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The myth of fibroid degeneration in the canine intervertebral disc

- A histopathological comparison of intervertebral disc degeneration in chondrodystrophic and non-chondrodystrophic dogs

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The myth of fibroid degeneration in the canine intervertebral disc – A histopathological comparison of disc degeneration in chondrodystrophic and non-chondrodystrophic dogs

Myten om fibroid degeneration i hundens intervertebraldisker – En histopatologisk jämförelse av diskdegeneration mellan chondrodystrofa och icke chondrodystrofa hundar

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SUMMARY

In the 1950's, the veterinary pathologist, Hans-Jörgen Hansen noticed several dissimilarities between so called chondrodystrophic and non-chondrodystrophic dog breeds regarding intervertebral disc degeneration and related diseases. Chondrodystrophic breeds are characterized by disproportionally short extremities due to disturbances in the endochondral ossification whereas non-chondrodystrophic breeds have normal bodily constitutions. The two breed types often differ regarding age of onset of disease, spinal location and severity of clinical signs. He also noted different histopathological patterns during the course of degeneration, foremost in the centre of the intervertebral disc, the nucleus pulposus. Due to this, he named the degenerative processes *chondroid metamorphosis* and *fibroid metamorphosis* in chondrodystrophic and non-chondrodystrophic breeds respectively. In general, chondroid metamorphosis refers to the replacement of the original cell type (the notochordal cell) with chondrocyte-like cells, whereas the fibroid metamorphosis refers to replacement of these cells with fibrocyte-like cells. This distinction has since then been an accepted distinction between the two breed types. However, more recent studies indicate that the histopathological patterns are more similar in the two breed types than previously described, and that the chondroid metamorphosis might be the degenerative process taking place in both breed types.

In this study the histopathological appearances of 42 intervertebral discs from 16 chondrodystrophic and 17 non-chondrodystrophic dogs in early stage intervertebral disc degeneration were examined. After midsagittal transection of the spines, the intervertebral discs were graded macroscopically between one and three on a five-grade scale (the Thompson grading scheme). Subsequently, each intervertebral disc was individually fixated in formalin and decalcified in EDTA. After decalcification, the intervertebral discs were embedded in paraffin, sliced with a microtome and stained with Hematoxilin and Eosin and Alcian Blue/Picrosirius Red. The histological samples were thereafter graded according to a histological grading scheme (the Boos grading scheme) after which the histological scores were compared between chondrodystrophic and non-chondrodystrophic dogs.

The results showed that there was a significant difference in total histological scores between chondrodystrophic and non-chondrodystrophic dogs in the two lowest macroscopical grades of degeneration. Chondrodystrophic dogs had a higher mean histological scoring, i.e. more histopathological degenerative changes could be observed in the intervertebral discs of the chondrodystrophic than in the non-chondrodystrophic dogs in these macroscopical grades. The histological parameters contributing the most to this difference were "chondrocyte proliferation of nucleus pulposus" and "presence of notochordal cells in nucleus pulposus". Chondrodystrophic dogs had significantly higher scores than non-chondrodystrophic dogs in these parameters.

No fibrocyte-like cells were found in the nucleus pulposus of any of the two breed types. The only cells found were notochordal and chondrocyte-like cells. This leads to the conclusion that a *chondroid metamorphosis* is the degenerative process taking place in both breed types and that the theory of a *fibroid metamorphosis* in non-chondrodystrophic breeds is inaccurate.

SAMMANFATTNING

På 1950-talet uppmärksammade den veterinärmedicinske patologen, Hans-Jörgen Hansen, flera olikheter mellan så kallade chondrodystrofa och icke chondrodystrofa hundraser gällande intervertebral diskdegeneration och sjukdomar relaterade till detta. Chondrodystrofa raser karakteriseras av oproportionerligt korta extremiteter vilket är en följd av en störd endochondral ossifikation medan icke chondrodystrofa raser karakteriseras av normala kroppsproportioner. De två

