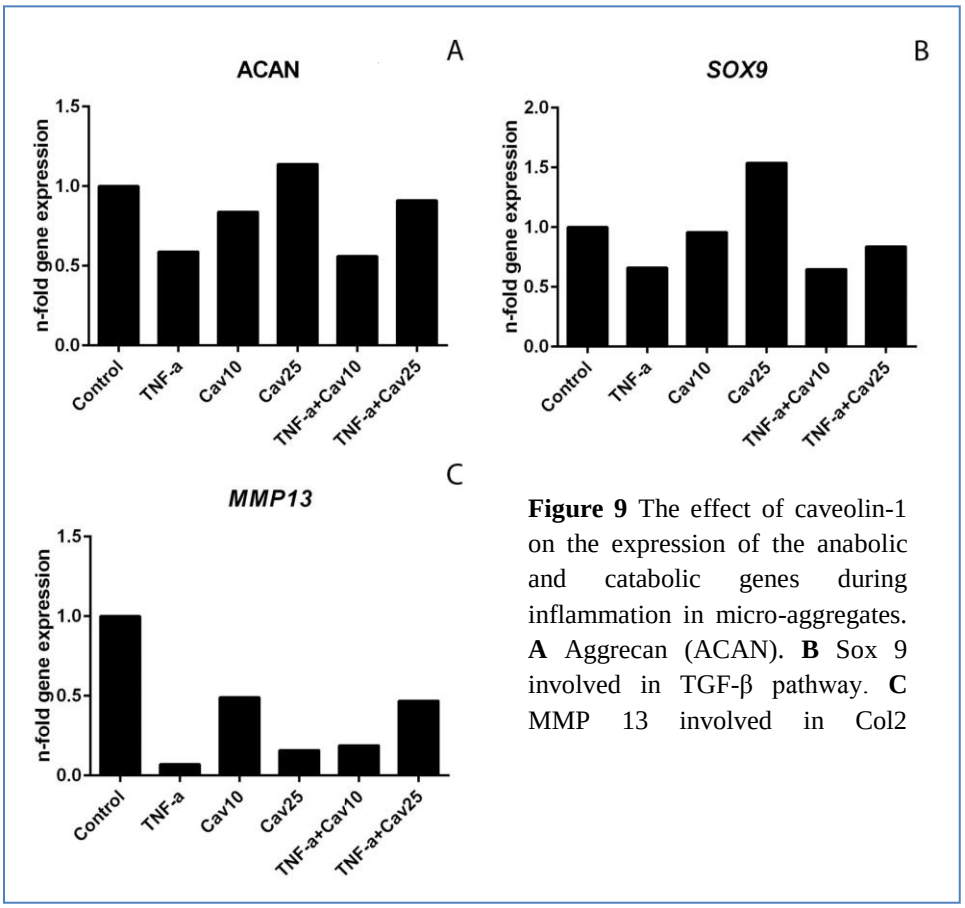

Figure 9 The effect of caveolin-1 on the expression of the anabolic and catabolic genes during inflammation in micro-aggregates. **A** Aggrecan (ACAN). **B** Sox 9 involved in TGF-β pathway. **C** MMP 13 involved in Col2

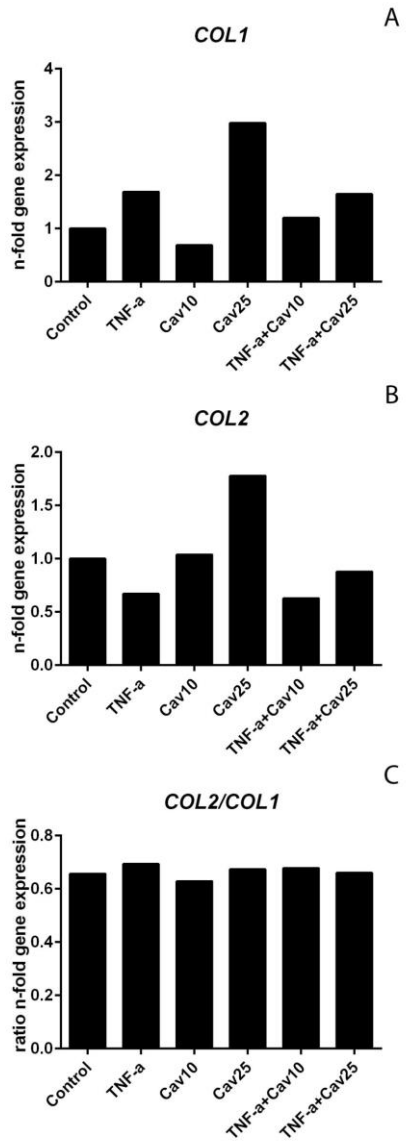

Figure 10 The effect of caveolin-1 on expression of collagen type I, type II and the ratio of Col2/Col1 during inflammation in micro-aggregates. **A** Collagen I (Col1) present in degenerated discs. **B** Collagen II (Col2). **C** Col2/Col1 ratio to determine whether the collagen I proportionally decreased.

In degeneration, collagen type I is increased. Hence, when aiming at regeneration a change from collagen type I to collagen type II is considered beneficiary.

Collagen type I was expressed in a greater amount in the TNF-α and cav25 than in the control group (figure 10A). Cav10 caused a decrease in collagen type I expression compared to the control group. TNF-α+cav10 decreased collagen type I mRNA expression compared to TNF-α alone.

Cav25 caused an increase in collagen type II compared with the control, while TNF-α+cav25 caused an increase in comparison with the TNF-α.(figure 10B)

The col2/col1 ratio (figure 10C) indicates which type of collagen is present in a relatively higher amount. The ratio was smaller than 1, which means the collagen type I is more present regardless of the treatment.

In experiment A, where the caveolin-1 was added on day 0 of culturing, the expression of the anabolic genes was increased compared to control group. The gene expression of the anti-apoptotic gene, Bcl2 (figure 11A) was decreased in the presence of cav10 and cav25 compared to the control group. The gene expression of the pro-apoptotic gene, BAX (figure 11B) was increased in the cav10 compared with the control, TNF-α+cav25 increased when compared with the TNF-α. The BAX/Bcl2 ratio (figure 11C) indicates the susceptibility for apoptosis of the cells. (50-52) In all conditions the gene expression of the pro-apoptotic BAX is relatively higher compared with the Bcl2. Casp3 (figure 11D) is a pro-apoptotic gene, which was increased in all conditions compared with the control group.

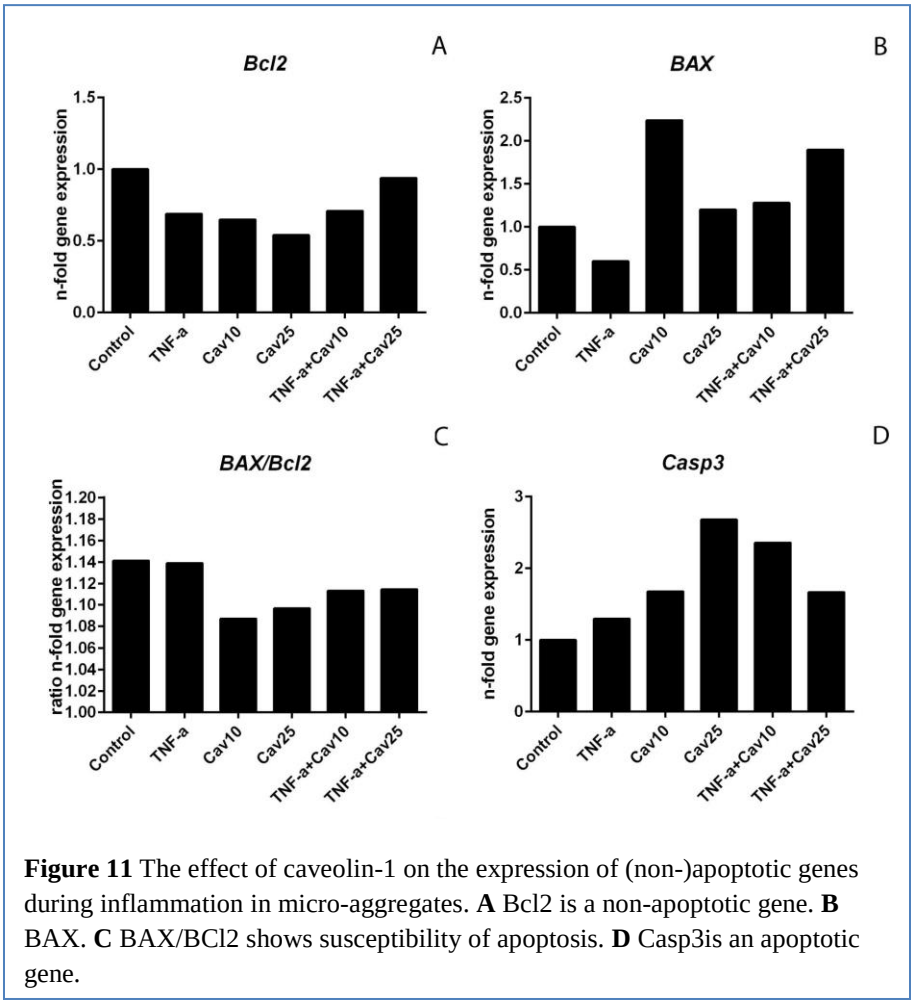

Figure 11 The effect of caveolin-1 on the expression of (non-)apoptotic genes during inflammation in micro-aggregates. **A** Bcl2 is a non-apoptotic gene. **B** BAX. **C** BAX/BCl2 shows susceptibility of apoptosis. **D** Casp3is an apoptotic gene.

Experiment B: prophylactic effect

The ACAN (figure 12A) expression was decreased in cav10 and cav25 compared with the control group. The conditions where the caveolin-1 was added in the presence of a pro-inflammatory stimulus showed an increased expression when compared with the TNF-α. SOX9 (figure 12B) expression was not influenced when only caveolin-1 was present; however its expression was increased when added to the TNF -α. The MMP13 (figure 12C) gene expression was decreased in the TNF-α, cav10 and cav25 compared to the control group. The TNF-α+cav10 and TNF-α+cav25 showed a higher expression of MMP13 compared with the TNF-α.

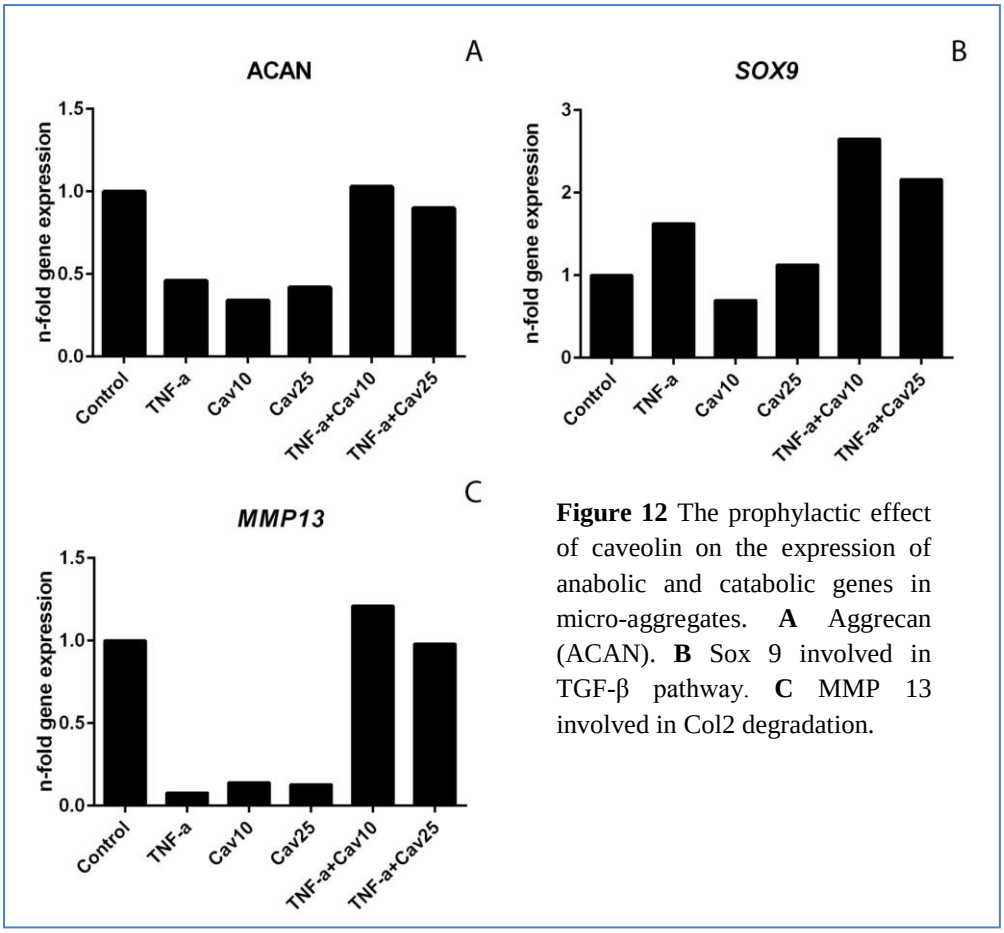

Figure 12 The prophylactic effect of caveolin on the expression of anabolic and catabolic genes in micro-aggregates. **A** Aggrecan (ACAN). **B** Sox 9 involved in TGF-β pathway. **C** MMP 13 involved in Col2 degradation.

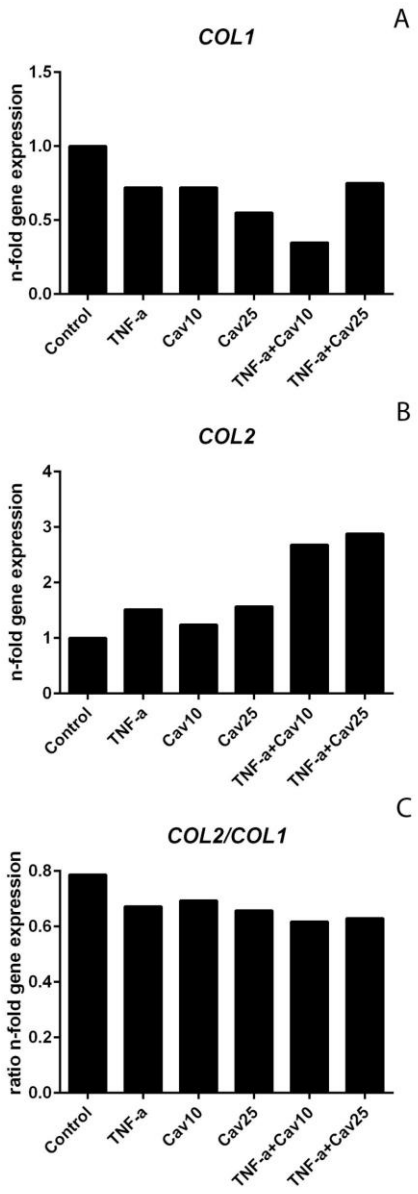

Figure 13 The prophylactic effect of caveolin-1 on the expression of collagen type I, type II and the ratio of Col2/Col1 in micro-aggregates. **(A)** Collagen I (Col1) present in degenerated discs. **(B)** Collagen II (Col2). **(C)** Col2/Col1 ratio to determine whether the collagen I proportionally

The collagen type I expression (figure 13A) decreased in TNF-α+cav10 in comparison with TNF-α. However in TNF-α+cav25 the Col1 expression was similar with the TNF-α.

