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[A Comparative study on MMP-2 activity and GAG concentration during subsequent stages of degenerated canine intervertebral discs.]

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Table of contents

Summary

Since 2006 intervertebral disc degeneration is defined as:

”*The process of disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerated disc is one with structural failure combined with accelerated or advanced signs of aging*”[1].

In order to come up with a justifiable model for intervertebral disc degeneration in humans, one has to quantify changes that take place on cellular and extracellular level inside the nucleus pulposus of the intervertebral disc during subsequent stages of intervertebral disc degeneration. The different stages of degeneration are graded using the five-category grading scheme according to Thompson[2]. A total of 123 individual nuclei pulposi samples were used, obtained from 13 randomly selected dogs older than one year of age, none of the 13 dogs was euthanized for reasons related to intervertebral disc degeneration.

For the quantification of the sulphated glycosaminoglycan content in the tissue samples, the Farndale (Dimethylmethylene Blue) assay was used. Two groups can be identified based on the GAG content. In Group A al samples that came from intervertebral discs with either a I or II on the Thompson scale are combined, whereas in group B holds all the samples that were graded III, IV or V. The Farndale assay showed that GAG concentration is significantly higher in group A when compared to in group B. For MMP-2 concentration a gelatin zymogram was used. For the MMP-2 activity there is a rise in activity for each step upwards on the Thompson scale, until samples that were graded with a III on the Thompson scale. The MMP-2 activity in samples originating from discs with a grade IV is significantly lower than those originating from discs with grade III.

1. Introduction.

1.1 Relevance and prevalence

As in humans, back problems in canines directly related to intervertebral disc degeneration (IVDD) is a major problem which seems to increase every year[3]. The first person to describe IVDD in dogs was Jansen in 1881[4]. Since then a lot of research has been done in the field of human and canine intervertebral disc disease[3-11]. There is evidence that the prevalence of disc degeneration related problems is increasing. About 40 years ago 0-1% of all patients seeking veterinary care were diagnosed with problems directly related to disc degeneration[4]. This prevalence climbed to 2-3% in the 1970's[12]. The increase in incidence could be due to an increased awareness of the disease among veterinarians combined with improved diagnostic methods, an increase of breeds prone to IVDD and an overall increase in age of our canine population due to improved overall animal health care[3].
So far there still is no consensus on what IVDD actually is and how it should be interpreted. In 2006, a research group reviewed existing literature to come up with a justifiable working definition of IVDD, they stated that:
"*The process of disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerated disc is one with structural failure combined with accelerated or advanced signs of aging*" [1].
In humans the magnitude of problems related to intervertebral disc disease, can be expressed in incidence and prevalence figures. For instance, in the European countries the yearly incidence of back pain due to intervertebral disc degeneration is as high as 5%. Whereas the yearly prevalence in European countries is somewhere between 25% and 40% of the total population, regional variance is clearly detectable. In the Netherlands for instance, the risk for a male to have problems of lower back pain at some point during his lifetime is 51%, for females this percentage is even higher (58%)[13].

1.2 Animal models for intervertebral disc degeneration.

Animals are widely used in an attempt to produce a representative model for human intervertebral disc degeneration. By using animal models scientists try to unravel the pathological mechanisms underlying IVDD and develop new therapeutic strategies to treat the disease.
In disc degenerative research, animal tissue and animals models are used for *in vitro, in silico* and *in vivo* studies[14].
With *in vitro* studies on intervertebral disc diseases, intervertebral disc tissue is grown under controlled laboratory conditions outside the living organism. In this way cellular behavior and extracellular tissue can be studied in a simple controlled model. This situation however differs a great deal from the physiological environment of intervertebral cells *in vivo.* A physiologically more relevant model to study tissue growth and/or behavior inside the disc is the so called *in vitro* explant culture model. In this model, the cells are not removed from their natural surrounding extracellular matrix. The explants can be composed out of one or more intervertebral disc(s), with or without their adjacent vertebral bodies and the ligaments that bind them together. With these models the cellular behavior is studied whilst placing the entire structure under different static and/or dynamic loads. In this way a situation is created that tries to resemble the environment of the intervertebral disc *in vivo*[15].
With *in silico* models, a computer based simulation is used to predict certain outcomes. With an ever increasing strength and speed of our computer capabilities this type of models will become more and more important in the future[16].

Both *in vitro* and *in silico* models however are in their current stage only capable of producing an adequate simulation for acute processes[14]. IVDD is a long-term process according to its definition. Therefore both *in vitro* as *in silico* models are not *yet* usable in the simulation of intervertebral disc degeneration.
It is also important to validate findings from *in vitro* or *in silico* models by using an *in vivo* animal model. Therefore *in vivo* models are still the most important model to study the pathological mechanism underlying IVDD. For an animal model to be applicable it must not only be ethical, controllable, reproducible and cost effective *but* it must also adequately simulate the human pathologic process that is under investigation[17].
In vivo models can be divided in three main categories: genetically engineered, naturally occurring and experimentally (surgically, physically or chemically) induced.
On a macroscopic level it appears that the anatomical structure, maturation as well as the degenerative process in canine and human intervertebral discs have a lot in common[6]. This is also reflected in a recent study where the macroscopic scoring scheme according to Thompson for grading pathological changes in human intervertebral discs is validated for use in canines[18].

2. The canine intervertebral disc

2.1 Development of the intervertebral disc

The tissue of the intervertebral disc develops embryologically from both the mesenchyme and the notochord. During embryogenesis the notochord is surrounded by mesenchymal tissue. This tissue begins to segment early in gestation[10]. Because of the segmentation, regularly spaced condensations arise from which the future annulus fibrosus of the intervertebral disc will originate. The non-condensed areas will form the osseous vertebral bodies[10]. After segmentation, cells accumulate in the developing annuli fibrosi. These long, thin and biconvex cells become highly orientated and lay down several(15-38) distinct layers of fibrous tissue, mainly collagen type I[19]. The notochordal cells persist in the future nucleus pulposus, these cells expand and produce the fluid, gelatinous tissue inside the nucleus. Notochordal cells can be found as tightly packed clusters in which the cells have small, densely packed nuclei and large cytoplasmic vacuoles.
In dogs, two principal types of breeds can be distinguished, the chondrodystrophic and the non-chondrodystrophic breeds[3, 19]. The chondrodystrophic breeds are characterized by their abnormal chondrocyte function, this results in the failure of normal enchondral ossification, leading to so called disproportionate dwarfism. Examples of these breeds are the beagle and the dachshund. One principal difference in their development is that in chondrodystrophic breeds, as in humans, the notochordal cells are lost from the nucleus pulposus at a young age, whereas in non-chondrodystrophic breeds the notochordal cells are lost eventually, but at a much older age[15].

2.2 Anatomy

The canine spinal column consists out of 26 separate intervertebral discs. They form a flexible, though durable linkage between two consecutive vertebral bodies. The intervertebral discs are widest in the cervical and lumbar regions, and more narrow in the thoracic spine[20, 21]. The only joint in the spinal column that does not hold an interposing disc is the atlanto-axial joint. Dorsally and ventrally each disk is bound by, and in some places continues with, the dorsal and ventral longitudinal ligaments. Each disk consists out of three distinct anatomical regions; a central gelatinous area - the nucleus pulposus, a surrounding fibrous envelope - the annulus fibrosus and a hyaline cartilaginous end-plate on the cranial and caudal side[3].