rastyperna skiljer sig ofta åt gällande anslagsålder, spinal lokalisation samt allvarlighetsgrad av sjukdomarnas kliniska manifestationer. Han uppmärksammade även att de degenerativa histopatologiska förändringarna skiljde sig åt, framförallt i diskens centrala del, den så kallade nucleus pulposus. I detta hänseende kallade han de degenerativa förändringarna för *chondroid metamorfos* hos de chondrodystrofa raserna och för *fibroid metamorfos* hos de icke chondrodystrofa raserna. Generellt menade han att chondroid metamorfos innebär att den, för nucleus pulposus, ursprungliga celltypen (notokordcellen) ersätts av kondrocytliknande celler medan den vid fibroid metamorfos ersätts av fibrocytliknande celler, vilket sedan dess har varit en vedertagen distinktion mellan de två rastyperna. Senare studier har dock indikerat att de histopatologiska förändringarna är mer snarlika mellan de två rastyperna än vad som tidigare beskrivits, och att den så kallade chondroida metamorfosen är den degenerativa process som äger rum oavsett rastyp.

I denna studie utvärderades de histopatologiska förändringarna i 42 intervertebraldisker från 16 chondrodystrofa och 17 icke chondrodystrofa hundar i tidiga stadier av degeneration. Ryggraderna sågades mittsagittalt varefter intervertebraldiskerna graderades makroskopiskt mellan ett och tre på en fem-gradig skala (Thompson). Därefter fixerades varje disk i formalin och dekalcifierades i EDTA. Efter dekalcifieringen bäddades materialet in i paraffin, snittades med en mikrotom och färgades med Hematoxilin och Eosin samt med Alcianblått/Picrosiriusrött. De histologiska preparaten graderades därefter enligt ett histologiskt graderingssystem (Boos graderingssystem) varefter de histologiska poängen jämfördes mellan chondrodystrofa och icke chondrodystrofa hundraser.

Resultaten av denna studie visar på en signifikant skillnad mellan chondrodystrofa och icke chondrodystrofa hundraser vad gäller histologisk gradering i de två lägsta makroskopiska degenerationsgraderna. Chondrodystrofa hundar uppnådde högre genomsnittliga histologiska poäng, det vill säga mer extensiva histopatologiska förändringar kunde observeras i diskerna hos chondrodystrofa hundar än hos icke chondrodystrofa hundar i de två lägsta makroskopiska degenerationsgraderna. De histologiska parametrar som bidrog mest till denna skillnad var ”kondrocytproliferation i nucleus pulposus” och ”närvaro av notokordceller i nucleus pulposus”. Chondrodystrofa hundar uppnådde signifikant högre histologiska poäng än icke chondrodystrofa hundar i dessa parametrar.

Inga fibrocytliknande celler kunde observeras i nucleus pulposus inom någon av de två rastyperna. De enda observerade cellerna var notokordceller och kondrocytliknande celler. Detta innebär att så kallad *chondroid metamorfos* är den degenerativa process som äger rum inom båda rastyperna och att teorin om en så kallad *fibroid metamorfos* hos de icke chondrodystrofa raserna sannolikt är felaktig.

CONTENT

ABBREVIATIONS

IVD	Intervertebral disc
CD	Chondrodystrophic
NCD	Non-chondrodystrophic
NP	Nucleus pulposus
AF	Annulus fibrosus
EP	Endplate
TZ	Transition zone

INTRODUCTION

The intervertebral disc (IVD) is an important and versatile part of the canine spine, providing both stability and flexibility during movement (Inoue, 1973, Hansen, 1952, Hukins, 1988). Degeneration of the IVD can be part of the normal aging process and degenerated IVDs are common incidental findings in clinically healthy dogs (Brisson, 2010, Hansen, 1952, da Costa et al., 2006). However, IVD degeneration is also highly associated with diseases like cervical and thoracolumbar IVD herniation, degenerative lumbosacral stenosis (DLSS) and cervical spondylomyelopathy (CSM) (Hansen, 1952, Meij and Bergknut, 2010, Burbidge, 1994, Bergknut et al., 2012a, da Costa et al., 2006).