Collagen type II expression (figure 13B) increased in cav10 and cav25 in comparison with the control group. Also the expression of collagen type II increased TNF-α+cav10 and TNF-α+cav25 in comparison with TNF-α.

The ratio of col2/col1 (figure 13C) in experiment B, as well as in experiment A, was less than 1, which showed collagen type I was still predominant in all culture conditions and did not differ between culture conditions.

The presence of cav10 and cav25 reduced the expression of Bcl2 compared with control (figure 14A). In TNF-α+cav10 and TNF-α+cav25 an increase in expression of the non-apoptotic Bcl2 was visible compared with control. The pro-apoptotic BAX (figure 14B) was increased in TNF-α+cav10 and TNF-α+cav25 when compared with TNF-α. The BAX/Bcl2 ratio (figure 14C) shows the susceptibility for apoptosis of the cells. (50-52) In all conditions the BAX was relatively more expressed compared to Bcl2. Casp3 (figure 14D), the apoptotic gene, decreased in cav10 compared with the control group. The expression was increased in TNF-α+cav10 and TNF-α+cav25 compared with TNF-α.

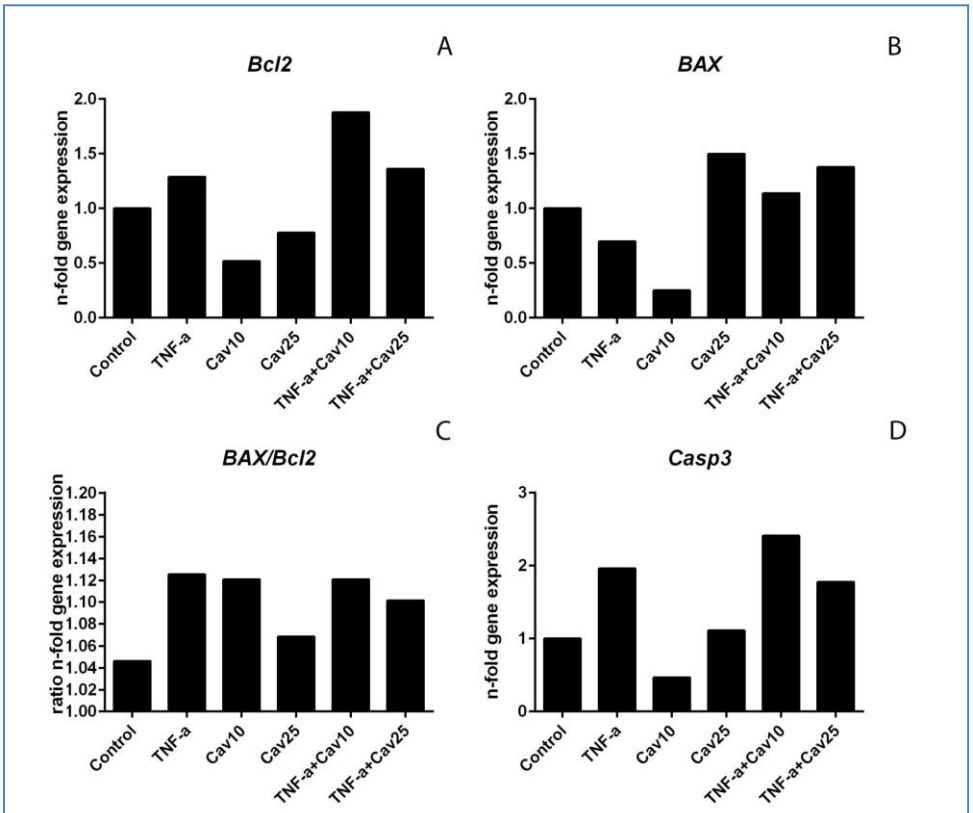

Figure 14 The prophylactic effect of caveolin-1 on the expression of (non-)apoptotic genes in micro-aggregates. **A** Bcl2 is a non-apoptotic gene. **B** BAX. **C** BAX/BCl2 shows susceptibility of apoptosis. **D** Casp3 is an apoptotic gene.

Safranin-O/Fast-Green staining

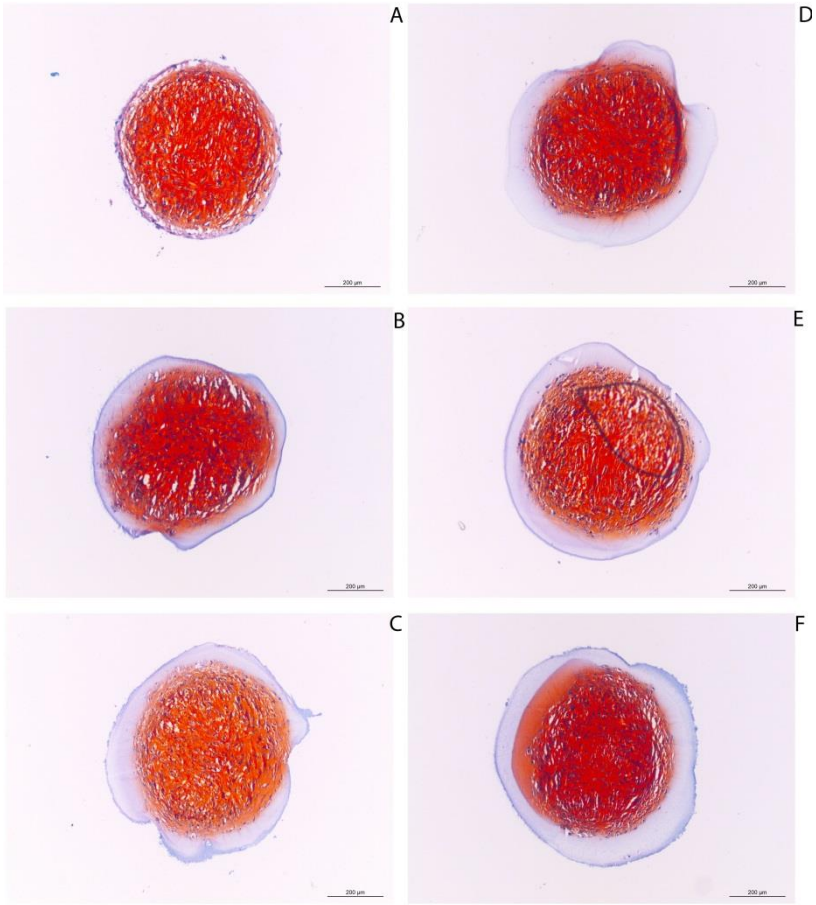

Figure 15 The effect of caveolin-1 on the matrix anabolism at histological level during inflammation in micro-aggregates. **A** Control with only TGF-β. **B** Caveolin 10µM. **C** Caveolin 25µM. **D** TNF-α. **E** TNF-α + Caveolin 10 µM. **F** TNF-α + Caveolin 25 µM.

The Safranin-O/Fast-Green staining (figure 15) stains the GAG molecules red. In this experiment, all different conditions did stain red while no clear differences were seen between the varying conditions. This was true for experiment A as well as for experiment B. All the sections contained great amounts of GAG. The amount of GAG was expected to reduce in TNF-α occurred, but this was not visible.

Collagen type I and II staining

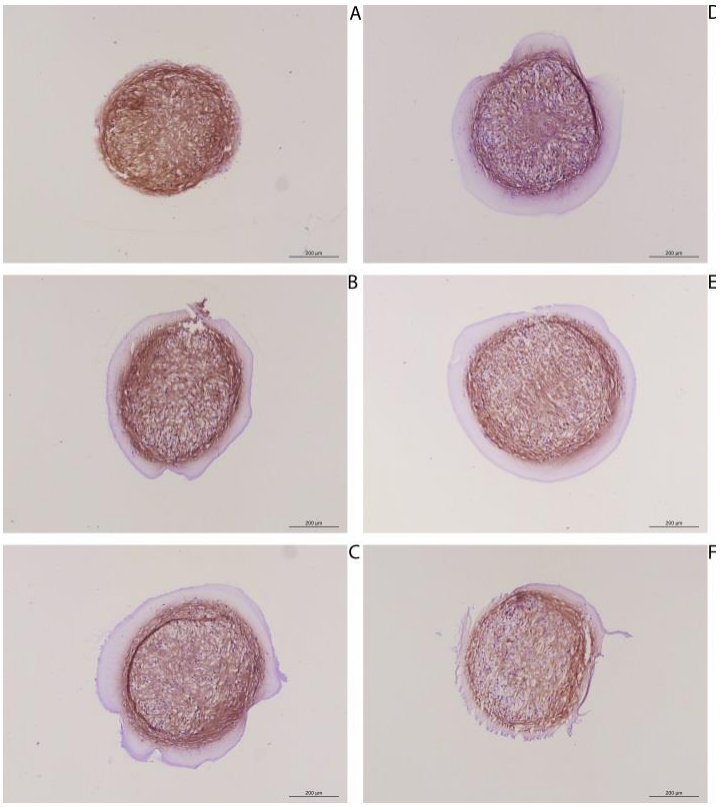

Figure 16 The effect of caveolin-1 on the collagen type I staining during inflammation in micro-aggregates. **A** Control with only TGF-β. **B** Caveolin 10µM.
C Caveolin 25µM. **D** TNF-α. **E** TNF-α + Caveolin 10 µM. **F** TNF-α Caveolin 25 µM.

In experiment A (anti-inflammatory effect), a decrease of collagen type I staining (figure 16) was seen in the TNF-α compared to the control group.
In experiment B (prophylactic effect), there was no visible mutual difference of staining for collagen type I.

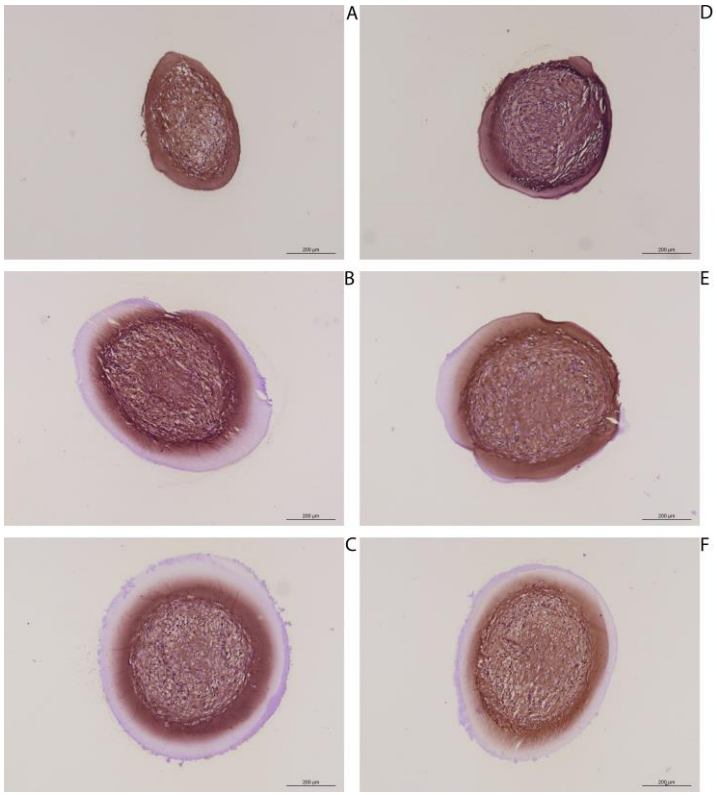

Figure 17 The prophylactic effect of caveolin-1 on the collagen type II staining in micro-aggregates. **A** Control with only TGF-β. **B** Caveolin 10µM.
C Caveolin 25µM. **D** TNF-α. **E** TNF-α + Caveolin 10 µM. **F** TNF-α Caveolin 25 µM.

For collagen type II (figure 17), there was no visible difference in staining for the varying sections, for both experiment A and experiment B.

Discussion

This study was performed to determine the anti-inflammatory effect of caveolin-1. In experiment A caveolin-1 was added after inflammation was already established with the aid of a pro-inflammatory stimulus to determine whether caveolin-1 could reduce inflammation. In experiment B, caveolin-1 was added from the start of the experiment when the inflammation was initiated to investigate the possible prophylactic effect of caveolin-1. In the different assays- DMMB, Qubit, qPCR and (immune)stainings- no clear role of caveolin-1 could be determined. As far as known, no earlier reports were available into the effect of caveolin-1 on the intervertebral disc, so therefore no comparisons could be made with other studies.

The GAG content in the medium decreased when caveolin-1 was added, while the GAG content of the micro-aggregates did not change. This indicates the micro-aggregate produced less GAGs in the presence of cav-1 when compared with the control group. In experiment B (prophylactic effect), the reduction of matrix components were visible in the cav10 and cav25 as well. However, in experiment A (anti-inflammatory effect), cav10 and cav25 seemed to show more gene expression of the matrix components.

Both types of collagen were represented in great amount, both when the prophylactic and the anti-inflammatory effect of caveolin-1 were determined. The collagen type II/collagen type I ratio showed which type of collagen was predominant. qPCR data showed a predomination of collagen type I in all conditions in both experiments. The effect of caveolin-1 was dose dependent, seen the fact a dose of 25µM sometimes showed a more preferable effect than a dose of 10µM, although it also presented itself the other way around. When degeneration occurs the total content of collagen increases(1), collagen 1 is predominant. Collagen type II is more flexible in comparison with collagen type I, which is more stiff. The collagen II is preferred to be predominant, however this effect was not seen in the current experiment in the qPCR. The stainings showed less collagen type I and more collagen type II in experiment B (prophylactic effect) when compared with experiment A (anti-inflammatory effect). Both type of collagen however were represented in great amounts.

Regarding to the BAX/Bcl2 the susceptibility to apoptosis reduced when caveolin-1 was added. BAX is known to transmit death signals while Bcl2 is known to transmit survival signals.(49) The caspase 3 in experiment A was increased in the inflammatory condition and in the conditions where caveolin-1 was present. Experiment B showed comparable expression of caspase 3 in the control group, cav10 and cav25. The casp3 expression in TNF-α+cav10 and TNF-α+cav25 were both comparable with the TNF-α condition. This indicated the incidence of apoptosis was increased when TNF-α was present, but the presence of caveolin-1 did not make a difference. The MMP13 did not show a clear pattern, in experiment A the expression was highest in the control group. In experiment B, the TNF-α+cav10 and TNF-α+cav25 show comparable expression with the control group. The rest of the conditions showed much lower expression of MMP13.

The main difference found in the qPCR between the two experiments was the Sox9 expression in the TNF-α condition when compared with the control. Experiment A and B differ in the moment of caveolin-1 addition. However, both experiments were equipped with TNF-α from day -5. This implicates the TNF-α was the same condition in both experiments. Despite this, the Sox9 expression did show a difference between those exactly the same conditions. Sox9 is involved in the TGF-β pathway, and thus of interest in anabolism. In experiment A(anti-inflammatory effect), the expression was reduced, while in experiment B (prophylactic effect), the expression was increased in comparison with the control. Collagen expression is known to be increased as a reaction to heal damage caused by TNF-α.(53) The collagen expression increases in order to try to heal the damage. When inflammation

occurs over a longer period of time, the collagen expression would still decrease due to the damage suffered from TNF-α. It is possible, since the collagen is able to increase in presence of TNF-α, Sox9 was able to do this as well. However, it would be a real coincidence this difference was found between two conditions which were supposed to be exactly the same.