2.3 Nucleus pulposus

The canine nucleus pulposus is the central gelatinous structure within each individual intervertebral disc. It is held in place by the annulus fibrosus which surrounds the nucleus along the entire length of the spinal column. The cranial and caudal borders are composed out of the hyaline cartilage end-plates[3]. The nucleus pulposus is made out of notochordal cells, chondrocyte like cells and extra-cellular matrix. The extra-cellular matrix is mainly made out of collagen (20% total dry-weight) and proteoglycan (50% total dry-weight). Note that the percentages given are those found in young individuals and that those ratios will be significantly different in older individuals [19].
The collagen found in a canine nucleus pulposus is mainly collagen type II, this is found in several load-bearing cartilaginous structures throughout the body (e.g. articular cartilage). It differs from other types of collagen by its hydroxylysine amino acid content, which on average is nine times higher in type II collagen when compared to other types of collagen. Hydroxylysine amino acid in type II collagen interacts readily with carbohydrate molecules in glycosaminoglycans, binding the

collagen content of the nucleus pulposus to the glycosaminoglycan side chains of the proteoglycan monomers[3].

A proteoglycan monomer is structurally formed by one protein backbone to which multiple polysaccharide subunits are covalently attached. The polysaccharide subunits, also called glycosaminoglycans (GAG) are long chains of monosaccharide subunits, mainly chondroitin-6-sulfate, keratan-sulfate and hyaluronic acid. The sulphated monosaccharide subunits, GAG's holds a strong negative charge which causes them to reject each other and stand out perpendicularly from the protein backbone creating the "bottle-brush phenomenon"[3]. The negative charge also results in a high osmotic gradient inside the nucleus attracting water which makes up 80% to 88% of the total content of the nucleus early on in life[3].

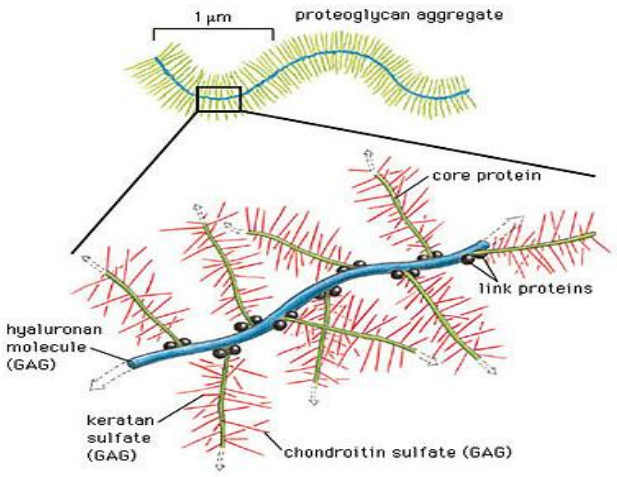

fig. 1 The structure of a proteoglycan-aggregate.
Source: http://www.rejuvenal.info/Terminology/proteoglycan-glucosaminoglycan-cartidea.html

So together the collagen substrate binding the highly hydrophilic proteoglycan molecules results in a high water content/pressure inside the nucleus pulposus giving it an excellent ability to resist deforming loads.

The cellular components of the nucleus continually shift during a dog's lifetime, in young dogs, the predominant cell type is the notochordal cell. Its concentration declines during a dog's lifetime whilst it is being replaced by fibrocytes during maturation[10].

Although the cell concentration inside the nucleus is very low, they play an important role in maintenance of the extracellular matrix composition by producing proteoglycans and collagens as well as proteases and their inhibitors[1, 10].

2.4 Annulus fibrosus

The outer surface of each individual nucleus pulposus is bounded by a though fibrous tissue called the annulus fibrosus. Its most important function is to bind two adjacent vertebrae tightly together. It is almost entire made out of fibrous tissue (extracellular matrix), collagen makes out approximately 70% of its total dry weight. It is mainly collagen type I that is found in the annulus fibrosus, making it exceptionally capable in resisting tensile loading.
The collagen bundles are produced by cells (fibrocytes) that are located between the individual fiber bundles. On microscopic view, the fibers are situated around the central nucleus pulposus in individual fibro-cartilage lamellae. The lamellae are laid down in different angles to one another and no evidence of any inter-lamellar connection is found up to date[3]. This is an important factor for it makes it possible for the individual lamellar layers to glide over each other during biomechanical loading forces[3].

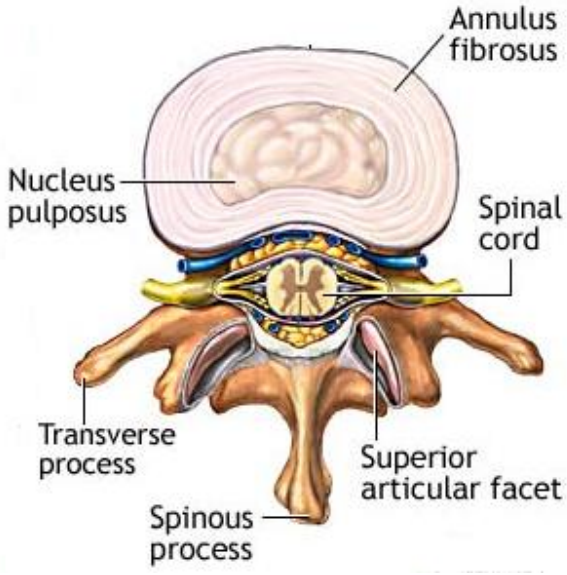

fig. 2 Intervertebral disc overview.
Source: www.nytimes.com/imagepages/2007/08/01/health/adam/19469Intervertebraldisk.html

2.5 Hyaline cartilage end-plates

As outlined above, the cranial and caudal borders of each individual intervertebral disc is bounded by the hyaline cartilage end-plates. The hyaline cartilage is approximately 1-2 mm. thick and tapers inward towards the central area opposite to where the nucleus pulposus is in closest contact with it. Fibers originating in both the annulus fibrosus and the nucleus pulposus intermingle with the collagen fibers of the end-plate, forming a stable connection between the three structures.
The cartilaginous end-plates make up the major nutritious pathway for the large a-vascular nucleus pulposus. It seems that only the thin central area is permeable for dissolvable nutrients by diffusion[3].

3. Matrix metalloproteinases and Glycosaminoglycans

3.1 Matrix metalloproteinases

Matrix metalloproteinase (MMP), also called matrixin, is an enzyme structure which is readily identified in vertebrate as well as in non-vertebrate animals. It is a proteinase, with the capability to degrade components of the extracellular matrix (ECM) as its main function[5]. The orderly breakdown of ECM is essential in many biological processes (e.g. embryogenesis, morphogenesis, reproduction and normal tissue remodeling and turnover) whereas disruption of the timely breakdown of ECM may result in pathological conditions.