Regarding IVD degeneration and IVD disease, there are some differences between so-called chondrodystrophic (CD) and non-chondrodystrophic (NCD) dog breeds, which were first described by Hansen (1952). CD breeds are characterized by a disturbed endochondral ossification, primarily in the diaphyses of the long bones, resulting in disproportionally short extremities, compared to the NCD dogs who have normal bodily constitutions (Hansen, 1952). These two dog types are dissimilar with regards to age of onset, prevalence and spinal location of IVD degeneration and IVD-related diseases (Hansen, 1952, Bergknut et al., 2012a, Priester, 1976).

Each IVD is composed of three distinct compartments: the central nucleus pulposus (NP), the outer annulus fibrosus (AF) and the cartilaginous vertebral endplates (EP) (Hansen, 1952, Bray, 1998). Degenerative changes can occur in all these parts and in the epiphyses of the vertebral bodies, although the most prominent changes taking place are those in the NP (Hansen, 1952). In the NP of a healthy IVD, the dominating cell type is the notochordal cell, which is derived from the embryonic notochord (Hansen, 1952). Hansen (1952) stated that during the degenerative process, the NP undergoes a so called chondroid metamorphosis in the CD breed types whereas it undergoes a fibroid metamorphosis in the NCD breed types. That means, that the notochordal cells are gradually replaced by chondrocyte-like cells in the CD breeds and by fibrocyte-like cells in the NCD breeds. However, recent studies have shown that degeneration of IVDs in NCD breeds involves replacement of the notochordal cells with chondrocyte-like cells, i.e. that the changes are similar to those taking place in the CD breeds (Bergknut et al., 2013a, Bergknut et al., 2012b, Kranenburg et al., 2013). This conflicts with Hansen's thesis of a fibrous type of metaplasia.

In this project the histopathological appearance of degenerated IVDs of CD and NCD dogs with the same gross morphological appearance were compared with respect to any histopathological differences during early stage IVD degeneration.

For the assasment of gross morphology, the five grade scheme according to Thompson et al. (1990) is used. This scheme was originally created for assessment of gross morphological changes in human lumbar IVDs, but it has recently been modified and validated for use in both CD and NCD dogs by Bergknut et al. (2011). To evaluate the histological changes during IVD degeneration, Boos et al. (2002) have developed a histological grading scheme for midsagittal sections of human lumbar IVDs. This grading scheme has been modified and validated for use in dogs of both breed types by Bergknut et al. (2013a) and was used in this thesis for assessment of the histological changes.

Aim

To evaluate if Hansen was correct in stating that IVD degeneration in chondrodystrophic and non-chondrodystrophic dogs follows different histopathological pathways, i.e. chondroid versus fibroid

degeneration. To do so, CD and NCD dogs with early stage degeneration (Thompson grade I-III) were compared with regards to histopathological differences.

Hypothesis

The hypothesis of this study is that IVDs assigned to the same Thompson grade have a similar histopathological appearance regardless if they are derived from CD or NCD dogs.

LITERATURE OVERVIEW

Anatomy of the intervertebral disc

The canine vertebral column consists of 7 cervical, 13 thoracic, 7 lumbar, 3 (fused) sacral, and a variable number of coccygeal vertebrae (Hansen, 1952, Dyce, 2010). From C2 to S1, and in the coccygeal spine there are intervertebral discs connecting all adjacent vertebrae (Dyce, 2010).

The IVDs occupy approximately one-third of the entire length of the spine (Hansen, 1952) and their purpose is to provide both stability and flexibility to the vertebral column (Inoue, 1973, Hansen, 1952). Each IVD is composed of three distinct compartments: the central nucleus pulposus (NP), the outer annulus fibrosus (AF) and the cartilaginous vertebral endplates (EP) (Hansen, 1952, Bray, 1998). Although, some authors (Johnson et al., 2010, Bergknut et al., 2013b) consider the transition zone (TZ) (the transitional area between the AF and the NP) to be a separate fourth anatomical compartment.

The healthy NP is a soft, translucent, oval shaped structure in the middle of the IVD (Hendry, 1958, Hansen, 1952). It consists of a highly hydrated proteoglycan-gel, and some collagen fibrils (Hukins, 1988). It is relatively eccentrically located since the surrounding AF is 2-3 times thicker ventrally than dorsally (Johnson et al., 2010, Hansen, 1952). This somewhat dorsal placement of the NP is believed to increase the risk of dorsal herniation, toward the vertebral canal, rather than ventrally (Brisson, 2010).