The TNF-α did not show a unanimous, sufficient inflammatory reaction, this was shown in the staining for the GAG content and the collagen type I and II. No clear differences were visible between the different conditions. In an *in vitro* study on rat IVD's sufficient inflammation was induced with TNF-α which caused an increase in gene expression of MMP13 and ADAMTS5.(54) This insufficient inflammation could be caused by the lack of efficacy of the inflammatory cytokine, but can also be caused by the overruling effect of the TGF-β which was present in a dose of 10 ng/mL, a dose comely used in 3D culture experiments of NP cells.(55,56) Therefore, the fact caveolin-1 did not seem to play a role, can be caused by the TNF-α or the TGF-β. In this pilot study, the main limitation was overall the low amount of samples in each condition for all assays.

This study was not successful in determining the anti-inflammatory effect of caveolin-1 on the canine NP cells, since no inflammation had occurred. Therefore, it is necessary to determine which inflammatory cytokine induces the best inflammatory environment. Le Maître found IL-1β causes the inflammation in human NP cells, it is possible this is also the case in canine NP cells.(13) Therefore, this pilot should be repeated to determine the anti-inflammatory effect of caveolin-1 on the canine NP cells with an improved study design. It is possible, stimulating the NP cells with IL-1β does simulate a sufficient inflammatory reaction. In subsequent experiments, TGF-β will be added in a concentration of 5 ng/mL instead of 10 ng/mL in order not to have the dominant anabolic effect. Since both TNF-α and IL-1β are known to induce intervertebral disc degeneration, the next experiment will investigate both those pro-inflammatory cytokines.(13,17,20)

Study 2: IL-1β or TNF-α as pro-inflammatory factor in IVDD for dogs *in vitro*

Aim of the study

The pilot concerning the anti-inflammatory effect of caveolin-1 did not show an inflammatory effect in the condition where TNF-α was supplemented. This makes it impossible to conclude whether caveolin-1 has an anti-inflammatory or prophylactic effect on the IVD. While TNF-α has been proposed to be a pro-inflammatory stimulus for NP cells(17), the group of Le Maître stated IL-1β is the main inflammatory factor in human in the degeneration of the intervertebral disc.(13) Therefore, the next pilot is intended for determination of the best pro-inflammatory factor in canine NP cells. Only when this is succeeded it is possible to investigate in a follow-up experiment the anti-inflammatory role of caveolin-1.

Study design

This pilot was performed both on NCD and CD pooled NP cells given that they present IVDD at a different life-stage and may differ in their response to pro-inflammatory stimuli.(9,57) Both pellets and micro-aggregates were created on the basis that the size of the pellet may influence the response of cells to a pro-inflammatory stimulus. The CD cells needed 4 days of expansion, while the NCD needed 7 days of expansion. This pilot will show which inflammatory factor is preferred to be used in the subsequent pilot into the anti-inflammatory effect of caveolin-1. Donors from table 1 and 2 were used. The further culture conditions for all cells are stated in figure 18.

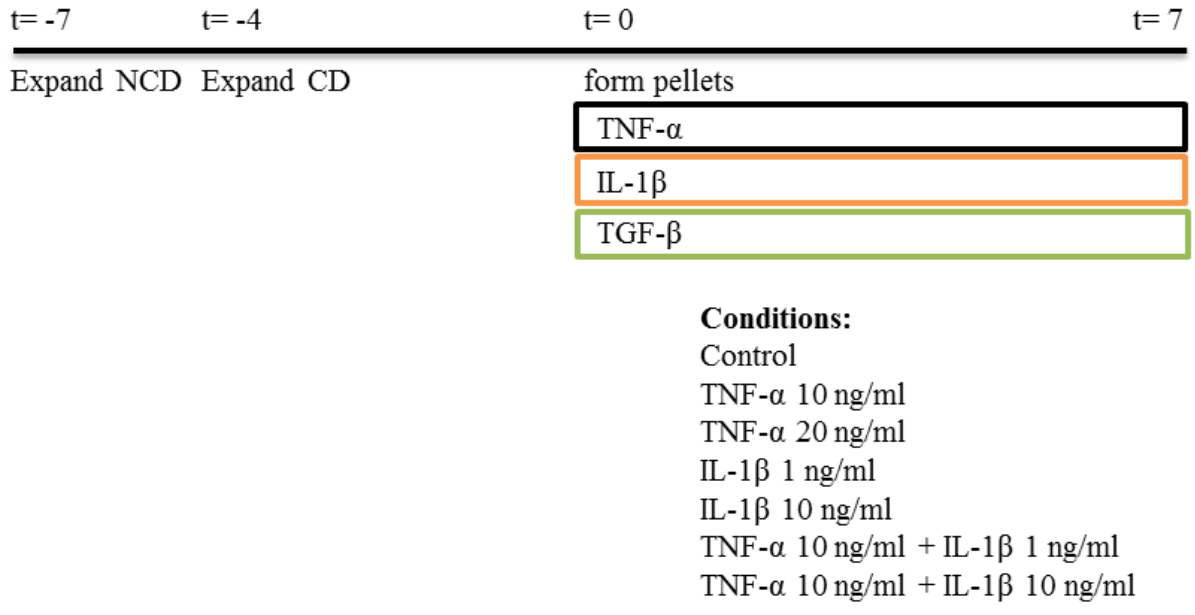

Figure 18 NP cells of degenerated discs were cultured with TNF-α (10 and 20 ng/ml) and IL-1β (1 and 10 ng/ml). TGF-β is used in culture with a dose of 5 ng/ml, to obtain a less dominant anabolic effect.

Aggrecan, collagen type I and II represented the structural genes. Sox9 is part of the TGF-pathway. ADAMTS5, caspase 3, MMP13, FasL, BAX and Bcl2 visualised the apoptotic markers. IL6 was measured as pro-inflammatory cytokine. $PTGES_1$ and $PTGES_2$ are part of the PGE_2 pathway. Two genes were added to the list from the earlier pilot, the cav-1 which is the main subject of interest in this research internship and the Pai1 which is a downstream target in the TGF-β pathway.

Results

GAG and DNA content

The data were collected from the micro-aggregates and the pellets after papain digestion, but also from the corresponding medium. No statistics were performed on these data since for each condition only one sample was available.

In both types of dogs, a pattern was present in the GAG content of the pellets (figure 19A). The control group of the NCD contained the highest amount of GAGs, the IL-1β 1 and IL-1β 10 treatment resulted in minor reduction of GAGs, but this was still comparable with the control group. The TNF-α 10 and 20 treated pellets contained much less GAG compared with control, where the TNF10+IL-1β 1 and TNF10+IL-1β 10 combination of contained the smallest amount of GAGs. The CD groups showed overall higher amounts of GAG when compared with the NCD groups, but the same pattern in the GAG content was present with respect to the application of a pro-inflammatory stimulus. The same pattern was present in the DNA content of the pellets (figure 19B). Remarkable was the higher DNA content of NCD in the inflammatory conditions with TNF-α than the control group, the conditions in which only IL-1β was present were comparable with the control group. The CD was over all the inflammatory conditions comparable with each other, all conditions were lower than the control group. The GAG content corrected for DNA showed no clear differences between the CD and the NCD (figure 19C). However, the GAG/DNA content of the IL-1β 1 and IL-1β 10 were comparable with the control group, while the TNF 10, TNF20, TNF10+IL-1β 1 and TNF10+IL-1β 10 contained less GAG/DNA compared to control.

In micro-aggregates, less clear differences could be distinguished in the different culture groups (figure 19). The GAG content was comparable over all conditions for both CD and NCD-dogs. The same was true for the DNA content of the micro-aggregates. The GAG/DNA of the micro-aggregates (figure 19C) showed a pattern between the different conditions. The IL-1β 1 and 10 were comparable with the control and the conditions which contained TNF-α did show a decrease in GAG/DNA.

Overall, the pellets contained more GAGs compared to the micro-aggregates. The DNA content was higher in the pellets as well. When the GAG/DNA ratio was compared, the pellets did still contain more GAG/DNA in the control group, while in the other conditions no clear differences were visible between the pellets and micro-aggregates (figure 19C).

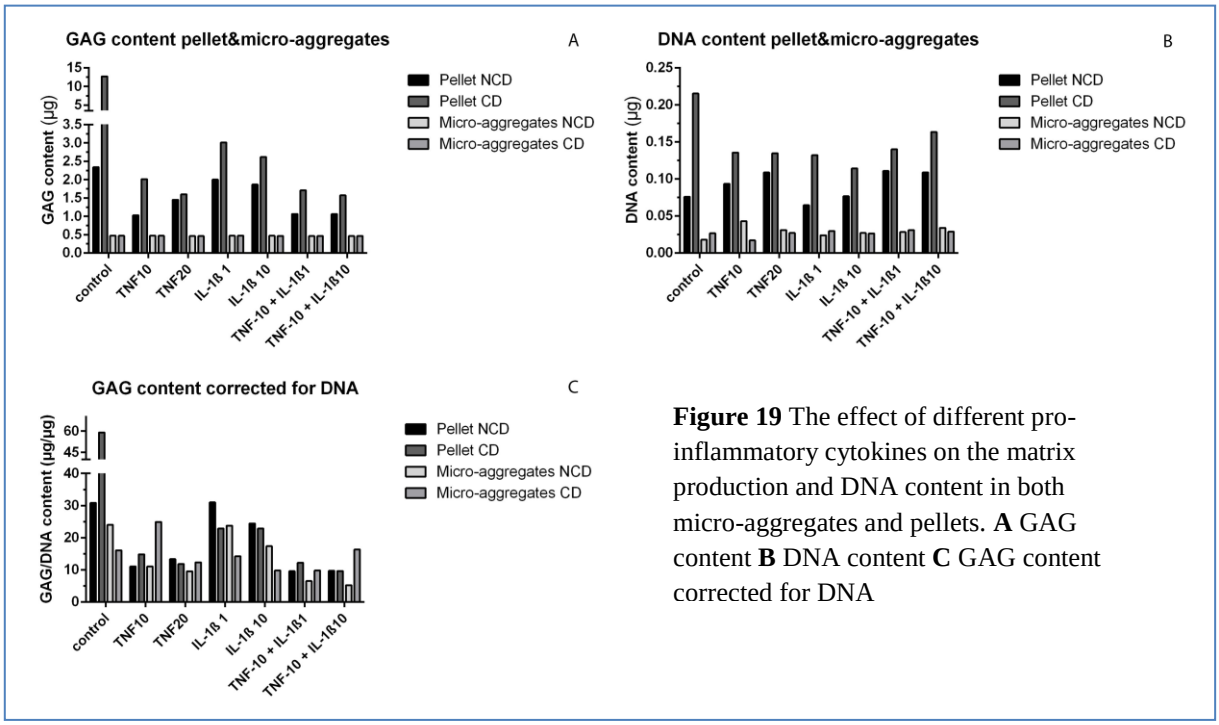

Figure 19 The effect of different pro-inflammatory cytokines on the matrix production and DNA content in both micro-aggregates and pellets. **A** GAG content **B** DNA content **C** GAG content corrected for DNA

The GAG content in the medium was measured in both the pellets and micro-aggregates on day 2, 4 and 6. In the medium of the pellets (figure 20A), in both the NCD and the CD the GAG content of the IL-1β conditions were comparable with the control group and the conditions with TNF-α contained less GAGs compared to both control and IL-1β conditions. The conditions of TNF10 had less GAGs in the NCD pellet when compared with the TNF20. The total amount of GAGs released in the medium was reduced in the conditions which contained TNF-α compared with control, but also the pellet content reduced in GAG content.

The medium of the micro-aggregates (figure 20B) on day 2 of both CD and NCD of the control and TNF10 was not collected due to management problems and thus could not be measured. The micro-aggregates of the CD and the NCD and the medium of day 6 of both dog types contained a comparable amount of GAGs, except for a few outliers. There did not seem to be a difference between the CD and the NCD. However the GAG content of a few conditions in the medium seemed to behave as outliers. This could not be verified, since there was one sample of each condition available for GAG and DNA content measurement.

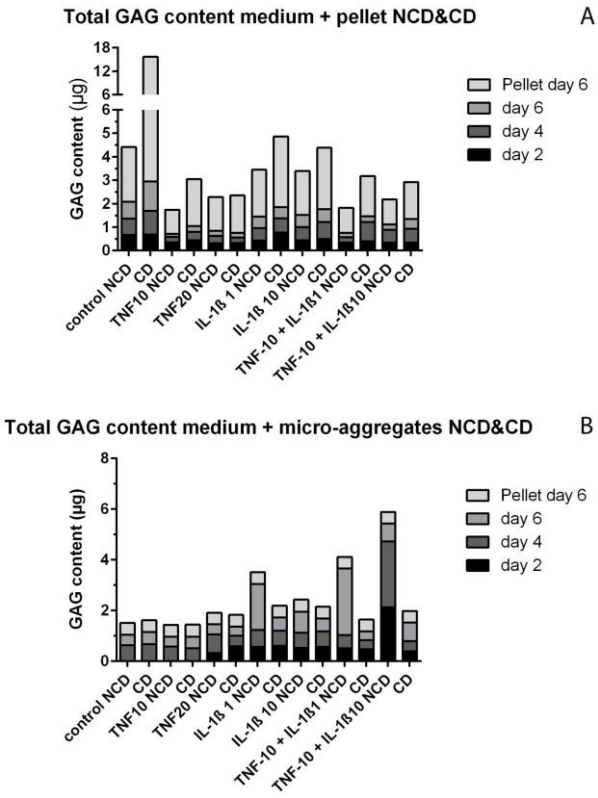

Figure 20 The effect of different pro-inflammatory cytokines on the total amount of GAG production of both pellets and micro-aggregates of CD and NCD. **A** GAG content in micro-aggregates and corresponding medium. **B** GAG content in pellets and corresponding medium.

Gene expression

The following results concern the pellets (n=3) of the non-chondrodystrophoid and chondrodystrophoid dogs. The micro-aggregates yielded insufficient amount of RNA and therefore could not be analyzed. In the figures below only the relevant significances of the CD-dogs are shown since these are the dogs of final interest for the follow-up studies.

Aggrecan (ACAN) is a structural matrix gene and was expressed in a higher amount in the IL-1β 1 condition in the NCD-dog compared to the CD (figure 21A). Both types of dogs had a decrease in the ACAN expression in the conditions where TNF-α was present when compared with the control group. In CD, the expression of ACAN was significantly higher in the control than in TNF10, TNF20 and TNF10 + IL-1β 1. The IL-1β 10 caused a significantly higher expression of ACAN compared to all the conditions in which TNF-α was present. In the NCD, all TNF-α conditions were lower than the control group. All conditions in which TNF-α was present were significantly lower compared to the IL-1β 1 and IL-1β 10.

ADAMTS5 degrades aggrecan (figure 21B). Expression of ADAMTS5 was overall, except for control and TNF-α, higher in the CD dogs compared to the NCD dogs. Almost all conditions were increased compared with the control group in both CD and NCD. The TNF-α seemed to have a dose dependent effect. In the NCD, the TNF10, TNF20 and TNF10 + IL-1β 1 caused a significantly higher expression of ADAMTS5 than the control group. IL-1β 1 of the NCD was lower when compared with TNF10 + IL-1β 1 and TNF10 + IL-1β 10.