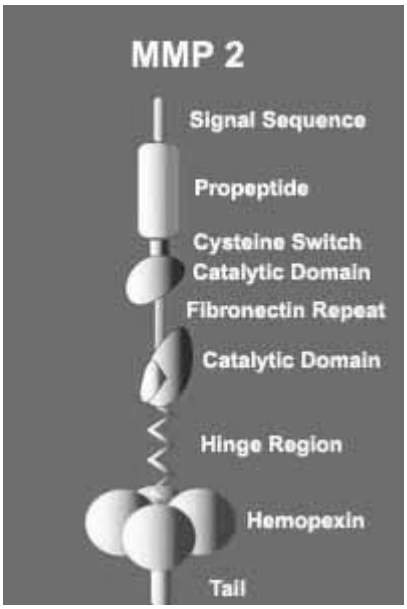

fig. 3 Matrix metalloproteinase 2
source: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/cell-signaling-enzymes/matrix-metalloproteinases.html

In our current understanding, the MMP-family comprise out of at least 24 members which can be found in vertebrate animals[22]. Members of the MMP-family have some common characteristics; they are secreted in an inactive form and need to be activated by proteinases or chemical agents. There are also many similarities in their amino acid sequence. They all contain a highly conserved sequence motif –PRCGXPD- which causes the MMP to remain in its zymogen form (proMMP). They share a zinc-binding site in their catalytic domain. They can be inhibited by specific tissue inhibitors of metalloproteinases or TIMPs and they all degrade a specific component of the ECM[22].
The MMP domain structure is generally composed out of three distinct regions (figure 3), a N-terminal pro-domain; a catalytic; and a hemopexin domain at the carboxy-terminal[23].
Because of the vital role MMPs play in physiological as well as pathological processes, their activation has to be regulated in a meticulous way. This activity is regulated in at least four different levels:
1) The transcription of genes coding for MMP is inducible by a wide range of bioactive agents including growth factors, cytokines and hormones.
2) Transcription can be down-regulated by other biological agents such as transforming growth factor β, steroids and retinoic acids.
3) Because MMPs are secreted as latent pro-enzymes (pro-MMP) they need to be activated. In vitro, secreted MMPs are activated by proteinases as well as non-proteolytic agents such as reactive oxygen and denaturants.
4) The activity of MMPs can be inhibited by specific TIMPs, of which four (TIMP 1 - 4) are identified until now[24].
Disruption of the regulatory balance can result in pathophysiological processes such as arthritis, tumor growth and metastasis which leads to suggest that therapeutic inhibition of MMPs could be a promising approach for treatment of some diseases [25].
In an immunohistochemical study of matrix metalloproteinase activity in human intervertebral discs, with different stages of IVDD, Roberts *et al.* demonstrated the occurrence of several MMPs(-1, -2, -3, -7, -8, -9, -12) in human intervertebral tissue. The frequency of staining for MMPs was positively correlated with the degeneration stage of the sample [26]. In intervertebral disc disease, degeneration is associated with the loss of ECM components, especially collagen type II and proteoglycans. The role of MMP-2 in intervertebral disc degeneration is of particular interest since several authors have reported that the amount of MMP-2 protein produced by nucleus pulposus cells increases with the degree of intervertebral disc degeneration both in *in vitro* and in *in vivo* studies.[27, 28] Kozaci *et al.*

also found that the pro-MMP-2 concentration levels were negatively correlated with the collagen levels in nucleus pulposus tissue[27].
MMP-2 differs from other members of the MMP family in its activation pathway. Unlike most of the members of the MMP family, proMMP-2 production and secretion are less responsive to the local cellular cytokine environment[29]. Furthermore, while most of the MMPs are activated by tissue or plasma proteinases outside the cell, the activation of pro-MMP-2 is thought to take place primarily on the cell surface[5]. *In vivo*, MMP-2 is selectively inhibited by its tissue inhibitor (TIMP-2), this proteinase inhibitor also binds to pro-MMP-2 forming a pro-MMP-2-TIMP-2 complex. This complex has the ability to bind and inhibit other enzymes of the MMP family, such as MMP-1, MMP-8 and MMP-9. This illustrates the complex involvement of this enzyme in the degeneration pathway of intervertebral disc[27].

3.2 Glycosaminoglycans

In order to resist compressive loads arising from body weight and/or muscle tension, the nucleus pulposus acts as a hydraulic tensioned cushion. The high water content inside the nucleus is achieved by a high osmotic environment leading to attraction and retention of water molecules. This high osmotic gradient is in part composed by the high anionic glycosaminoglycan(GAG) content[1]. The predominant GAGs inside the intervertebral nucleus pulposus are chondroitin-6-sulfate and keratan sulfate[11]. The GAGs are covalently linked to a core protein to form the principle proteoglycan monomers of the extracellular matrix. The main proteoglycans in the mammalian intervertebral disc are similar to the large aggrecan molecule found in articular cartilage. However some structural differences exists; as 80% of the proteoglycans in cartilage tissue can form aggregates with hyaluronic acid, only about 30% of the proteoglycans in nucleus pulposus samples exhibit this property[30].
Chondoitin-6-sulfate consists of alternating disaccharide amino acid subunits; 1-4 *N*-acetylgalactosamine and 1-3 glucuronic acid, and is sulfated at C-6 of the *N*-acetylgalactosamine. It has a molecular weight (MW) of $2x10^4$. Each disaccharide unit carries a double negative charge [22].
Keratin sulfate consists of repeating disaccharide units of *N*-acetylglucosamine and galactose. Two types of keratan sulphate have been recognized based on their linkage site with the core protein. Both can be found in mammalian nucleus pulposus[31]. It has a low MW of $5x10^3$ to $20x10^3$ and holds only a single negative charge[3].
In an amino-terminal to carboxy-terminal orientation the core protein can be divided into several structural domains (figure 4). Binding of chondroitin-6-sulphate mainly occurs at the distal SC-1 and SC-2 regions while keratan sulphate is usually concentrated about the proximal end of the protein backbone[32]. *In vivo*, the globular G1 domain of the proteoglycan monomer will bind with hyaluronic acid, a process known as aggregation. By this aggregation large molecules are produced that are capable of stabilizing the extracellular matrix.

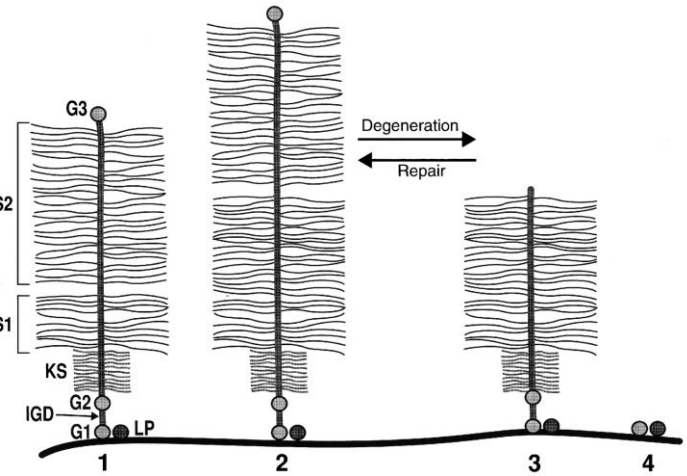

fig. 4 The structure of aggrecan, G1 domain is responsible for interaction with hyaluronic acid.
Source: http://www.biochemsoctrans.org/bst/030/0869/bst0300869f01.htm?resolution=HIGH

Normal aging of the canine nucleus pulposus is associated with several changes in the quality *and* quantity of the proteoglycan content. There is a shift from chondroitin-6-sulphate as predominant GAG towards keratan sulphate. Remember, keratan sulphate holds only a single negative charge. The result of the relative increase in Keratan sulphate in the aggrecan monomer is a decrease in osmotic gradient inside the nucleus pulposus. The absolute concentration of proteoglycan also decreases with aging, from an immature level of 40% to only 33% by eight years of age[3].