The AF is a fibrous tissue, with 70% of it's dry weight consisting of collagen, mainly type I (Bray, 1998). The AF surrounds the NP ventrally, dorsally and laterally and consists of multiple fibrous lamellae which are able to glide over each other during mechanical loading, thereby making them able to resist stretching/tearing forces (Hansen, 1952, Bray, 1998). These lamellae have been shown to increase in thickness from the inner to the outer layers of the AF (Inoue, 1973). In human IVDs, the fibrous lamellae of the inner one third of the AF are interconnected with the EP whereas the lamellae of the outer two thirds are anchored directly into the vertebral bodies, i.e the bone tissue (Inoue, 1981) although some authors consider this to be calcified cartilage (Roberts et al., 1989). The TZ is defined as the area between the AF and the NP where distinct annular fibres no longer can be distinguished (Johnson et al 2010) and the tissue transcends into a more cartilaginous/mucoid structure (Butler, 1988).

The cartilage EP is interposed between the NP and the cancellous bone tissue of the vertebral

epiphyses and thus makes up the cranial and caudal borders of the IVD. It consists of a thin hyaline cartilage layer composed of chondrocyte-like cells embedded in a matrix of proteoglycan molecules reinforced with collagen fibrils arranged parallel to the subchondral bone (Maroudas et al., 1975, Inoue, 1981, Hansen, 1952).

Function of the intervertebral disc

The function of the IVD is to regulate the stability and the flexibility of the spine in flexion-extension, lateral bending, and axial rotation. It is also suggested that the IVD acts as a shock-absorber between adjacent vertebrae during cranio-caudal compression of the IVD, as the energy is absorbed through radial displacement of the NP and secondary dilation of the AF (Inoue, 1973, Takeuchi et al., 1999, Bray, 1998, Hansen, 1952). There are however other publications indicating that the IVD has limited shock absorbing function and that this action is mainly achieved by the epaxial musculature (Hukins, 1988).

The high intradiscal pressure within the NP (responsible for maintaining disc height) is created through highly negatively charged proteoglycans osmotically attracting water and thereby increasing the hydrostatic pressure. The NP contains a higher proportion of proteoglycans than the AF and therefore the NP attracts more water than the AF. (Hukins, 1988) Early in life, water constitutes about 80-88 % of the total NP content (Bray, 1998). Hence, the NP plays a greater role than the AF in withstanding compressive loading of the IVD (Hukins, 1988).

The strength of the IVD comes from the oblique collagen fibres, making up the lamellae of the AF (Hansen, 1952, Hukins, 1988). During twisting and bending of the spine, the outer AF lamellae provide strength through stretching of the collagen fibres. The inner lamellae of the AF mainly provide strength during compression of the IVD when the hydrostatic NP pressure increases, causing circumferential stress. Compression leading to increased pressure in the NP also causes pressure on the EPs and through stretching of the collagen fibrils in the EPs this causes the EPs to bulge into the subchondral cancellous bone of the vertebral bodies. (Hukins, 1988)

Nutrition of the intervertebral disc

The IVD is the largest avascular structure in the body (Urban et al., 1977, Maroudas, 1988). Only the outermost part of the AF has direct blood supply while the reminder of the AF and the NP are believed to receive nutrients, like oxygen and glucose, from the EP via diffusion from nearby vertebral epiphyseal arteries. Out of these two, the EP route is considered the main mechanism of solute transport. (Maroudas et al., 1975, Urban et al., 1977, Holm et al., 1981) The diffusion of small molecules and metabolites into the IVD is believed to be mainly a passive process, and “pumping” of fluid through physical activity with compression of the IVD does not seem to have a significant effect on diffusion of small solutes (Urban et al., 1982). For large solutes with lower diffusion rates (e.g. growth factors, proteases and their inhibitors) however, the “pumping mechanism” may contribute significantly to their transport through the IVD matrix (Urban et al., 2004). In a study of human IVDs it was found that in the region of the NP, the effective area through which solute transport takes place constitutes approximately 85% of the bone/disc interface. In the inner region of the AF, this area is reduced to 35% whereas the outer part of the AF is almost completely impermeable (Urban et al., 1977).