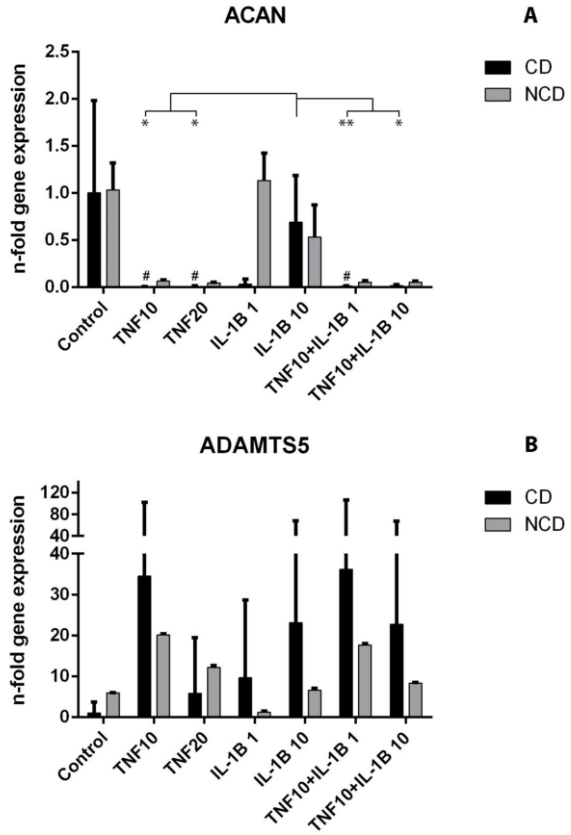

Figure 21 Effect of different pro-inflammatory cytokines on the gene expression of ACAN and ADAMTS5 in pellets. **A** ACAN. **B** ADAMTS5

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005.

The collagen type I gene expression (figure 22A) decreased in all the inflammatory conditions in comparison with the control group. However, the gene expression of collagen type I in IL-1β 1 and IL-1β 10 was higher compared to all conditions which contained TNF-α. This was true for both NCD and CD dogs. In the NCD, the TNF10 and TNF20 both caused a lower gene expression of the collagen type I compared to the control group. The IL-1β 1 and IL-1β 10 in the NCD were both higher than TNF10 and TNF20.

Collagen type II (figure 22B) showed no visible gene expression for the conditions in which TNF-α was present. These conditions were comparable for both dog types. In the CD, IL-1β 1 and IL-1β 10 were significant higher than the TNF10. The IL-1β 1 and IL-1β 10 were significantly higher than the TNF10. IL-1β 10 was also higher compared to both conditions in which the cytokines were combined. The IL-1β 1 and IL-1β 10 in the NCD, were both higher than TNF10 and TNF20. In the NCD TNF10 and TNF20 were both significantly lower than the control.

In CD and NCD, the ratio of col2/col1(figure 22C) was higher in the control, IL-1β 1 and IL-1β 10 compared with the TNF10, TNF20, TNF10+IL-1β 1 and TNF10+IL-1β 10.

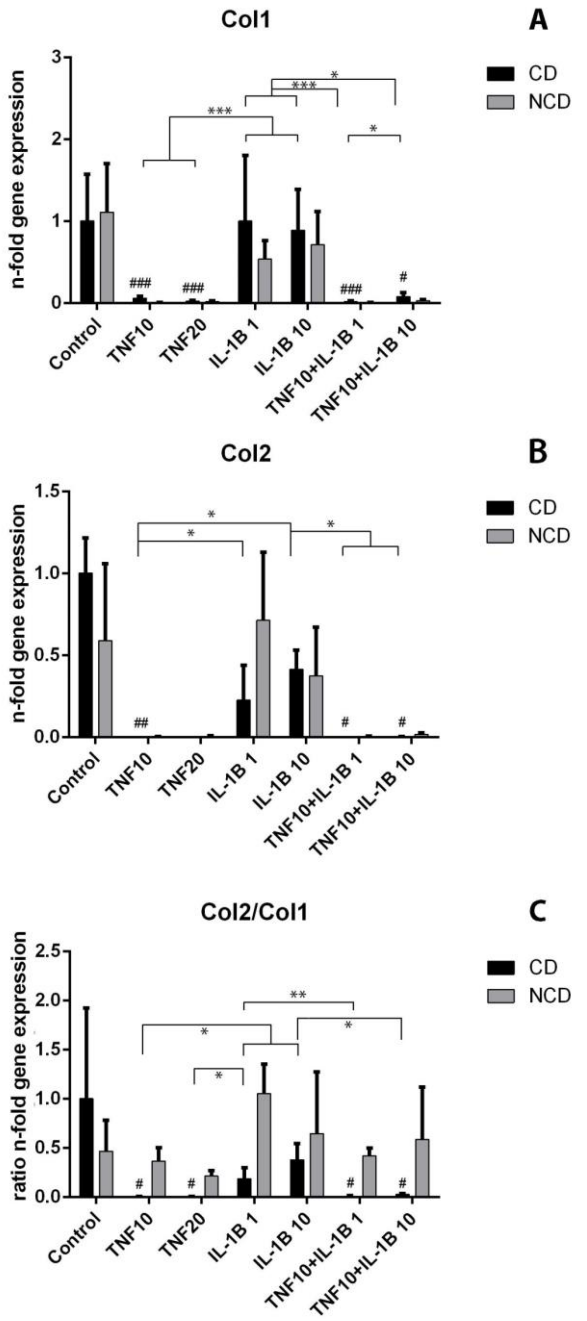

Figure 22 Effect of different pro-inflammatory cytokines on the gene expression of different collagen types in pellets. **A** Collagen type I. **B** Collagen type II. **C** Ratio of collagen type II and type I.

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005.

Sox9 (figure 23) is involved in the TGF-β pathway. The different conditions of the NCD were generally comparable with the control group, while CD showed lower expression of Sox9 in the conditions where TNF-α was present. In the conditions where IL-1β was present a dose dependent effect could be seen, with a higher dose of IL-1β, a higher expression of Sox9 was measured. The TNF-α conditions were significantly lower than the control group and the IL-1β 10. In the NCD these significances were found as well. The IL-1β 1 and TNF1+ IL-1β 1 showed a lower expression compared to the control group.

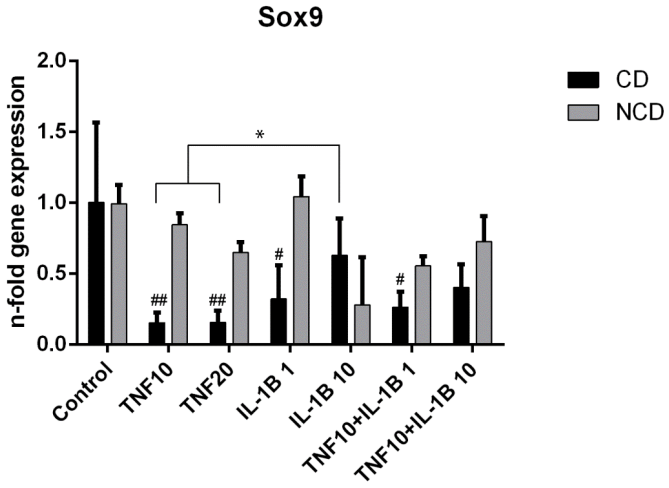

Figure 23 Effect of different pro-inflammatory cytokines on the gene expression of SOX9 in pellets.

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005.

Pai1 (figure 24) is a downstream target of TGF-β and was more expressed over all conditions in the NCD. No significances were found for both CD and NCD.

MMP13 expression (figure 25) was different between the two dog types. The TNF20 and IL-1β 1 conditions of CD showed almost no expression, while the NCD for the same conditions showed more expression of the MMP13 than in the control group. The gene expression was highly variable and not significantly different between the conditions.

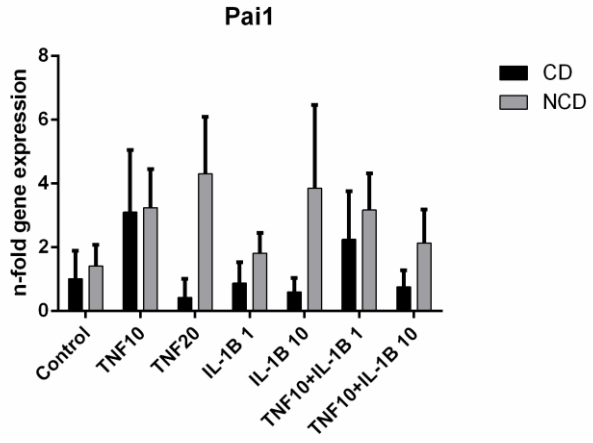

Figure 24 Effect of different pro-inflammatory cytokines on the gene expression of Pai1 for both CD and NCD in pellets.

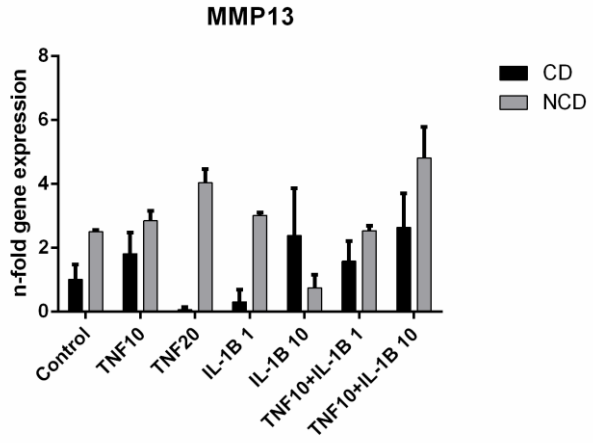

Figure 25 Effect of different pro-inflammatory cytokines on the gene expression of MMP13 in both CD and NCD in pellets.

The caspase 3 (figure 26) reflex' the apoptotic incidence. No clear differences could be determined between the different conditions for the different dog types. The TNF10 and TNF10+IL-1B 1 of the CD caused a significantly higher expression compared to the control group. The TNF1+ IL-1β 1 showed a higher gene expression compared with the TNF+ IL-1β 10.

IL6 gene expression (figure 27) was increased in the TNF-α treated pellets compared to control. In both CD and NCD, a higher dose of TNF-α caused a higher expression of IL6. However, NCD overall expressed more IL6 compared to CD. The IL-1β1 and 10 were lower than the TNF+ IL-1β 1 and TNF+ IL-1β 10. In the NCD, all conditions which contained TNF-α expressed a significantly higher IL6 compared to the control group.

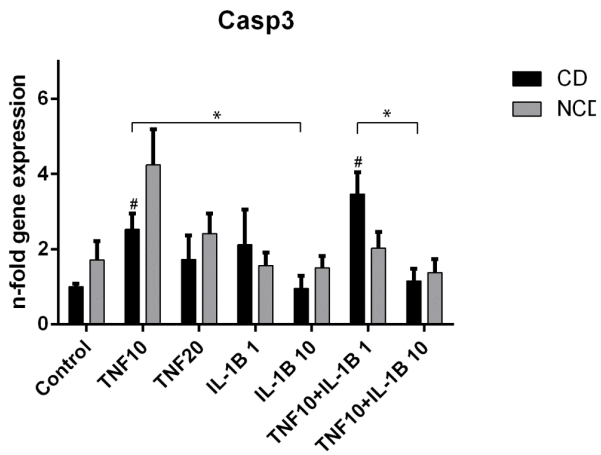

Figure 26 Effect of different pro-inflammatory cytokines on the gene expression of Casp3 in both CD and NCD in pellets.

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005.

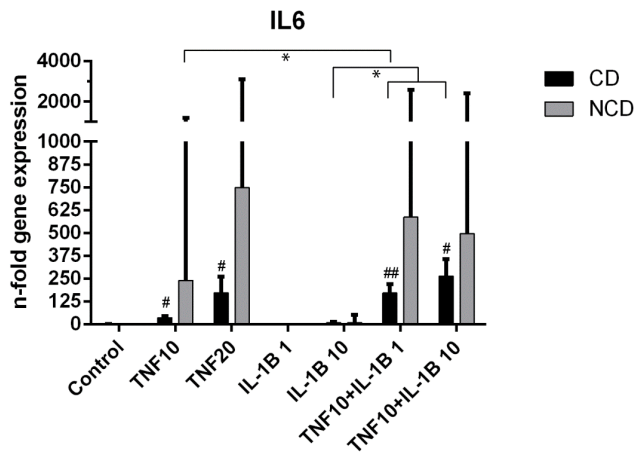

Figure 27 Effect of different pro-inflammatory cytokines on the gene expression of IL6 in both CD and NCD in pellets.

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005.

BAX (figure 28A) is a marker for apoptosis and its expression was comparable for both dog types in all conditions. There seemed to be a dose dependent effect concerning the TNF-α, an increase in TNF-α dose caused an increase in BAX expression. Significances were only found for the NCD. The TNF20 and TNF10+IL-1β 10 were both significantly increased compared with the control group. The IL-1β 1 and IL-1β 10 were significantly lower expressed than the conditions in which TNF-α was present.

Bcl2 (figure 28B) is an anti-apoptotic gene and showed a clear difference between the NCD and the CD. The NCD was expressed more outspoken in the conditions in which TNF-α was present. For the NCD, all conditions in which TNF-α was present were significantly increased in comparison with the control group. Thereby, these conditions were also significantly increased when compared with IL-1β 1 and IL-1β 10. For the CD, the TNF-α conditions were significantly lower than the control group.

The ratio between BAX and Bcl2 showed the susceptibility for apoptosis of the cells (figure 28C). NCD was generally more susceptible for apoptosis when pro-inflammatory stimuli were present. The TNF20 of the CD was significantly different with all other conditions. The BAX/Blc2 ratio was significantly higher in the IL-1β 1 compared to TNF10+IL-1β 1 and TNF10+IL-1β 10. In the NCD, the IL-1β 10 was significantly higher compared with the control and with all conditions in which TNF-α was present.

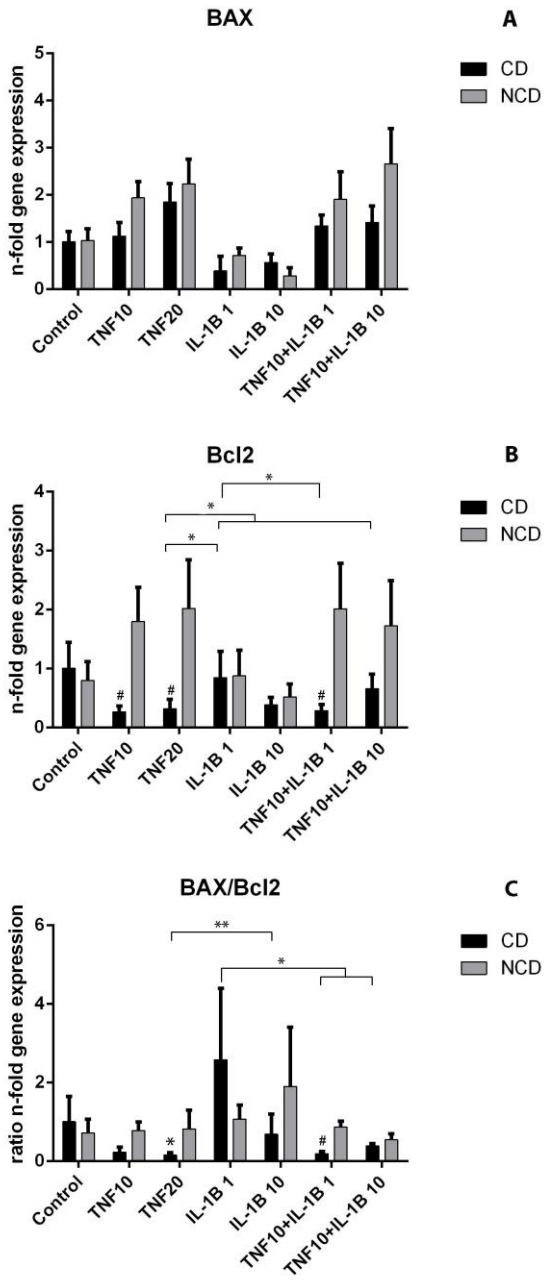

Figure 28 Effect of different pro-inflammatory cytokines on the gene expression of apoptotic and non-apoptotic genes in pellets. **A** BAX. **B** Bcl2. **C** BAX/Bcl2 ratio.