4. Intervertebral disc degeneration

4.1 What is intervertebral disc degeneration?

A lot of research has been done on the subject of intervertebral disc disease. The subject has been analyzed from different points of view by researchers from many different fields. This is of course a good thing for the general increase of knowledge in this subject, but this has made it difficult to obtain a proper, justifiable working definition for intervertebral disc degeneration. However, Adams and Rougly recently reviewed and reinterpreted existing literature and proposed the following definition for IVDD:
'The process of disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerate disc is one with structural failure combined with accelerated or advanced signs of aging. Early degenerative changes should refer to accelerated age-related changes in a structurally intact disc. Degenerative disc disease should be applied to a degenerate disc that is also painful'[1].
This definition distinguishes healthy aging discs from those subjected to pathology. It also states that intervertebral disc degeneration alone *can* be a natural manifestation of aging (tissue remodeling) and not a disease on itself.

4.2 Changes in aging and degenerating discs

With aging several biochemical, histological, metabolic and functional changes take place inside the canine intervertebral disc. In general these changes are progressive and irreversible. The differences are due to changes in cellular biology and/or extracellular matrix composition inside the intervertebral disc.
Changes in extracellular matrix (ECM) composition comprise in part of fragmentation of the proteoglycan content. The size of the aggrecan monomer and the amount of proteoglycans that form the aggrecan monomer declines with time. There is also a shift in quality of the proteoglycan content inside the nucleus pulposus. In Greyhounds for instance, by 5 years of age, the predominant glycosaminoglycan molecule is keratan sulphate instead of chondroitin-6-sulphate[3]. As a result of these combined changes the osmotic pressure gradient decreases, resulting in a declining water content/pressure inside the nucleus pulposus. At the same time there is a corresponding increase in collagen content. With a shift from predominantly collagen type II to collagen type I inside the nucleus pulposus. As the discs mature there is an increase in non-enzymatic glycation. This leads to an increase in cross-linkage between components of the extracellular matrix, mainly collagen, which inhibits matrix turnover and repair, making it more susceptible to mechanical failure[3, 11].
Cellular changes include signs of degeneration characterized by pyknotic and disintegrated nuclei. As the notochordal cells begin to degenerate, the absolute number of cells inside a canine nucleus pulposus seem to increase during aging and degeneration. Whether the notochordal cells develop into chondrocyte like cells or are replaced by cellular migration is unknown[3, 10].
Differences exist in ECM composition and cellular biology between dogs of non-chondrodystrophic and chondrodystrophic breeds. While the concentration of collagen keeps rising in the latter throughout its life(25% collagen at the age of 11 moths), the collagen concentration inside the NP of non-chondrodystrophic breeds remains less than 5% for most of the dogs life, only to rise at a very old age(>100moths)[9].
The proteoglycan composition of the chondrodystrophic breeds is of lower quality at birth and continues to decrease faster throughout life than in its counterpart.

As stated before, on a cellular level, the difference between the two are the loss of notochordal cells at an early age in chondrodystrophic breeds a situation which is also the case in humans, whereas the non-chondrodystrophic dogs retain their notochordal cells throughout much of their adult life[9, 15].
Hansen tried to describe the two types of degeneration histologically by stating that in the non-chondrodystrophic breeds the degenerative changes resemble fibrous metaplasia. Whereas in the chondrodystrophic breeds chondroid metaplasia is the result of degenerative changes[9]. In the non-chondrodystrophic breeds degeneration is mostly confined to a single disc along the spinal column. However in the chondrodystrophic breeds degenerative changes can be seen simultaneously along the entire length of the vertebral column. In addition, the degenerative changes start at a much younger age in the latter[9].
The above mentioned differences lead to an incidence of clinical signs of disk disease that is far from uniform. The dachshund for instance is predisposed for the disease, accounting for 45% to 70 of all canine cases, other breeds that are in higher risk of showing signs of IVDD are mostly chondrodystrophic, and predisposed to developing a Hansen type I intervertebral disc protrusion at some stage during their lifetime[9].

5. Aim and Hypothesis

5.1 *Aim*

The aim of this project was to investigate how the MMP-2 activity and the glycosaminoglycan content in canine intervertebral discs relate to the different degeneration grades described by Thompson et al.

5.2 Hypotesis

The hypothesis of this study was that the glycosaminoglycan content in canine intervertebral discs would decrease with increasing Thompson grades of disc degeneration. It was also hypothesized that the degree of MMP-2 activity would increase with increasing Thompson grades of disc degeneration up to grade IV and then decrease again in Thompson grade V discs[33-35].

6. Materials and methods

6.1 Sample acquisition

The samples used in this research project were obtained from 13 randomly selected dogs. The donor animals ranged in age from 16 to 120 months (mean 48,6). They were composed out of several chondrodystrophic and nonchondrodystrophic breeds. The dogs had different backgrounds and origins (table 1); 5 dogs were used for educational purposes; 2 dogs were used for research purposes and the remaining 6 dogs had been used for hunting purposes.
As signs of IVDD are most likely to be encountered in the intervertebral discs between the eleventh thoracic(T11) and first sacral(S1) vertebrae, these are the spinal segments that were used for this project. None of the dogs showed clinical signs of IVDD (back pain, incontinence, pelvic limb lameness and weakness), and were all euthanized due to other reasons than spine related problems.

Dog	Breed	Age(months)	Weight(kg)	Sex	Purpose	Achondroplastic
1	Kerry Beagle	36	29	♂	Hunting	0
2	Kerry Beagle	36	29	♂	Hunting	0
3	Beagle	96	31	♂	Hunting	1
4	Beagle	117	12,1	♀	Education	1
5	Beagle	120	9,5	♀	Education	1
6	Foxhound	84	39	♀	Hunting	0
7	Foxhound	120	44	♂	Hunting	0
8	Foxhound	108	37	♀	Hunting	0
9	Mongrel	17	22,2	♀	Research	0
10	Mongrel	16	20	♀	Research	0
11	Mongrel	28	11,7	♀	Education	0
12	Beagle	25	11	♀	Education	1
13	Beagle	25	11,2	♀	Education	1

Table 1. Donor animals signalements and origins.

The spinal segments were dissected at the department of pathology at the Faculty of Veterinary Medicine, Utrecht University. Immediately after dissection they were wrapped in moist towels and placed in plastic bags to prevent dehydration. The sample material was processed in several ways to be studied as part of the Phd project performed by DVM N. Bergknut.
For the first part of the project T2-weighted sagittal MR images were performed using a 0.2 Tessla MRI. The specimens were then cut down the midline by the use of a belt saw and macroscopic photographs were taken of the mid sagittal surface of each intervertebral segment. The acquired data was evaluated and compared in an effort to validate a macroscopic scoring and to correlate that with the results of the low field magnetic resonance imaging.
One half of each intervertebral segment was fixated in 4% neutral buffered formaline, decalcified in EDTA and used for histopathological examination and grading.
The remaining half of the transected spinal segments was used for biochemical analysis as part of this project. The nucleus pulposus of all the individual intervertebral discs were removed and immediately snap frozen in liquid nitrogen. They were stored at minus 80° Celsius[18].

6.2 Nucleus processing

The nuclei were allowed to thaw before they were divided into at least two parts, depending on the quantity of tissue that could be retrieved. One part was used for the quantification of glycosaminoglycans, the other to assess the MMP-2 activity inside the intervertebral nucleus pulposus. During the separation, parts of the annulus fibrosus still attached to the nucleus pulposus were carefully separated since the focus of this project lies purely on changes of GAG concentration and MMP-2 activity inside the nucleus pulposus.