Intervertebral disc degeneration and disease in chondrodystrophic and non-chondrodystrophic breeds

IVD degeneration can be part of the normal aging process and degenerated IVDs are common incidental findings in clinically healthy dogs of all breeds (Brisson, 2010, Hansen, 1952, da Costa et al., 2006). However IVD degeneration is also highly associated with diseases like cervical and thoracolumbar IVD herniation, DLSS and CSM (Hansen, 1952, Meij and Bergknut, 2010, Burbidge, 1994, Bergknut et al., 2012a, da Costa et al., 2006). All these diseases involve IVD compression of neural tissue, which may lead to clinical signs of disease like gait abnormalities, postural deficits, pain, paresis, paralysis and lameness (Kranenburg et al., 2013, Brisson, 2010, Burbidge, 1994, Sharp, 2005).

Regarding IVD degeneration and IVD disease, Hansen was the first to distinguish differences in IVD degeneration between so-called chondrodystrophic (CD) and non-chondrodystrophic (NCD) dog breeds (Hansen, 1952). CD breeds are characterized by a disturbed endochondral ossification, primarily in the diaphyses of the long bones, resulting in disproportionally short extremities, compared to the NCD dogs that have normal body constitutions (Hansen, 1952).

Signs of IVD degeneration occurs earlier in life and in all discs in CD breeds, compared to NCD breeds, where degeneration has a later onset and only one or a few discs are affected (Hansen, 1952, Braund et al., 1975). Macroscopic signs of degeneration are mainly seen in the NP, making the delineation between the NP and the AF less evident (Inoue, 1973, Hansen, 1952). It has been shown, from a macroscopic view, that the majority of the CD dogs have lost their mucoid NP already at the age of one year, with the tissue being replaced by a more chondroid structure, with semi-elastic consistency and a greyish-white or grey-yellow colour. This is to be compared with the NCD dogs, where the probability to find a fibrocartilaginous NP rather than a mucoid one becomes grater later on in life (generally after the age of seven years). The NP in the NCD dogs at this stage becomes milk-white, but often still has a mucoid, gelationous consistency. (Hansen, 1952)

Furthermore, CD and NCD breeds are dissimilar regarding the age of onset and the spinal locations of IVD related diseases. In CD breeds, IVD related diseases in the cervical or thoracolumbar spine are most common and typically have their onset relatively early in life (3-7 years of age) (Hansen, 1952, Hoerlein, 1953, Brisson, 2010). This is in contrast to the NCD breeds, where IVD related diseases have a later, often progressive, onset (around 6-8 years of age) and mainly affect the lower cervical or lumbosacral spine, although the thoracolumbar spine can also be affected (Brisson, 2010, Hansen, 1952, Meij and Bergknut, 2010, Cudia and Duval, 1997).

When herniations occur, they also differ somewhat between the two breed types. Hansen described two types of herniations (Hansen, 1952). Hansen type I is defined as an extrusion of NP material through a rupture of the AF and the dorsal longitudinal ligament, often in a dorsomedial or dorsolateral direction, leading to deposition of NP material into the epidural space. These herniations were described to predominantly occur in comparatively young CD breeds, often with acute and severe clinical signs. Hansen type II herniations were described as smaller protrusions of NP material through partial ruptures of the AF, causing smaller, intradiscal herniations, in a dorsomedial or dorsolateral direction, with bulging of the AF and the longitudinal ligament. This type of herniation was stated to occur in all breeds, but later in life, with less severe or no clinical signs. (Hansen, 1952)

Another phenomenon occurring in IVD degeneration is calcification of the IVD, which is common in CD dogs and relatively rare in NCD dogs (Hansen, 1952). The process of NP calcification can be seen as early as five months of age in CD breeds and although the prevalence increases with age (Hansen, 1952), it seems like it has reached a steady, or a maximum level at about 24-27 months of age (Jensen

and Arnbjerg, 2001). Calcifications are usually seen in the periphery of the NP and in the perinuclear layers of the AF and are considered to be of a dystrophic nature (Hansen, 1952, Stigen and Christensen, 1993).