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05; ##: p<0.01; ###: p<0.005. A single * above a bar illustrates a significance of p<0.05 with all the other conditions.

The $PTGES_1$ (figure 29A) did show an increase of expression in the conditions of NCD where TNF-α was present. This was also true for CD, but in smaller quantity, in general the NCD expressed more $PTGES_1$ compared to CD. In the NCD, the TNF20, TNF10+IL-1β 1 and TNF10+IL-1β 10 caused a significantly higher expression compared to the control. The IL-1β 1 was lower than all the conditions in which TNF-α was present.

The expression of $PTGES_2$ (figure 29B) was comparable between the two dog types. For both CD and NCD, no significances were found.

To see what happened to the caveolin-1 in the presence of a pro-inflammatory stimulus, the gene expression levels were also determined. (figure 30). The inflammatory condition did not influence the gene expression of caveolin-1. Overall, caveolin-1 seemed to be expressed more in NCD than in CD.

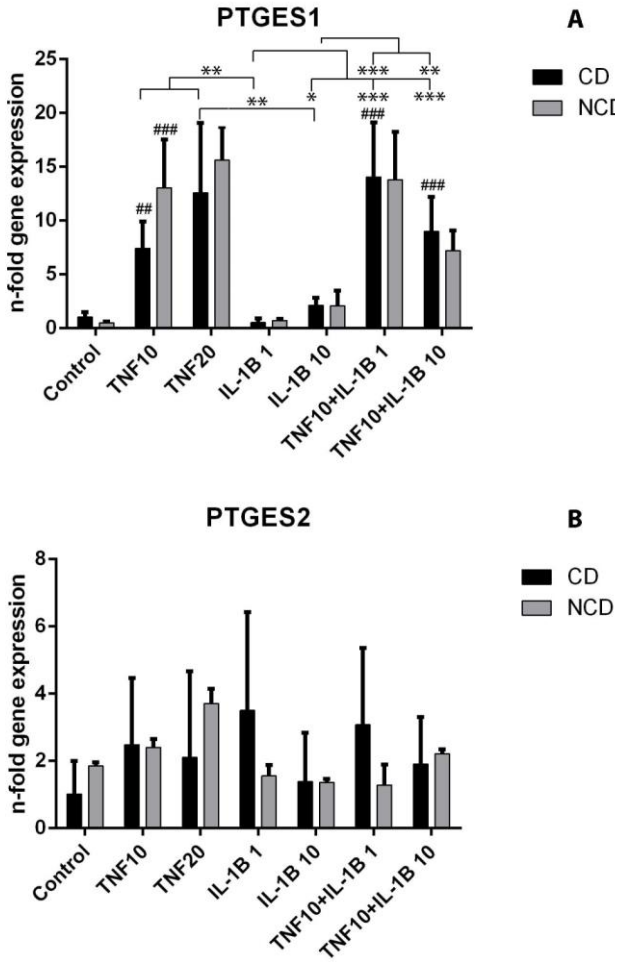

Figure 29 Effect of different pro-inflammatory cytokines on the gene expression in pellets of **(A)** PTGES1 and **(B)** PTGES2

Significances are visualized with *: p<0.05; **: p<0.01; ***: p<0.005. If significances were found in comparison with the control group, this was illustrated with #: p<0.05;

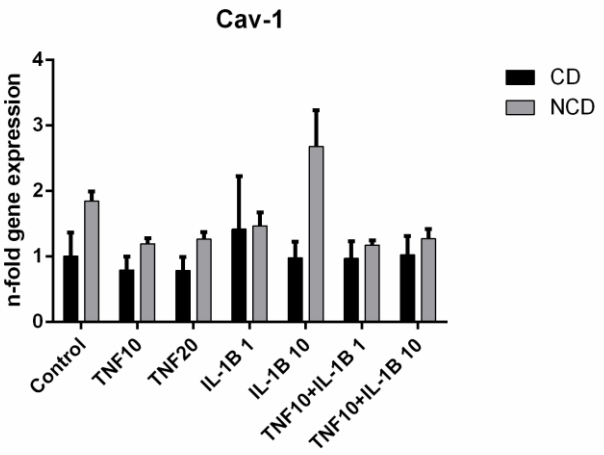

Figure 30 Effect of different pro-inflammatory cytokines on the gene expression of caveolin-1 in pellets.

Safranin-O/Fast-Green

The control and the IL-1β 10 of the micro-aggregates got lost during the culture process. The micro-aggregates were much smaller than the pellets (figure 31). The Safranin-O/Fast-Green stained the GAG content red and everything else became blue. All the control groups showed blue with red on the edges, while none of the TNF conditions showed any red, same applies for the conditions where the inflammatory factors were combined. The different doses of IL-1β did show red/blueish, which indicates GAG presence. Differences between negative and positive cells are shown in figure 32.

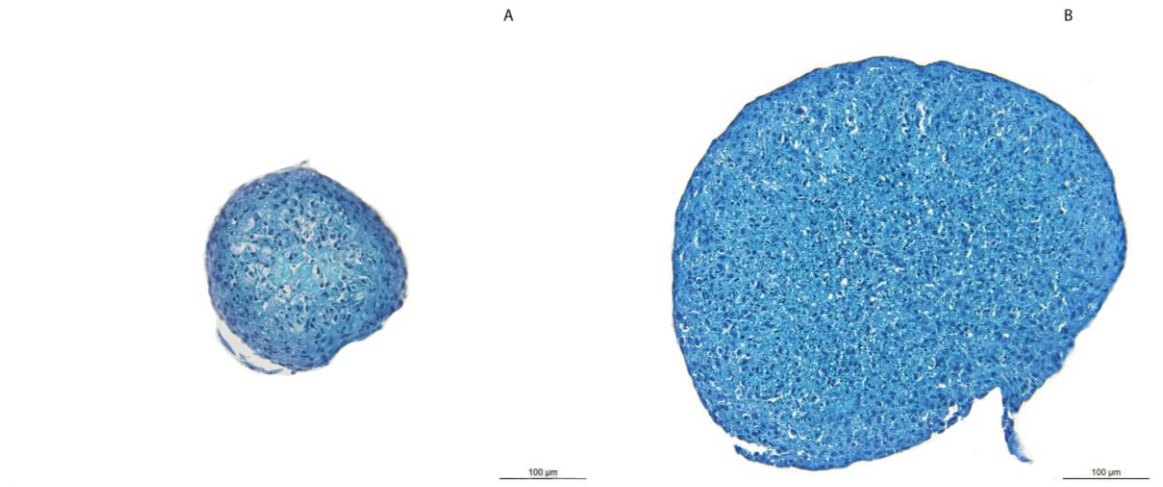

Figure 31 Differences in size of micro-aggregates (35.000 cells) and pellets (200.000 cells). **(A)** Smell micro-aggregate CD, TNF10+IL-1β 10 **(B)** Bigger pellet CD, TNF10+IL-1β10

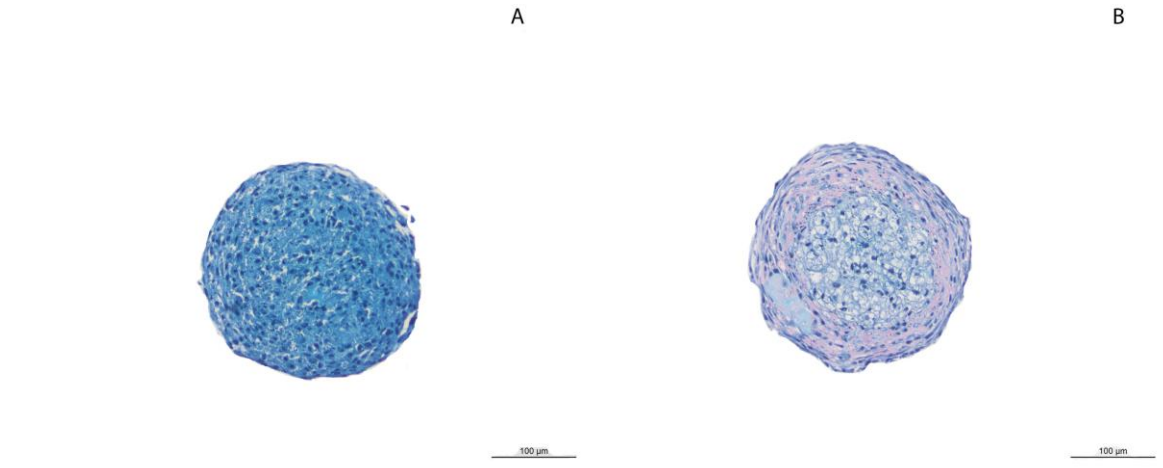

Figure 32 Effect of different pro-inflammatory cytokines on Safranin-O/Fast-Green staining. **(A)** micro-aggregate CD, TNF10+IL-1β 10. Negative stained cell. **(B)** micro-aggregate CD, IL-1β 10. Positive stained

Discussion

The aim of the present study was to investigate whether TNF-α and/or IL-1β showed a better inflammatory effect, since the previous study into the anti-inflammatory effect of caveolin-1 did not show a sufficient inflammatory effect of the TNF-α alone. Importantly, in the previous pilot, all the groups contained TGF-β in a dose of 10 ng/mL. This could mask the effect of TNF- α. Therefore, the TGF-β was reduced to 5 ng/mL in this pilot. Both IL-1β and TNF-α are known to play a role in development of IVDD.(13,17) Therefore, both these pro-inflammatory cytokines were tested in micro-aggregates and pellet cultures in order to determine whether size of the pellet may influence the induction of inflammation.

TNF-α did seem to induce the best inflammatory reaction at both gene expression level as GAG production and histology. The qPCR showed a decreased expression of matrix genes (ACAN, Col1, Col2) with an increase of catabolic genes (ADAMTS5, Casp3, BAX) in presence of TNF-α. Moreover, it was shown the susceptibility of apoptosis was increased in presence of TNF-α. In TNF-α, the collagen type I was predominant over the collagen type II, which was in line with our expectation.(58) TNF-α stimulated the expression of a pro-inflammatory gene, IL-6, more than IL-1β did. Furthermore, the GAG content in the medium and in the pellet was reduced in TNF10 and TNF20, this indicates the pellet produced less GAG when compared with the control group. The GAG/DNA content was clearly decreased in the TNF-α conditions in the pellets, which was also confirmed on histology. The stainings where the GAGs were designated show GAG formation in the conditions where IL-1β was present. The degradation of extracellular matrix was also found on gene level by Liu *et al* (2015). (59)

In the present study, the effect of IL-1β on the canine NP cells was investigated. MMP13 was found to be increased in the condition where the cells were cultured with IL-1β, this was found in human NP cells by Le Maître *et al* (2005) as well. The ADAMTS5 was not altered in the current study which is in line with the studies of the Le Maître group. ACAN was not significantly changed in the present study, but in the study of Le Maître, downregulation was found. However, Le Maître *et al* (2005) found a downregulation of Sox9, while in the current study no significant difference was found. The collagen type II was downregulated in research of Le Maître *et al* (2005), in the current research, a difference was found between the CD and the NCD where the CD was downregulated and the NCD was not significantly changed. The differences between the study of Le Maître (2005) and the present study might be explained due to the fact the group of Le Maître performed the experiment up to 48 hours. Moreover, the group of Le Maître performed the studies on human cells, so there might be species differences.(18)

There was a clear difference between the CD and the NCD cells concerning the BAX/Bcl2 ratio, CD's were more susceptible to apoptosis. From the BAX/Bcl2 ratio could be concluded the CD were more susceptible for apoptosis. Since inflammation and apoptosis are the origin of the degeneration of the IVD(1,9,14,60), this increased susceptibility of apoptosis in the CD's correspond.

This study showed caveolin-1 was expressed more in the NCD than in the CD, however no statistics were performed to determine the significances between the CD and NCD as it was not within the scope of the present study. Caveolin-1 is more expressed in discs where the NCs are dominant, which was the case in NCD.(61) In the NCD, degeneration occurs at a later age when compared with the CD due to the low amount of NC cells.(24) However, no NCs were present in the culture of NCD, this could be seen due to the fact no big vacuolated cells were visible in histology. Both literature and this pilot study found caveolin-1 was more expressed in NCD than in CD.

Aggrecan and collagen type I and II were increased in presence of the IL-1β. In line with the present study, Phillips *et al* (2015) report an effect on ECM remodeling in response to very low IL-1 stimulation. Based on this, the authors postulated that, IL-1β may have a biological role in the healthy IVD.(18) Dysregulated expression of IL-1β might be harmful.(62) It is possible the low dose in which the IL-1β was added has caused the effect of the higher expression of the structural genes.

In this pilot study, the main limitation was the number of samples in each condition. Since the qPCR did not show sufficient data in the IL-1β conditions for almost all genes, this made it hard or impossible to draw a conclusion from these conditions. This problem could easily be solved when the number of samples is increased in order to increase the input of material for RNA isolation.

The aim of the study was to determine the optimal inflammatory factor to simulate the best inflammatory reaction in the intervertebral disc so the experiment in which the anti-inflammatory effect of cav-1 was determined could be optimized. It seemed clear TNF-α was the best factor to induce inflammation in the canine NP cell in both CD and NCD dogs. Pellets of 200.000 cells were the best size in which the inflammation process could be simulated based on gene expression, GAG release in the media and deposition in the pellet.

Study 3: Caveolin-1 as anti-inflammatory factor on pellets

Aim of the study

With the earlier performed studies, the question whether caveolin-1 functions as an anti-inflammatory factor remained unanswered. Based on the second study we chose to treat the pellets with 5 ng/mL TGF-β and 10 ng/mL TNF-α given that TNF-a provided a distinct inflammation component in the *in vitro* culture system. Furthermore, in order to simulate the *in vivo* situation and able signaling pathways FBS (fetal bovine serum) was also added to the chondrogenic medium.

Study design

Pellets of a CD donor pool containing 200.000 cells were employed (table 1). TGF-β was present in 5 ng/mL and a pro-inflammatory stimulus was provided with 10 ng/mL TNF-α throughout the first 5 days of culture (figure 33). Thereafter, caveolin-1 was supplemented at two different concentrations, 10 and 25 µM to determine its anti-inflammatory effects.