6.3 Glycosaminoglycan assay:

For the quantification of the sulphated glycosaminoglycan content in the tissue samples, the Farndale (Dimethylmethylene Blue) assay was used. The assay is based on spectrometric detection of a metachromatic change of the solution of 1.9-dimethylmethylene blue, in other words it is bases on the property of the 1.9-dimethylmethylene blue sample solution to appear as different colors depending upon the wavelength of light under which it is viewed at[36].
First the tissue samples were placed in 1,5 ml eppendorf cups and the weight of an the empty cup was subtracted from the combined weight of the cup plus sample, in this way the precise weight of the sample was acquired.
Protein digestion of the samples was performed overnight by 56° Celsius in a Proteinase K digestion buffer. The reaction buffer contained: 50mM TRIS (pH 7.6) dissolved in 100ml. Millique, 1mM EDTA, 1mM iodoacetamide. Before the digestion buffer was used, 10µg/ml Pepstatin A and 1mg/ml Proteinase K was added. After incubation overnight at 56°C the samples were heated to 100°C for 10 minutes to inactivate the proteinases K.
After inactivation a series of 6 dilutions, ranging from 1:500 to 1:2000, were prepared from each sample by diluting it in PBS/EDTA. The composition of the PBS/EDTA (pH 6.5) was; 0,1 M Na_2HPO_4 and 0,01 M EDTA. Then 100µl of each dilution was pipetted into a 96 well, flat bottom, microtitre plate(microplate, 96w, PS, flat, 656191, Greiner bio-one, Frickenhausen, Germany).
The results obtained after spectrofotometric analysis of our samples were compared to the spectrofotometric results of a standard line composed out of standard dilutions of Chondroitin Sulphate C(CSC) (shark cartilage sodium salt, C-4384, SIGMA-ALDRICH, St. Louis, MO, USA).
The Dimethylmethylene Blue solution was prepared by dissolving 2.37g. NaCl and 3.04 g Glycine in 1L of Millique, to this 16 mg of Dimethylmethylene Blue (03610, Polysciences, Warrington, PA, USA) in 5ml. ethanol was added.
Just before the microtitre plates were analyzed spectrofotometricly, 200µl of filtered DMB solution was added to each well (standard and sample dilutions). The plate was read using light with the following wavelengths: 530nm. and 590nm.

6.4 MMP-2 activity by gelatin zymography

For the detection of MMP-2 activity a substrate zymography was performed. The identification of MMP-2 in this technique is based on the molecular weight of this enzyme. Quantification of the activity of MMPs is assessed by comparing the amount of degradation of the preferred substrate by the enzyme (MMP-2)[37]. Because MMP-2 readily degrades gelatin (gelatinase), a gelatin zymography was used to detect MMP-2 activity.
Because the MMP-2 activity in tissue samples taken from different intervertebral discs was going to be compared, it was necessary to first perform a protein assay of our tissue samples. By assessing the protein content, it was possible to load the exact same amount of protein into each slot of the gels that were going to be used for the quantification of the MMP-2 activity.

Protein assay:
For the quantification of the protein content of each tissue sample, the DC protein assay from Bio-Rad laboratories is used. This assay is a colorimetric assay for protein concentration following detergent solubilization. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are two steps in this protocol which lead to color development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. As with any colorimetric assay, the color formation of the different protein contents will be quantified colorimetrically and compared to a standard Bovine serum albumin solution.
The tissue samples were prepared by dissolving them in a lysis buffer (250 µl) containing 0,1 M TRIS, 0,1% Triton X-100 in Millique (MQ). The samples were gently mixed for 18 hours at 4°C. After overnight protein extraction, the samples were centrifuged at 1400 rpm for 10 minutes. The maximum amount of the supernatant was extracted for further analysis.
To measure all the samples 3,5 ml of working reagent was prepared by adding 80µl of reagent S to 3,5 ml of reagent A, now called reagent A*.
Bovine serum albumin (A-9647, SIGMA-ALDRICH, St. Louis, MO, USA) was used to create a standard protein concentration line. 5 Dilutions were prepared containing from 0.0 mg/ml to 1,5 mg/ml in PBS. After this, 5 µl of the standard and all of the samples were pipette into a clean and dry 96 well microtiter plate. After all the samples and standards were loaded, 25µl of reagent A* was added to each well. Prior to colorimetric analysis, 200 µl of reagent B was pipetted to each well. Although the plate reader had a mixing function, care was taken that all the components were adequately mixed by pipetting the solutions back and forth. Before the plate was entered in the microtiter plate-reader, any existing bubbles were popped by the use of clean pipette tips.
Absorbance was read in the microplate reader (DTX 880 multimode detector, Beckman-coulter INC., Fullerton, USA) with the wavelength set to 650 nm.

MMP-2 zymogram:
Since MMP-2 activity was compared between different samples, the exact same amount of protein(µg) had to be loaded into each slot of the gelatin gels. Maximum loading volume of the individual slots is 15µl this corresponded to 10µg of protein in each well. Each sample was dissolved in 10µl sample buffer, containing 240mM Tris-HCl, 8% SDS, 40% glycerol and 0,02% Broomphenol Blue.
Appropriate volumes (15µl) of each individual sample solution were loaded on the gel(345-0081, Bio-Rad, Hercules, CA, USA) and the proteins of the nucleus pulposus extract were separated during 75 minutes at 150 volts inside a criterion cell(165-6001, Bio-Rad, Hercules, CA, USA). During electrophoresis, the SDS causes the MMP-2 enzymes to denaturate and become inactive[37].

After electrophoresis the gels were washed twice for 15 minutes in a wash-buffer containing 2,5% Triton X-100 in MQ. The Triton X-100 is exchanged with SDS, causing a partial renaturation and recovery of the MMP-2 activity[37].
The gels were incubated for 21 hours in a incubation-buffer(200ml/gel) containing 50 mM Tris, 10mM $CaCl_2$, 0,05% Brij (35%), pH 7,4 in MQ at 37°C. After incubation the gels were stained with Coomassie brilliant blue (25% CH_3OH, 15% CH_3COOH, 0,1% Coomassie Brilliant Blue) for 1 hour. After the gels were destained using MQ, they were analysed densitometric with the use of the Molecular Imager Gel Doc XR (Bio-Rad, Hercules, CA, USA) and Quantity One software(Bio-Rad).

6.5 Data analysis
6.5.1 Glycosaminoglycan-assay

The concentration of sulphated glycosaminoglycans in our tissue samples is calculated by using the curve obtained from the standard (CSC) dilutions as a reference. The values for the standard curve were corrected for the blank results using the following calculation: y=([C]530nm/[C]590nm)-([C]530nm$_{blank}$/[C]590nm$_{blank}$).
By using this standard curve as a reference the concentration of each of the sample dilutions is obtained as µg/ml. These values were then multiplied by their dilution factor and total volume of the starting sample (300µl) by doing this the total amount of sulphated glycosaminoglycans per sample is obtained.
The last step in the calculation is to divide the amount of sulphated glycosaminoglycans by the original weight of the sample that was used in order to produce the concentration of sulphated glycosaminoglycans(µg) per mg of sampled tissue.

6.5.2 MMP-2 zymogram

Protein assay:
Calculations were made by comparing the absorbance of the samples of interest with the absorbance that was found for the Bovine serum albumin standard line, for the absorbance decreased linearly with an increase in protein concentration. In excel the absorbance data from our standard solutions of Bovine Serum Albumin was converted into a chart with absorbance set on the y-axis versus protein concentration set on the x-axis. The equation that predicts the slope of the trend line was used to calculate the protein concentration in the samples. By substituting y with the absorbance value from each individual sample, the x-value ([protein], µg/µl) was calculated.

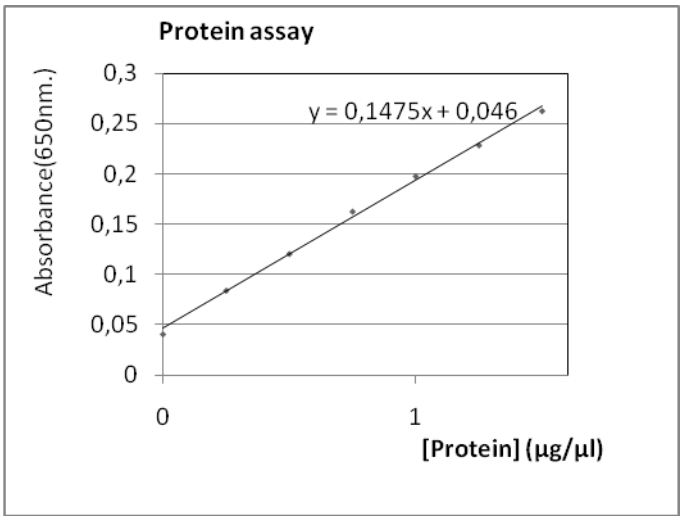

Fig. 5 Standard line protein content and equation.

Zymogram.

In the chemidoc module, high resolution pictures were taken of the gels in which the activity of the MMP-2 enzyme is shown as a clear band against a darker background. By using the Quantity One software, a feature called 'volume contour tool' made it possible to pinpoint a certain pixel on the gel after which the software includes all the pixels with a lower density in the vicinity of this point and joins them together. The software calculates the density inside the selected region, the density per mm^2 and the density of the selected region after the background density is subtracted. By selecting a large square on each single gel, the background density can be corrected so different MMP-2 density/activity within and between gels can be compared.

The following figures will be compared:

Relative MMP-2 activity = (mean density$^{\text{inside selection}}$ - mean density$^{\text{selected square}}$)/surface$^{\text{selection}}$

The higher the activity of the MMP-2 enzymes, the lower the density of the bands will be as more of the gelatin substrate has been degraded. It is also possible to give an indication of the MMP-2 concentration as the higher its content, the larger the individual bands will show up in the gelatin substrate. The higher amount of enzyme is shown as a larger area where the gel is degraded and subsequently destained.

While all samples were tested, only the samples that showed signs of degradation of the substrate on macroscopic view were analysed by the Quantity One software(table 2.)

	N	Minimum	Maximum	Mean	Std. Deviation
Activity	67	-17,318452	323,362903	106,21462194	72,372943237
No Activity	56	0	0	0	0

Table 2. Number of samples with/without macroscopic visible activity

7. Results

7.1 Distribution of the Thompson Score

The Intervertebral discs from which our samples were originating, were graded by four individual observers. Pictures taken from the discs were presented blind and in duplicate to investigate the intra-observer agreement as well as the inter-observer agreement. For the observer reliability, Cohens weighted Kappa analysis was performed which indicated a very high repeatability of the system.

Grade	Nucleus	Annulus	End-Plate	Vertebral body
I	Bulging gel	Discrete fibrous lamellas	Hyaline, uniformly thick	Margins rounded
II	White fibrous tissue peripherally	Mucinous material between lamellas	Thickness irregular	Margins pointed
III	Consolidated fibrous tissue	Extensive mucinous infiltration; loss of annular-nuclear demarcation	Focal defects in cartilage	Early chondrophytes or osteophytes at margins
IV	Horizontal(vertical) clefts parallel to end-plate	Focal disruptions	Fibrocartilage extending from subchondral bone; irregularity and focal sclerosis in subchondral bone	Osteophytes less than 2 mm.
V	Clefts extend through nucleus and annulus		Diffuse sclerosis	Osteophytes greater than 2 mm.

Table 3. The description of morphological grade according to Thompson

Thompson score		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1	42	34,1	34,1	34,1
	2	48	39,0	39,0	73,2
	3	23	18,7	18,7	91,9
	4	10	8,1	8,1	100,0
	Total	123	100,0	100,0	

Table 4. Distribution of Thompson scores across all samples**.**

Table 4, shows the distribution of Thompson grade in the collection of samples.
The aim of the study was to investigate *if* the sulphated glycosaminoglycan concentration significantly decreased and *if* the MMP-2 activity significantly increased with increasing Thompson grades.
In all 123 samples, only one sample was given a Thompson grade V. For statistical purposes this sample was added to the group of Thompson grade IV.

7.2 Statistical analysis.

Results obtained during this research project will be analyzed statistically with the use of SPSS 15.0 software for windows. Descriptive statistics will be used in an attempt to asses if there are any correlations between GAG content, MMP-2 activity and Thompson score of the individual canine discs. For analysis the Kruskal-Wallis one-way analysis of variance(ANOVA) will be used, comparing equality of medians between the different groups(Thompson grades).

7.3 Sulphated glycosaminoglycan concentration.

GAG concentration in relation to Thompson scores (Figure 6). The mean of the GAG concentration decreases for every one-step increase in Thompson score. When viewing the data, two main groups can be composed based on the GAG concentrations. The first group (A) with a high GAG concentration is composed out of all the samples which were designated to having a Thompson grade of either I or II. Whilst the other group(B) is composed out of all the samples which were given a Thompson grade of III, IV(+V). Differences in GAG concentration between separate morphological degeneration grades were analyzed by Kruskal–Wallis non-parametric one-way analysis of variance (ANOVA), significance output was Bonferroni corrected. When comparing the GAG concentration between the separate degeneration grades, the GAG concentration did not significantly differ within the A group (Th.1-Th.2, p = 1,000), nor did they significantly differ within the B group (Th.3-Th.4, p = 1,000). When comparing the GAG concentration between the two groups, a significant difference was found between grade I and grade III (p = 0,00) and between grade II and grade III (p = 0,01).(Table 5.)

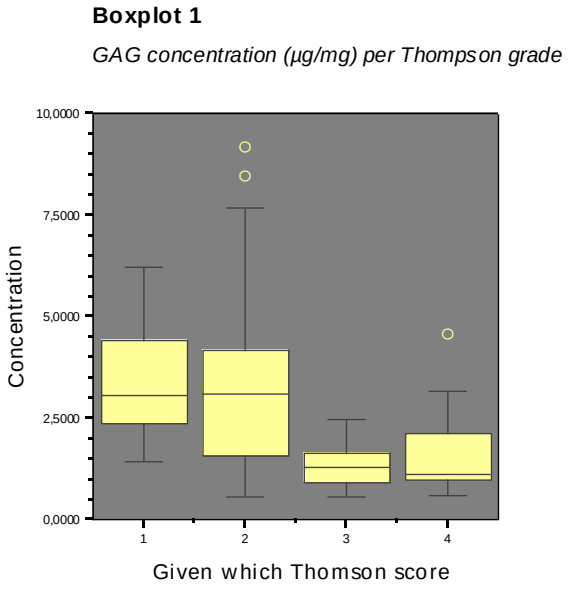

fig. 6 GAG concentration per Thompson score.