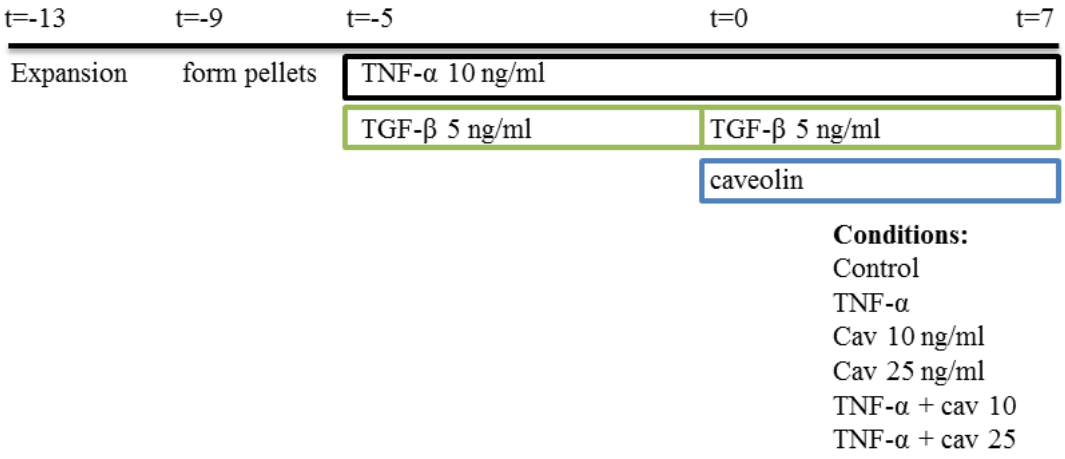

Figure 33 NP cells of degenerated discs were cultured with TNF-α (10 and 20 ng/ml) and IL-1β (1 and 10 ng/ml). TGF-β is used in culture with a dose of 5 ng/ml, to obtain a less dominant anabolic effect.

Aggrecan, collagen type I and type II represented the structural genes. Sox9 is part of the TGF-pathway. ADAMTS5, caspase 3, MMP13, FasL, BAX and Bcl2 visualised the apoptotic markers. IL6 and IL-1β were measured as inflammatory cytokines. CAV1 is the gene which corresponds with the caveolin-1 protein, which is of final interest. Thereby also the GAG and DNA content were measured and histology is visualized with the Safranin-O/Fast-Green staining.

Results

GAG and DNA content

Statistics were not significant due to the low number of samples (n=3), however borderline significant trends (p=0,100) were observed.

The GAG content of the pellets (figure 34 A) on day 7 was increased in the cav10 and cav25 compared to the control. Moreover, the GAG content was borderline significantly (p=0.100) 5-times higher in the control condition compared with all the conditions in which TNF-α was present.

The DNA content of the pellets (figure 34 B) was decreased in all conditions at day 7 in comparison to day 0. The DNA content of the pellets on day 7 was decreased in the TNF-α treated pellets when compared with the control group. The DNA content of the pellets was increased in the cav25 compared to the control group.

The GAG/DNA content in the pellets (figure 34 C) was clearly reduced in the inflammatory conditions compared with the respective control groups (borderline significance). Cav10 seemed to increase GAG/DNA when compared with the control group.

The amount of GAGs released in the medium was reduced in the TNF-α+cav10 and TNF-α+cav25 compared with the TNF-α (figure 34D). In the pellets, the amount of GAGs was increased or at least stable, which implicates that the pellets were producing the same or even a greater amount of GAG in presence of the caveolin-1.

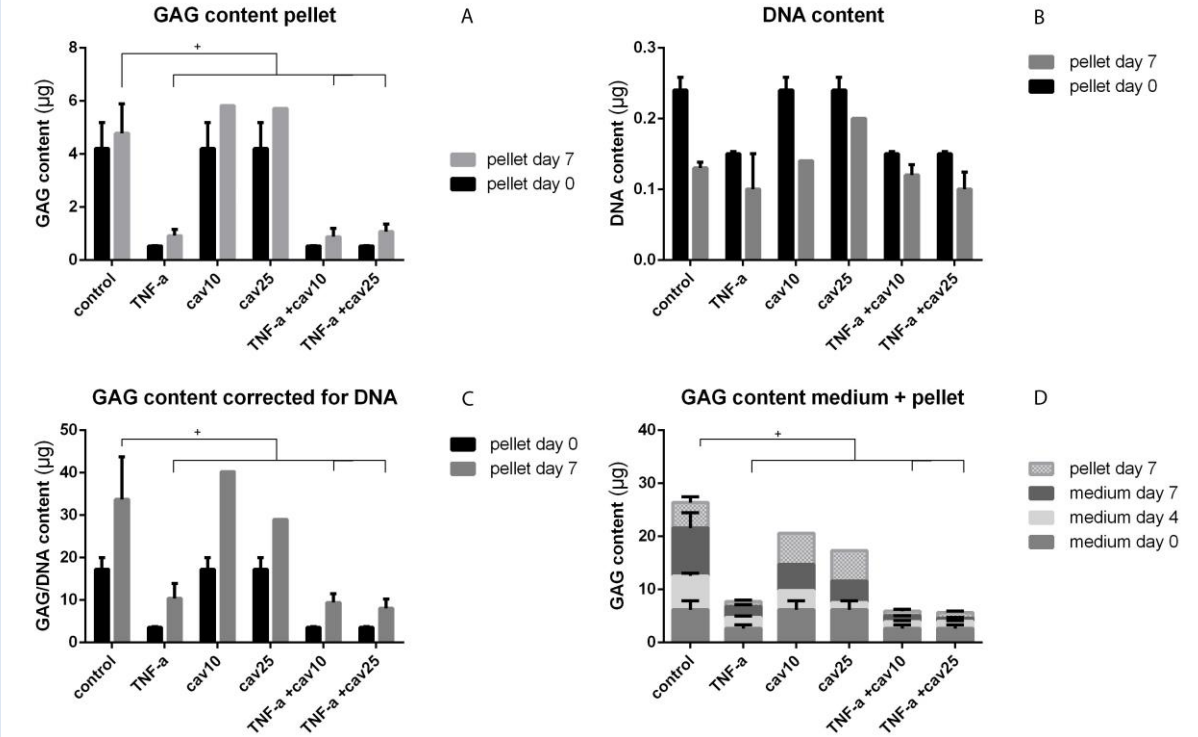

Figure 34 The effect of caveolin-1 on the matrix production and DNA content during inflammation in pellets. (A) GAG content in pellets **(B)** DNA content in pellet **(C)** GAG content corrected for DNA in pellet **(D)** GAG medium (d0+d4 +d7) + pellet (d7)

The total amount of GAG released in the medium (figure 34D) decreased in the TNF-α+cav25 when compared with the TNF-α. In the presence of TNF-α release of GAG in the medium seemed to decrease in time during culture (figure 35). In the control, cav10 and cav25, there was no comparable trend.

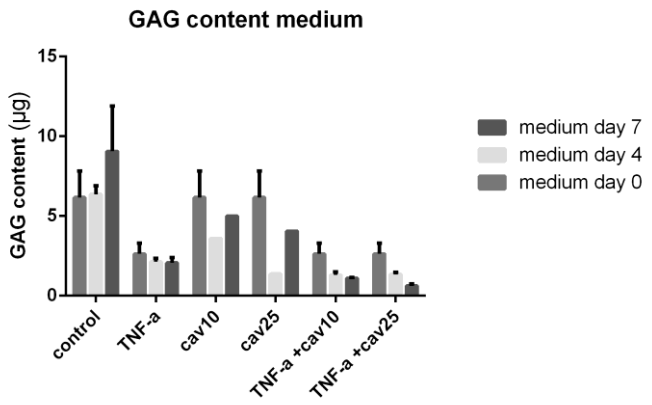

Figure 35 The effect of caveolin-1 on the GAG release in medium in pellets. Cumulative image of total GAG release in medium.

Gene expression

qPCR was performed only on the control, TNF-α, TNF-α+cav10, TNF-α+cav25, no statistics were performed because n=2.

The ACAN (figure 36 A) expression was 5-fold lower in the TNF-α compared to the control group. The TNF-α+cav10 and TNF-α+cav25 showed a slightly higher expression of ACAN when compared with the TNF-α.

The ADAMTS5 (figure 36 B), which breaks down aggrecan, was expressed much higher in the TNF-α compared with the control. The TNF-α+cav25 showed a lower expression than the TNF-α, less aggrecan breakdown took place, however there was a lot of variability. The MMP13 was reduced 1.5-fold in the TNF-α+cav10 and TNF-α+cav25 when compared with the TNF-α.

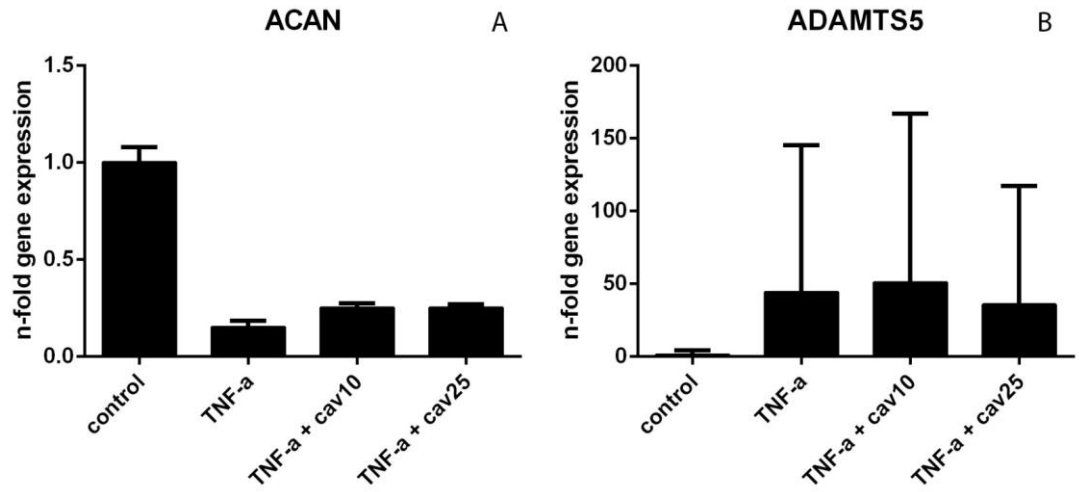

Figure 36 The effect of caveolin-1 on the gene expression in pellets during inflammation. **(A)** Aggrecan **(B)** ADAMTS5

Collagen type I expression (figure 37 A) was increased in the TNF-α+cav10 compared to TNF-α. Collagen type II expression (figure 37 B) was reduced in TNF-α in comparison with the control group. The TNF-α+cav10 and TNF-α+cav25 caused an slight increase in expression of collagen type II compared to the TNF-α. However, the col2/col1 ratio still showed a dominance of collagen type I in all conditions.

Sox9 showed a 2-fold decrease in expression in the TNF-α compared to the control group, in the TNF-α+cav10 a 1.2-fold increase in comparison with this TNF-α was observed.

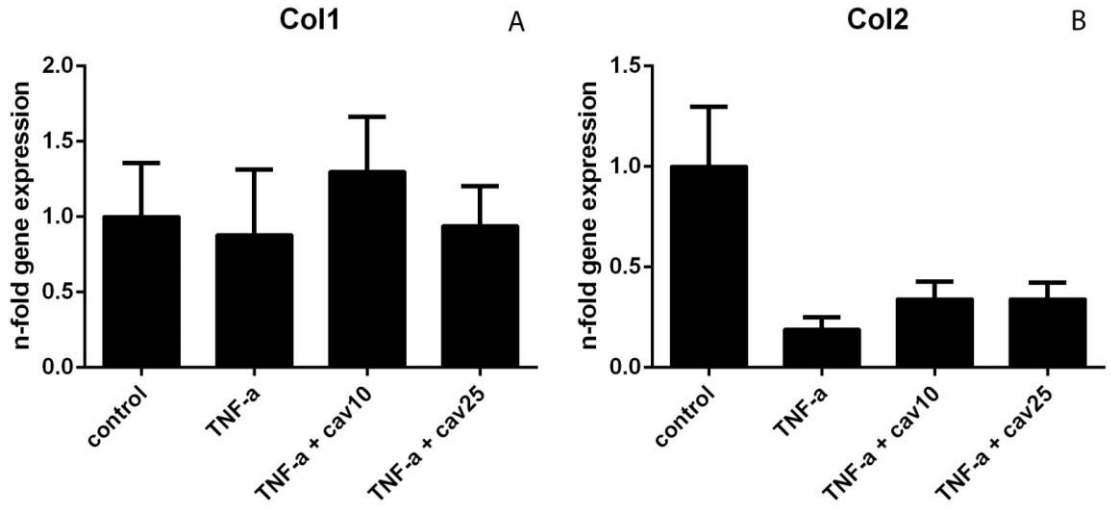

Figure 37 The effect of caveolin-1 on the gene expression of different collagen types in pellets during inflammation.**(A)** Collagen type I **(B)** Collagen type II

The gene expression of IL6 (figure 38) was increased in the TNF-α when compared with the control. The TNF-α+cav10 caused an even higher expression of the inflammatory cytokine compared to TNF-α. The TNF-α+cav25 however caused a comparable expression of IL6 with TNF-α.

The expression of IL-1β was comparable for the control, TNF-α+cav10 and TNF-α+cav25. The samples did show sufficient data, however the TNF-α showed a 2- fold reduced expression compared to the control group.

The BAX/Bcl2 increased in TNF-α+cav10 and TNF-α+cav25 when compared with the TNF-α. In these conditions the ratio was greater than 1.

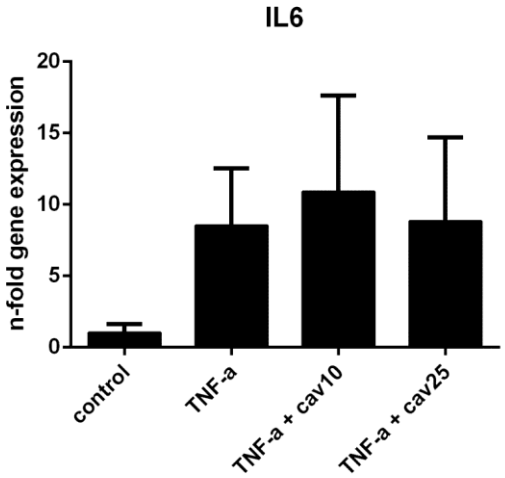

Figure 38 The effect of caveolin-1 on the gene expression of interleukin (IL-) 6 in pellets

Caveolin-1 expression was increased 1.1-fold in TNF-α compared to control, the TNF-α+cav25 showed a comparable expression with the control. The TNF-α+cav10 caused a 1.4-fold expression increase compared with the TNF-α.

Safranin-O/Fast-Green staining

In the control group (figure 39 A) the matrix stained red and the pellet was surrounded by a blue rim of matrix containing fibroblasts. In the center of the pellet pyknotic nuclei were present and matrix contained empty lacunae. In the TNF-α (figure 39 B) treated pellets the periphery of the pellet contained primarily fibrotic cells embedded in GAG-rich matrix. Pyknotic nuclei were present in the center of the pellet. Between these two distinct layers, there was a transition layer that was GAG-poor and contained primarily apoptotic cells. The TNF-α+cav10 (figure 39 C) did show the same layered pattern as the TNF-α did, but less distinct. The TNF-α+cav25 (figure 39 D) did show an apoptotic nucleus and a gradient, but both were less present. Less pyknotic cells were present and the pellet was less damaged. When further sections of the pellet of the TNF-α+cav25 were stained, there was indeed an apoptotic nucleus of the pellet (figure 40).