Thompson Score(I)	Thompson score(J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
		Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound
1	2	,2612790	,4334806	1,000	-,901951	1,424509
	3	2,3003798(*)	,5310328	,000	,875372	3,725388
	4	1,9259315	,7189197	,051	-,003264	3,855127
2	1	-,2612790	,4334806	1,000	-1,424509	,901951
	3	2,0391009(*)	,5169301	,001	,651937	3,426265
	4	1,6646526	,7085665	,123	-,236760	3,566066
3	1	-2,3003798(*)	,5310328	,000	-3,725388	-,875372
	2	-2,0391009(*)	,5169301	,001	-3,426265	-,651937
	4	-,3744483	,7721119	1,000	-2,446383	1,697487
4	1	-1,9259315	,7189197	,051	-3,855127	,003264
	2	-1,6646526	,7085665	,123	-3,566066	,236760
	3	,3744483	,7721119	1,000	-1,697487	2,446383

* The mean difference is significant at the .05 level.

Table 5. Multiple comparisons (ANOVA) of absolute GAG concentration per Thompson score.

7.4 MMP-2 Activity

Figure 7 shows a box plot in which the relative MMP-2 activity of the samples is plotted against the various Thompson grades. It shows that the mean relative MMP-2 activity of all samples increases with increasing Thompson scores. This is true for samples with a Thompson score of I, II and III. Results showed that for samples with a Thompson score higher than III, the overall MMP-2 activity declines. As with the GAG concentration the differences in MMP-2 activity between separate morphological degeneration grades were analyzed by Kruskal–Wallis non-parametric oneway analysis of variance (ANOVA), significance output was Bonferroni corrected.

Table 6 shows the difference in mean relative MMP-2 activity and *if* the difference is significant at the 0,05 level.

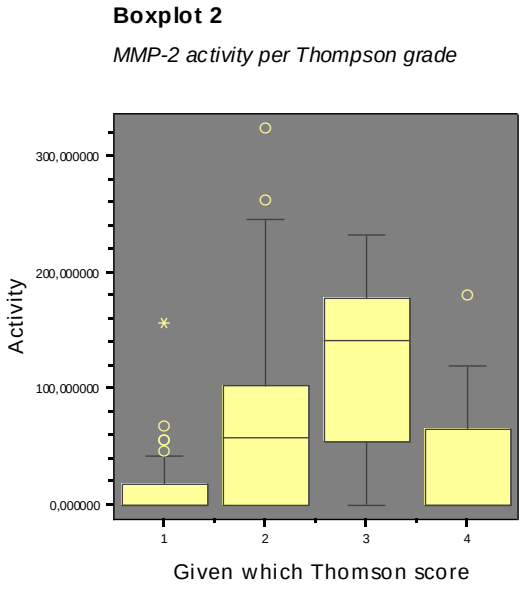

fig. 7 Relative MMP-2 activity per Thompson score.

Thomson score (I)	Thomson Score (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
		Lower Bound	Upper Bound	Lower Bound	Upper Bound	Lower Bound
1	2	-56,035 (*)	13,866	,001	-93,23817592	-18,83271705
	3	-103,752(*)	17,023	,000	-149,4255925	-58,07803840
	4	-27,105	23,091	1,000	-89,06016624	34,84968487
2	1	56,035(*)	13,866	,001	18,83271705	93,23817592
	3	-47,716(*)	16,642	,029	-92,36862958	-3,06410842
	4	28,930	22,812	1,000	-32,27553953	90,13595114
3	1	103,752(*)	17,023	,000	58,07803840	149,42559257
	2	47,716(*)	16,642	,029	3,06410842	92,36862958
	4	76,647(*)	24,858	,015	9,95179166	143,34135794
4	1	27,105	23,091	1,000	-34,84968487	89,06016624
	2	-28,930	22,812	1,000	-90,13595114	32,27553953
	3	-76,647(*)	24,858	,015	-143,3413579	-9,95179166

* The mean difference is significant at the .05 level.

Table 6. Multiple comparisons (ANOVA) of relative MMP-2 activity per Thompson score.

For instance, the amount of MMP-2 activity was significantly lower in samples graded Thompson grade I when compared to grade II or III (p = 0,001 and 0,000, respectively). No significant difference was found between samples graded Thompson grade I when compared to Thompson grade IV (p = 1,000).

Samples with a Thompson grade of II had a significant higher MMP-2 activity than samples with grade I (p = 0,001), a significant lower activity when compared to samples which were given a grade III on the Thompson scale(p = 0,029) but did not differ significantly from samples with grade IV.

Samples with grade III had a significant higher MMP-2 activity than samples with a Thompson grade of I, II and IV(p = 0,000; 0,029 and 0,015 respectively).

And the MMP-2 activity in samples that were given a Thompson grade IV did not differ significantly from samples that were given a grade of I or II, but were significantly lower than samples that were given a grade III on the Thompson scale(p = 0,015).

Justification of the statistical approach.

In our statistical analysis of the correlation between Thompson grades and GAG concentration or MMP-2 activity we did not take the age, weight or breed of the dogs into account. Although we know that intervertebral disc degeneration is a normal part of aging and that dogs of chondrodystrophic breeds show signs of disc degeneration at a younger age than their counterparts. The intention of this study was to find out if there was a correlation between Thompson grades and GAG concentration as well as the MMP activity in canine intervertebral discs.

8. Discussion

The purpose of this research project was to find out *if* and if so, *in what way* the glycosaminoglycan content and MMP-2 enzyme activity changes during successive stages of canine intervertebral disc degeneration. The gross morphologic grading scheme according to Thompson was used in the project as a 'gold standard' for different stages of disc degeneration. In a previous research project, the intervertebral discs, from which the samples in this study originated, were graded according to Thompson by several observers. The results of that study were tested for inter- and intraobserver reliability and the results showed that the Thompson scoring system could reliable be used in canine intervertebral discs[18].

In our results it is clearly visible that there is correlation between the first three stages of degeneration and increasing MMP-2 activity as well as decreasing GAG concentrations. A similar correlation for MMP-2 activity and degeneration was found in research that has been done in human tissue samples[35]. Although the shape of the activity curve is shifted to the right in humans.

Rutges found that in human intervertebral discs that the delay between time of death and time of tissue collection and snap freezing did not correlate with MMP-2 activity or concentration within a 24 h period[35]. All samples used in this study were harvested and frozen well within that time limit hence that time laps is not thought to have influenced the MMP-2 activity. However in the process of protein extraction and determining the protein content of the samples they went through three freeze-thaw cycles before they were used for the MMP-2 zymogram. These cycles could have decreased the protein stability resulting in an overall decrease in MMP-2 activity. But since all the samples received the same treatments it is not thought to have influenced the relationship between the Thompson grades. The mean amount of activity per sample is thus likely to be underestimated and this could account for the large number of samples that did not show any visible activity.

There were several outliers that could not be explained other than caused by some sort of laboratory error (e.g. pipetting error, interpretation error). For the GAG assay we found one sample (nr. 84) that was extremely high in GAG content (17,2 µg/mg), this is a difference of more than 5 times the standard deviation of the samples given a grade I on the Thompson scale.

For the GAG concentration a Farndale assay was used, which compares the sulphated glycosaminoglycan content to a previously known chondroitin sulphate standard. Because some of our samples had a very high GAG concentration, we had to dilute the samples so that the output from the spectrophotometer was within the range of the standard line. This meant that some of our samples had to be diluted up to 1:2000. These dilutions were created as serial dilutions, by making serial dilutions an increased risk for pipetting errors occurs.

Most of the technical challenges arose in the quantification of the MMP-2 enzyme activity. In order to compare activity between different samples every slot of the Criterion gel was loaded with the same amount of protein. This was however not possible for all samples since the protein concentration was found to be so low that even when the total slot volume (15µl) was filled with only the sample solution, a total protein concentration of 10µg could not be achieved. For these samples (n=7) we loaded the highest possible volume into each slot of the Criterion gel. For example if only 6µg of protein could be loaded out of the intended 10µg, we multiplied the measured MMP-2 activity by 10/6=1 ⅔. *This method was justified by proving that MMP-2 activity rises linearly with the amount of protein.*

Two of the total of five gels came out with unevenly distributed bands and lanes for the MMP-2 activity. The MMP-2 enzyme protein should present itself as clear bands at 68 kDa, but in these two gels, clear bands were formed in several places, with a lot of variance between different samples (figure 8). We can only speculate over possible causes for this inconsistency. It could have been an uneven contact of the Criterion Cell electrodes with the running buffer or a human error in setting up

the Criterion Cell properly. The samples that were in these gels were retested and the second time they came out with a normal appearance.
Another technical problem with the running phase of the criterion gel was the fact that after the staining procedure, some slots in the gel showed up as clear lanes instead of only clear bands at 68 kDa (figure 8). This is probably due to a part of the MMP-2 enzymes being active whilst being separated through the gel, degrading the gelatin gel on their way through the gel. This could be counteracted by cooling the running buffer during the electrophoresis and thus lowering the enzyme activity of the MMP-2 enzymes during electrophoresis.

Figure 8. Uneven distribution of MMP-2 activity after electrophoresis.

The largest discrepancy we came across was that some of the MMP-2 samples did not de-stain the gel, although they came from intervertebral discs with high Thompson grades. The analysis of the MMP-2 activity was done by dividing the local density of the clear bands by a large portion of the background of the entire gel.

Thompson Score	Visible	Not visible
I	14	28
II	30	18
III	19	4
IV+V	4	6

Table 7. sample distribution of samples that showed macroscopic MMP-2 activity

Some of the samples showed a very low activity which could not be quantified with the "Quantity one" software although faint bands were visible to the naked eye. So only the samples that showed obvious de-staining were used to measure the MMP-2 activity. This resulted in 67 of the total of 123 samples. The remaining samples were given an activity of 0 but were still included in the statistical analysis.
The samples that did not show any activity on the gel were all expected to have a low Thompson score however that was not the case (table 7). So for at least 28 samples with no visible MMP2 activity (grade II, III and IV) we would have expected to see at least some activity. Why there is no visible activity in these samples remains unclear. By comparison, a recent study done by J. Rutges, states that in humans the amount of active MMP-2 negatively correlated significantly with degeneration grade, although the activity levels seem to stabilize between grade IV to V[35]. In our canine samples we initially see the activity of the MMP-2 increases with an increase in Thompson grade, followed by a decrease in activity in samples coming from intervertebral discs given a grade IV on the Thompson scale. We should keep in mind that the results of MMP-2 activity in the samples with a grade IV on the Thompson scale came from a total of only 4 samples. One possible explanation for the low MMP-2 activity in canine intervertebral discs with a grade IV is that the substrate for MMP-2 degradation, which is components of the extracellular matrix, has *decreased* as a result of degeneration.
We do however realize that there are significant ultrastructural differences between chondrodystrophic and non-chondrodystrophic breeds. For instance, the proteoglycan concentration

in the chondrodystrophic intervertebral disc of all ages is below that reported for similarly aged, non-chondrodystrophic discs.[33, 34] This could mean that the GAG concentration could be lower in a chondrodystrophic breed than it is in a non-chondrodystrophic breed, even though they were given the same Thompson grade. No research has been done on the difference of MMP-2 activity chondrodystrophic and non-chondrodystrophic dogs, although discs from both types of dogs were included in this study, we could not evaluate this difference due to the fact that there were too many variable factors between the dogs (age, sex, different breeds, weights and origins) combined with the fact that the sample size was not large enough.
Although the gross morphological scoring scheme according to Thompson has been validated for canine intervertebral discs it should be recognized that there still are many differences between dogs and humans and that dogs might not be the best model for human IVDD research.
There are marked differences between chondrodystrophic and non-chondrodystrophic dogs which to some extent opens the possibility for two different canine models for human intervertebral disc degenerative research.

Some major differences are however noted between human and canine intervertebral discs and the pathogenesis of disc degeneration:

1. The human discs contain a limited amount of notochordal cells at birth. The number of notochordal cells starts to decrease soon after birth and this continues until the age of 4-10 years when no more notochordal cells can be detected in the human nucleus pulposus. All dogs can be divided into either chondrodystrophic breeds e.g. Beagle and Dachshund or into non-chondrodystrophic breeds. The chondrodystrophic dogs lose their notochordal cells shortly after birth, resembling the pattern in man. Non-chondrodystrophic breeds retain their higher number of notochordal cells until the age of 5-6 years[38]. These notochordal cells are believed to be important progenitor and/or organizer cells inside the nucleus pulposus, directly secreting extracellular matrix proteins. Because of the functionality of these cells, the relevance of research done on a non-chondrodystrophic breed model could be limited, depending on the question one is trying to answer.
2. In humans the weight of the upper body exerts an increased pressure on the lumbar spine the case in quadrupeds. Although in quadrupeds the weight of the upper body is somewhat replaced by muscle contraction and ligament tension that acts on the spine. The loading patterns of human and canine discs have in fact been shown to be quite similar [3, 8].
3. The size of the disc needs to be considered when animal models are used, since some aspects of disc biology (e.g. solute transport, relative loading) are size-dependant.
4. A final difference that should be taken into account is the fact that the experience of pain in animals is difficult to quantify, yet a great part of the therapeutic goal in humans is to reduce this symptom as the intervertebral disc degenerates[15].

It is obvious that when using an animal model to provide information about intervertebral disc degeneration similarities *and* differences have to be taken into account before conclusions about the gathered information can be drawn

So is it possible to use non-chondrodystrophic breeds as models for human intervertebral disc degeneration? We think it is, but one should be aware that there is not one model that can truly mimic human intervertebral disc degeneration. By using canine models, a compromise is created and the optimum model to be used depends on which questions one is trying to answer.

8. Conclusions

The aim of this project was to investigate if GAG concentration and MMP-2 activity correlated with the different stages of intervertebral disc degeneration in dogs. The macroscopic grading system according to Thompson for grading of IVDD was used as a gold standard.
It was found that GAG concentrations were generally decreased with increasing Thompson grades although the only significant difference was between discs graded Thompson grade I or II compared to discs given a grade III or IV. Looking at our data, we have got reason to believe that the GAG concentration remains high until a certain stage of degeneration is reached, after which it falls and remains at a lower concentration for the rest of the process.
For the MMP-2 activity there is a rise in activity for each step upwards on the Thompson scale, until a Thompson grade of III. The MMP-2 activity in samples originating from discs with a grade IV is significantly lower than those originating from discs with grade III.

9. Acknowledgements

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Thanks for that Niklas!

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