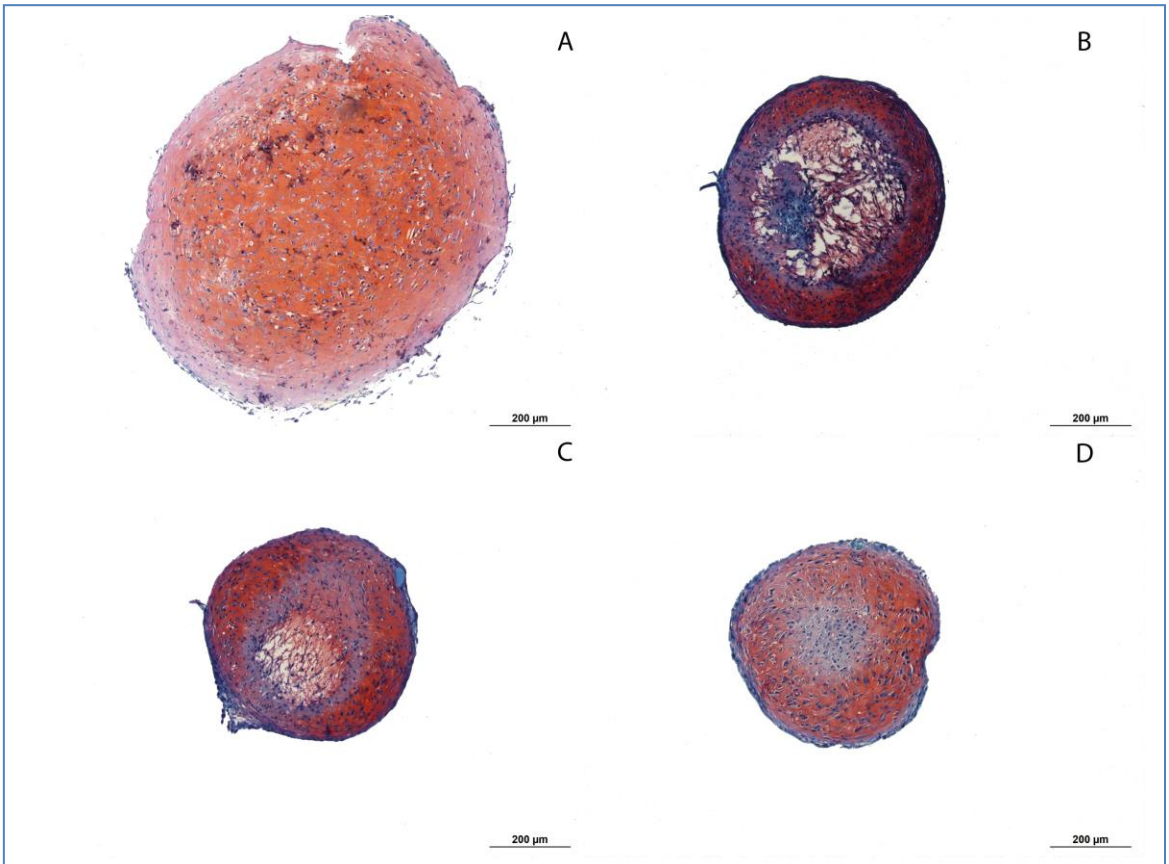

Figure 39 The effect of caveolin-1 on the Safranin-O/Fast-Green staining of pellets. **(A)** Control with TGF-β **(B)** TNF10 with an apoptotic center and gradient **(C)** TNF-α +cav10, in which the apoptotic center and gradient were less present **(D)** TNF-α +cav25 in which no apoptotic center seemed to be

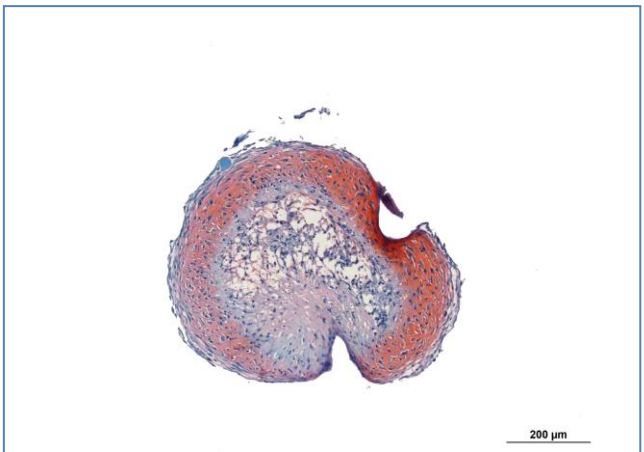

Figure 40 The effect of caveolin-1 on the Safranin-O/Fast-Green staining of the TNF-α+cav25 showed an apoptotic nucleus after all.

Discussion

This study was performed to determine the anti-inflammatory effect of caveolin-1 on NP cells isolated from degenerated IVDs in the presence of a pro-inflammatory stimulus.

By comparing the control and the TNF-α concerning the GAG content of the pellet, it could be concluded TNF-α as a pro-inflammatory cytokine indeed induced a distinct catabolic effect. The inflammatory component is visible in the increase of IL-6 gene expression but not in the expression of IL-1β. The latter seems to play a less prominent role in inflammation in this *in vitro* setting which is in line with the findings of the previous study, where 10 ng/mL IL-1β alone did not elicit an inflammatory response in a similar *in vitro* set up. Based on earlier literature, it was suspected IL-1β would have shown an increase in expression of IL-1β when TNF-α was present.(17) Thereby, the TNF-α caused a BAX/Bcl2 ratio greater than 1, which implicated the susceptibility of apoptosis is greater in the conditions in which TNF-α was present.

The GAG content of the pellets treated with TNF-α+cav25 was increased compared with TNF-α alone, while GAG release in the medium was decreased in the TNF-a+cav25 compared with TNF-a alone. This implicates an increased incorporation of GAGs by the pellets, which would be a positive aspect in the regenerative application of caveolin-1. However, this increased incorporation of GAGs by the pellets of TNF-α+cav25 was not significant compared to the TNF-α. When the experiment would be prolonged, this effect might become more evident. In line with this observation, qPCR and Safranin-O/Fast-Green staining indeed showed an increase in GAGs deposition as well. Notably, a gradient was visible in the staining of the TNF-α, with more pyknotic nuclei and matrix devoid of GAGs in the center of the pellets where the nutrient could not reach the center.

In this pilot, not only the expanding medium, but also the chondrogenic medium contained FBS (fetal bovine serum). Since the exact content of the FBS is unknown, the exact function is also unknown, but it is speculated that it may mediate also other signaling pathways than the TGF-β signaling pathway alone. We cannot exclude that the apoptotic center may be due to the presence of FBS. The presence of FBS may also have affected the measurements GAG and DNA . Literature shows an increased GAG/DNA ratio in NCs which are cultured in presence of FBS.(63) It was observed the DNA content of the pellets decreased during the present study, while the DNA content increased in time in the previous studies of this research internship. This increase was seen in earlier pilot studies among this research team as well.

In this pilot study, the main limitation was the small group size, and in case of the qPCR and staining the lack of two entire conditions cav10 and cav25. This was due to an insufficient number of cells expanded, by this reason the experiment was forced to be reduced.

The aim of the study was to determine the anti-inflammatory effect of caveolin-1. The DMMB assay showed a decrease in GAG release in the medium and an increase in GAG content in the pellet itself in the presence of caveolin-1. In the TNF-α+cav10 and TNF-α+cav25 an increase of IL-1β expression in comparison with the control was visible. Supplementation of caveolin-1 in the presence of a pro-inflammatory stimulus resulted in a slight increase of ACAN and decrease in ADAMTS5 compared with the TNF-α which further support the biochemical findings of the present study. On a histological level, caveolin-1 did not have a clear anti-inflammatory effect.

General discussion

This research internship focused on the possible anti-inflammatory effect of the caveolin-1 on the nucleus pulposus cells of CD dog breeds. During this internship different co-founding effects were encountered and further investigated. For example, the insufficient differences in the first pilot study between TNF-α treated micro-aggregates and controls, could be caused by a lack of a well performing pro-inflammatory cytokine and/or by the dominating effect of the growth factor TGF-β. The first pilot was performed on micro-aggregates. Therefore, in the second pilot, both micro-aggregates of 35.000 cells and pellets of 200.000 cells were used to determine which size of the clumps was best for simulating the inflammation. To determine the possible difference between CD and NCD, the second pilot was performed on both dog types.

The balance between anabolic and catabolic stimuli. In the first pilot the TNF-α did not seem to induce a catabolic effect. TGF-β is known to enhance anabolism by stimulating the proliferation and matrix synthesis in the IVD cells. The presence of TGF-β at 10 ng/mL may have caused a possible dominating anabolic effect, which resulted in the absence of TNF-α catabolic effect.(14) The dominant anabolic effect can be averted by supplementing it in a dose of 5 ng/mL.

TNF-a seems to be the most potent pro-inflammatory stimulus in canine NP cells. Both TNF-α and IL-1β were tested since both are shown to be involved in developing IVDD.(17) The pro-inflammatory cytokines were tested in different doses and combined with each other. It was seen that TNF-α initiated a more clear catabolic effect when compared with IL-1β. This was in line with the research on human NP cells by Walter *et al* (2015) (17), but was contradictory with the research of Hoyland *et al* (2008).(64) This inconsistency between Hoyland and the current research might be due to the fact the experiments of Hoyland lasted up to 48 hours. A higher dose of TNF-α however, did not unanimous showed a more sufficient inflammation. This may be caused by the possibility of cleavage of TNF-α receptors which then can become soluble in the cytoplasm. When TNF-α binds to these soluble receptors, no inflammation occurs.(65,66)

Nutrient supply may affect the catabolic effect of inflammation. Pellets of 200.000 cells are bigger compared with micro-aggregates of 35.000 cells. In pellets, there may be more nutrient and oxygen deprivation by diffusion due to the larger size, which may have induced the more clear catabolic effect which was visible in the pellets. This nutrient deprivation is more credible due to the fact *in vitro* culture with mesenchymal stem cell (MSC) pellets of $5*10^5$ or $10*10^5$ cells also show an apoptotic center.(67,68) This may be a good model, due to the fact that difficulty in the nutrient diffusion is a factor in development of IVDD.(5,8,69)

The anti-inflammatory effect of Caveolin-1. With substitution of caveolin-1 in 10 µM to an inflammatory pellet, the apoptotic center in the pellet became less prominent. The 25µM substitution of caveolin-1 to the inflammation did not seem to improve the apoptotic center. Gene expression showed an increase of matrix components and a decrease of catabolic components when caveolin-1 was substituted to the inflammation. Collagen type I however was still predominant over collagen type II. Caveolin-1 did seem to have a mild effect on stimulating matrix components and counteracting the catabolic effects caused by the presence of a pro-inflammatory stimulus. In line with these findings, Wang *et al* (2006) already reported that caveolin-1 had a protective role for inflammation by suppression of pro-inflammatory cytokine TNF-α in macrophages. It is known cav-1 interacts with TLR4, this receptor is shown to be present in human IVD's, dependent on the degree of IVDD. TNF-α can induce TLR4 expression which mediates inflammation. When cav-1 is decreased, the interaction of cav-1 and TLR4 does not occur and thereby inflammation is potentiated. (36,43,70) Besides the TLR4, caveolin-1 is also known to interact with EGFR and the TGF-β receptors.(35-37) The

interaction with the TGF-β receptor might be the cause of the only slight anti-inflammatory effect which was found in this study. The anabolic effect of the TGF-β might also be inhibited.

The protective role of caveolin-1 is however by other disputed. Caveolin-1 is known to influence the development of IVDD. Cav-1 knock out is related with increased apoptosis and decreased self-renewal of NC's and NP stem cells.(24) However, other sources state that upregulation of caveolin-1 is involved in SIPS, senescence which is known to be involved in IVDD.(19,21) Altogether, it seems that caveolin-1 is indeed involved in development of IVDD being both down- and upregulated. (19,21,24)

Caveolin-1 is related with tumorigenesis in different organs (31) and therefore prior to further translation additional studies are needed. Reduced expression can cause breast-, lung-, colon-, ovarian- and cervical cancer, while overexpression of caveolin-1 can cause prostate- and kidney cancer.(71-78) It is associated with tumor progression and reduced survival time, poor tumor differentiation and blood vessel invasion and invasion and metastasis of bladder cancer.(71,76,77) Other research however shows cav-1 suppresses tumors.(79) Effects on tumor growth in the spine are not yet known, therefore, those effects on the intervertebral disc should be researched.

Limitation of the study. This research internship consisted out of three pilot studies, so only data up to day 7 were available. It is unknown what the effects of caveolin-1 might be in a longer experiment. Thereby, all pilots were performed with maximum 3 samples in each condition group. Also, in the second pilot, no statistical differences could be found with SPSS, due to the low sample size.

Conclusion.

The best pro-inflammatory cytokine involved in simulating IVDD in pellets, composed by NP cells of CD dog breed *in* vitro, is the TNF-α with substitution of TGF-β in a dose of 5 ng/mL. Inflammatory pellets treated with caveolin-1 showed a slight increase GAG amount in the pellets. The matrix components did show an increased gene expression and the catabolic genes showed a decreased gene expression in the caveolin-1 treated inflammatory pellets. *In vitro* supplementation of caveolin-1 to inflammatory pellets composed of NP cells from CD dog breed did seem to have a mild anti-inflammatory effect.

References

(1) Bergknut N, Smolders LA, Grinwis GCM, Hagman R, Lagerstedt A-, Hazewinkel HAW, et al. Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. Veterinary Journal 2013;195(3):282-91.

(2) Freemont AJ, Watkins A, Le Maitre C, Jeziorska M, Hoyland JA. Current understanding of cellular and molecular events in intervertebral disc degeneration: implications for therapy. J Pathol 2002;196:374-379.

(3) OpenStax College. The vertebral column. In: CNX Anatomy and Physiology, editor. Anatomy & Physiology. 6.27th ed.: OpenStax College; 2013.

(4) Anderson G, Albert TJ. The Molecular Basis of Intervertebral Disk Degeneration. Seminars in Spine Surgery 2003;15(4):352-60.

(5) Le Maitre CL, Freemont AJ, Hoyland JA. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. J Pathol 2004;204(1):47-54.

(6) Adams MA, Dolan P. Intervertebral disc degeneration: Evidence for two distinct phenotypes. J Anat 2012;221(6):497-506.

(7) Lama P, Le Maitre CL, Dolan P, Tarlton JF, Harding IJ, Adams MA. Do intervertebral discs degenerate before they herniate, or after? Bone Joint J 2013;95-B(8):1127-33.

(8) Iwata M, Aikawa T, Hakozaki T, Arai K, Ochi H, Haro H, et al. Enhancement of Runx2 expression is potentially linked to beta -catenin accumulation in canine intervertebral disc degeneration. J Cell Physiol 2015;230(1):180-90.

(9) Smolders LA, Bergknut N, Grinwis GCM, Hagman R, Lagerstedt A-, Hazewinkel HAW, et al. Intervertebral disc degeneration in the dog. Part 2: Chondrodystrophic and non-chondrodystrophic breeds. Veterinary Journal 2013;195(3):292-9.

(10) Jeffery ND, Levine JM, Olby NJ, Stein VM. Intervertebral disk degeneration in dogs: Consequences, diagnosis, treatment, and future directions. Journal of Veterinary Internal Medicine 2013;27(6):1318-33.

(11) Burke JG, Watson RWG, McCormack D, Dowling FE, Walsh MG, Fitzpatrick JM. Intervertebral discs which cause low back pain secrete high levels of proinflammatory mediators. J Bone Joint Surg Br 2002;84(2):196-201.

(12) Weiler C, Nerlich AG, Bachmeier BE, Boos N. Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: A study in surgical specimen and autopsy controls. Spine 2005;30(1):44-53.

(13) Le Maitre C, Hoyland JA, Freemont AJ. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile. Arthritis Res Ther 2007;9:R77.

(14) Feng C, Liu H, Yang Y, Huang B, Zhou Y. Growth and differentiation factor-5 contributes to the structural and functional maintenance of the intervertebral disc. Cell Physiol Biochem 2015;35(1):1-16.

(15) Ulrich J, Liebenberg E, Thuillier D, Lotz J. ISSLS prize winner: repeated disc injury causes persistent inflammation. Spine 2007;32(25):2812-9.

(16) Grang L, Gaudin P, Trocme C, Phelip X, Morel F, Juvin R. Intervertebral disk degeneration and herniation: the role of metalloproteinases and cytokines. Joint Bone Spine 2001;68(6):547-53.

(17) Walter B, Purmessur D, Likhitpanichkul M, Weinberg A, Cho S, Qureshi S, et al. Inflammatory Kinetics and Efficacy of Anti-inflammatory Treatments on Human Nucleus Pulposus Cells. Spine 2015;40(13):955-63.

(18) Le Maitre C, Freemont A, Hoyland J. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. Arthritis Res Ther 2005;7(4):R732-45.

(19) Heathfield S, Le Maitre C, Hoyland J. Caveolin-1 expression and stress-induced premature senescence in human intervertebral disc degeneration. 2008;10(4):87.

(20) Walter B, Likhitpanichkul M, Illien Junger S, Roughley P, Hecht A, Iatridis J. TNFa transport induced by dynamic loading alters biomechanics of intact intervertebral discs. PLoS ONE 2015;10(3):e0118358.

(21) Dai SM, Shan ZZ, Nakamura H, Masuko-Hongo K, Kato T, Nishioka K, et al. Catabolic stress induces features of chondrocyte senescence through overexpression of caveolin 1: possible involvement of caveolin 1-induced down-regulation of articular chondrocytes in the pathogenesis of osteoarthritis. Arthritis Rheum 2006;54:818-831.

(22) Le Maitre C, Freemont A, Hoyland J. Accelerated cellular senescence in degenerate intervertebral discs: a possible role in the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther 2007;9(3):R45.

(23) Mayer JE, Iatridis JC, Chan D, Qureshi SA, Gottesman O, Hecht AC. Genetic polymorphisms associated with intervertebral disc degeneration. Spine Journal 2013;13(3):299-317.

(24) Smolders LA, Meij BP, Onis D, Riemers FM, Bergknut N, Wubbolts R, et al. Gene expression profiling of early intervertebral disc degeneration reveals a down-regulation of canonical Wnt signaling and caveolin-1 expression: Implications for development of regenerative strategies. Arthritis Research and Therapy 2013;15(1):1-19.

(25) Kalichman L, Hunter DJ. The genetics of intervertebral disc degeneration.Associated genes. Joint Bone Spine 2008;75(4):388-96.

(26) Mercier I, Jasmin J-, Pavlides S, Minetti C, Flomenberg N, Pestell RG, et al. Clinical and translational implications of the caveolin gene family: Lessons from mouse models and human genetic disorders. Lab Invest 2009;89(6):614-23.

(27) Zou H, Stoppani E, Volonte D, Galbiati F. Caveolin-1, cellular senescence and age-related diseases. Mech Ageing Dev 2011 0;132(11–12):533-42.

(28) Weiss CR, Guan Q, Ma Y, Qing G, Bernstein CN, Warrington RJ, et al. The Potential Protective Role of Caveolin-1 in Intestinal Inflammation in TNBS-Induced Murine Colitis. PLoS ONE 2015;10(3).

(29) Li X, Gu X, Boyce TM, Zheng M, Reagan AM, Qi H, et al. Caveolin-1 increases proinflammatory chemoattractants and blood–retinal barrier breakdown but decreases leukocyte recruitment in inflammation. Invest Ophthalmol Vis Sci 2014;55(10):6224-34.

(30) Takizawa N, Sawada S, Chosa N, Ishisaki A, Naruishi K. Secreted caveolin-1 enhances periodontal inflammation by targeting gingival fibroblasts. Biomed Res 2013;34(1):1-11.

(31) Luan T, Zhu T, Cui Y, Zhang G, Song X, Gao D, et al. Expression of caveolin-1 is correlated with lung adenocarcinoma proliferation, migration, and invasion. Med Oncol 2015;32(7):207.

(32) Galbiati F, Volonte D, Brown AMC, Weinstein DE, Ben-Ze'ev A, Pestell RG, et al. Caveolin-1 expression inhibits Wnt/ß-catenin/Lef-1 signaling by recruting ß-catenin to caveolae membrane domains. J Biol Chem 2000;275(30):23368-77.

(33) Li S, Song KS, Koh SS, Kikuchi A, Lisanti MP. Baculovirus-based expression of mammalian caveolin in Sf21 insect cells: A model system for the biochemical and morphological study of caveolae biogenesis. J Biol Chem 1996;271(45):28647-54.

(34) Tuma PL, Hubbard AL. Transcytosis: Crossing cellular barriers. Physiol Rev 2003;83(3):871-932.

(35) Gvaramia D, Blaauboer ME, Hanemaaijer R, Everts V. Role of caveolin-1 in fibrotic diseases. Matrix Biology 2013 8/8;32(6):307-15.

(36) Wang XM, Kim HP, Song R, Choi AM. Caveolin-1 confers antiinflammatory effects in murine macrophages via the MKK3/p38 MAPK pathway. Am J Respir Cell Mol Biol 2006;34(4):434-42.

(37) Lee YD, Yoon S-, Park CK, Lee J, Lee ZH, Kim H-. Caveolin-1 regulates osteoclastogenesis and bone metabolism in a sex-dependent manner. J Biol Chem 2015;290(10):6522-30.

(38) Liu P, Rudick M, Anderson RG. Multiple functions of caveolin-1. J Biol Chem 2002;277:41295-8.

(39) Pelkmans L, Bürli T, Zerial M, Helenius A. Caveolin-Stabilized Membrane Domains as Multifunctional Transport and Sorting Devices in Endocytic Membrane Traffic. Cell 2004 9/17;118(6):767-80.

(40) Zemans R, Downey GP. Role of caveolin-1 in regulation of inflammation: Different strokes for different folks. Am J Physiol Lung Cell Mol Physiol 2008;294(2):175-77.

(41) Brown G, Wallin C, Tatusova T, Pruitt K, Murphy T, Maglott D. Gene Help: Integrated Access to Genes of Genomes in the Reference Sequence Collection. 2014:1- 53.

(42) Mineo C, James GL, Smart EJ, Anderson RG. Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. J Biol Chem 1996;271(20):11930-5.

(43) Tang H, Pang S, Wang M, Xiao X, Rong Y, Wang H, et al. TLR4 activation is required for IL-17-induced multiple tissue inflammation and wasting in mice. J Immunol 2010;185(4):2563-9.

(44) Hu G, Ye RD, Dinauer MC, Malik AB, Minshall RD. Neutrophil caveolin-1 expression contributes to mechanism of lung inflammation and injury. Am J Physiol Lung Cell Mol Physiol 2008;294(2):L178-L186.

(45) Handley C, Goldschlager T, Oehme D, Ghosh P, Jenkin G. Mesenchymal stem cell tracking in the intervertebral disc. World J Stem Cells 2015 Jan 26;7(1):65-74.

(46) Bach FC, Willems N, Penning LC, Ito K, Meij BP, Tryfonidou MA. Potential regenerative treatment strategies for intervertebral disc degeneration in dogs. BMC Veterinary Research 2014;10:1-12.

(47) Catalán V, Gómez-Ambrosi J, Rodríguez A, Silva C, Rotellar F, Gil MJ, et al. Expression of caveolin-1 in human adipose tissue is upregulated in obesity and obesity-associated type 2 diabetes mellitus and related to inflammation. Clin Endocrinol 2008;68(2):213-9.

(48) Wang H, Liu H, Zheng Z, Zhang K, Wang T, Sribastav S, et al. Role of death receptor, mitochondrial and endoplasmic reticulum pathways in different stages of degenerative human lumbar disc. Apoptosis 2011;16(10):990-1003.

(49) Das M, Gherghiceanu M, Lekli I, Mukherjee S, Popescu L, Das D. Essential role of lipid raft in ischemic preconditioning. Cell Physiol Biochem 2008;21(4):325-34.

(50) Salakou S, Kardamakis D, Tsamandas A, Zolota V, Apostolakis E, Tzelepi V, et al. Increased Bax/Bcl-2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. In Vivo 2007;21(1):123-32.

(51) Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74(4):609-19.

(52) Yang E, Korsmeyer SJ. Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 1996;88(2):386-401.

(53) Miosge N, Hartmann M, Maelicke C, Herken R. Expression of collagen type I and type II in consecutive stages of human osteoarthritis. Histochem Cell Biol 2004;122(3):229-36.

(54) Ishibashi H, Tonomura H, Ikeda T, Nagae M, Sakata M, Fujiwara H, et al. Hepatocyte growth factor/c-met promotes proliferation, suppresses apoptosis, and improves matrix metabolism in rabbit nucleus pulposus cells in vitro. J Orthop Res 2015.

(55) de Vries, Stefan A H, Potier E, van Doeselaar M, Meij B, Tryfonidou M, Ito K. Conditioned medium derived from notochordal cell-rich nucleus pulposus tissue stimulates matrix production by canine nucleus pulposus cells and bone marrow-derived stromal cells. Tissue Eng Part A 2015;21(5-6):1077-84.

(56) Pelle D, Peacock J, Schmidt C, Kampfschulte K, Scholten D, Russo S, et al. Genetic and functional studies of the intervertebral disc: a novel murine intervertebral disc model. PLoS ONE 2014;9(12):e112454.

(57) Willems N, Tellegen A, Bergknut N, Creemers L, Wolfswinkel J, Freudigmann C, et al. Inflammatory profiles in canine intervertebral disc degeneration. BMC Vet Res 2016;12(1):10.

(58) Frazer A, Bunning RA, Thavarajah M, Seid JM, Russell RG. Studies on type II collagen and aggrecan production in human articular chondrocytes in vitro and effects of transforming growth factor-beta and interleukin-1beta. Osteoarthr Cartil 1994;2(4):235-45.

(59) Liu C, Fei H, Sun Z, Tian J. Bioinformatic analysis of the microarray gene expression profile in degenerative intervertebral disc cells exposed to TNF-a. Eur Rev Med Pharmacol Sci 2015;19(18):3332-9.

(60) Risbud M, Shapiro I. Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol 2014;10(1):44-56.

(61) Smolders LA, Meij BP, Riemers FM, Licht R, Wubbolts R, Heuvel D, et al. Canonical Wnt signaling in the notochordal cell is upregulated in early intervertebral disk degeneration. Journal of Orthopaedic Research 2012;30(6):950-7.

(62) Phillips KLE, Cullen K, Chiverton N, Michael ALR, Cole AA, Breakwell LM, et al. Potential roles of cytokines and chemokines in human intervertebral disc degeneration: interleukin-1 is a master regulator of catabolic processes. Osteoarthr Cartil 2015;23(7):1165-77.

(63) Gantenbein B, Calandriello E, Wuertz Kozak K, Benneker L, Keel MJB, Chan SCW. Activation of intervertebral disc cells by co-culture with notochordal cells, conditioned medium and hypoxia. BMC Musculoskelet Disord 2014;15:422.

(64) Hoyland JA, Le Maitre C, Freemont AJ. Investigation of the role of IL-1 and TNF in matrix degradation in the intervertebral disc. Rheumatology (Oxford) 2008;47(6):809-14.

(65) Watts AD, Hunt NH, Madigan MC, Chaudhri G. Soluble TNF-alpha receptors bind and neutralize over-expressed transmembrane TNF-alpha on macrophages, but do not inhibit its processing. J Leukoc Biol 1999;66(6):1005-13.

(66) Bathon J. Anti-TNF Therapy for Rheumatoid Arthritis. 2016; Available at: http://www.hopkinsarthritis.org/arthritis-info/rheumatoid-arthritis/ra-treatment/anti-tnf-therapy-for-ra/. Accessed 01/12, 2016.

(67) Vidal M, Robinson S, Lopez M, Paulsen D, Borkhsenious O, Johnson J, et al. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. Vet Surg 2008;37(8):713-24.

(68) Murdoch A, Grady L, Ablett M, Katopodi T, Meadows R, Hardingham T. Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of scaffold-free cartilage. Stem Cells 2007;25(11):2786-96.

(69) Sudo H, Yamada K, Iwasaki K, Higashi H, Ito M, Minami A, et al. Global identification of genes related to nutrient deficiency in intervertebral disc cells in an experimental nutrient deprivation model. PLoS ONE; 2013 2013;8(3).

(70) Klawitter M, Hakozaki M, Kobayashi H, Krupkova O, Quero L, Ospelt C, et al. Expression and regulation of toll-like receptors (TLRs) in human intervertebral disc cells. Eur Spine J 2014;23(9):1878-91.

(71) Fong A, Garcia E, Gwynn L, Lisanti M, Fazzari M, Li M. Expression of caveolin-1 and caveolin-2 in urothelial carcinoma of the urinary bladder correlates with tumor grade and squamous differentiation. Am J Clin Pathol 2003;120(1):93-100.

(72) Kato T, Miyamoto M, Kato K, Cho Y, Itoh T, Morikawa T, et al. Difference of caveolin-1 expression pattern in human lung neoplastic tissue. Atypical adenomatous hyperplasia, adenocarcinoma and squamous cell carcinoma. Cancer Lett 2004;214(1):121-8.

(73) Bender FC, Reymond MA, Bron C, Quest AF. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res 2000;60(20):5870-8.

(74) Wiechen K, Diatchenko L, Agoulnik A, Scharff KM, Schober H, Arlt K, et al. Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 2001;159(5):1635-43.

(75) Razani B, Altschuler Y, Zhu L, Pestell RG, Mostov KE, Lisanti MP. Caveolin-1 expression is down-regulated in cells transformed by the human papilloma virus in a p53-dependent manner. Replacement of caveolin-1 expression suppresses HPV-mediated cell transformation. Biochemistry 2000;39(45):13916-24.

(76) Yang G, Truong LD, Wheeler TM, Thompson TC. Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. Cancer Res 1999;59(22):5719-23.

(77) Horiguchi A, Asano T, Asakuma J, Sumitomo M, Hayakawa M. Impact of caveolin-1 expression on clinicopathological parameters in renal cell carcinoma. J Urol 2004;172(2):718-22.

(78) Bouras T, Lisanti M, Pestell R. Caveolin-1 in breast cancer. Cancer Biol Ther 2004;3(10):931-41.

(79) Deb M, Sengupta D, Kar S, Rath S, Parbin S, Shilpi A, et al. Elucidation of caveolin 1 both as a tumor suppressor and metastasis promoter in light of epigenetic modulators. Tumour Biol 2014;35(12):12031-47.