The above-mentioned dissimilarities indicate that there might be differences in the pathogenesis and histopathological appearance of IVD degeneration in CD and NCD dog breeds. This aspect will be discussed more extensively below.

Normal histology of the intervertebral disc

The AF is composed of an outer layer of densely packed collagen fibres in a fibrous matrix with a narrow inner layer of fibrocartilage located adjacent to the NP (Hansen, 1952, Braund et al., 1975). The outer part of the AF is composed of elongated fibroblast and fibrocyte-like cells whose long axes run parallel to the collagenous lamellae, surrounded by a fibrous matrix. Towards the inner parts of the AF, the cell population changes into a mixed population of elongated fibrocytes and rounded chondrocyte-like cells. (Braund et al., 1975)

In the NP of a healthy IVD, the dominating cell type is the notochordal cell, which is derived from the embryonic notochord (Hansen, 1952). These large cells are characterized by cytoplasmic vesicles or vacuoles, which accounts for a significant volume of the cell (Hunter et al., 2003, Hunter et al., 2004a). These vacuoles may have a role in regulating cell volume and tonicity during rapid osmotic stress and thereby protecting the cells from potentially damaging increasing pressures (Hunter et al., 2007). The notochordal cells appear in large clusters (Hunter et al., 2004a) containing 4 to 426 cells (Hunter et al., 2003, Hunter et al., 2004b), which are interconnected through functional gap junctions. Enzymatic disruption of these interconnections affects the long-term survival of the notochordal cells and causes premature cell-death. Therefore, clustering is thought to play an important role in survival and functioning of these cells (Hunter et al., 2004b). The notochordal cells produce a basophilic extracellular matrix consisting of highly hydrated proteoglycans wherein collagen type II fibres are embedded (Hansen, 1952, Cappello et al., 2006).

Histopathology in chondrodystrophic and non-chondrodystrophic breeds

Even in pups, some histological differences between CD and NCD breeds are visible in the IVD, where the transition from the perinuclear layer of the AF to the NP is more distinct in the NCD than in the CD breeds where this transition zone is wider and occupies a larger portion of the AF (Hansen, 1952, Braund et al., 1975). Already at 2-9 months of age, the IVDs of CD dogs are showing signs of degeneration (Hansen, 1952). In these dogs, Hansen described cells appearing in pairs or in small groups, resembling cartilage in the perinuclear layer of the AF (Hansen, 1952).

The size of the NP differs between the two breed types. In CD dogs it occupies a relatively small portion of the whole IVD compared to the NCD dogs (Hansen, 1952, Johnson et al., 2010). In a study on cervical IVDs from dogs younger than 3 years, the NP of CD dogs accounted for <16% of the total disc surface area whereas it accounted for nearly 28% in the NCD breeds (Johnson et al., 2010). In CD dogs, the notochordal cells in the NP are starting to be replaced by chondrocyte-like cells surrounded by a fibrocartilaginous matrix already at 3 months of age (Hansen, 1952, Cappello et al., 2006). This process, which was termed *chondroid metamorphosis* by Hansen (1952), begins in the perinuclear layers of the AF or in the periphery of the NP and then extends throughout the whole NP (Hansen, 1952). In CD dogs, this transition proceeds very rapidly, and at the age of 9 months to 1 year the so-called chondroid metamorphosis is usually completed in all IVDs, with an entirely chondroid NP in

most dogs, although isolated foci of notochordal remnants might still be apparent at times (Hansen, 1951, Braund et al., 1975).

The above mentioned degeneration process is different to the process taking place in the NP of NCD breeds. In these breeds, the notochordal cell remains the main cell type of the NP during the majority of the adult life (Hansen, 1952, Braund et al., 1975, Cappello et al., 2006). At the age of 1-7 years, Hansen (1952) described a collagenization of the NP, which was either spread diffusely over the NP or tended to divide it into lobuli, a process that was beginning in the periphery of the NP. This separation of notochordal cells into lobuli have also been described by Braund et al. (1975) in a 9 months old Greyhound. Hansen (1952) described this as a process of slow maturation, although he also described degenerative changes in some of the notochordal cells, which were isolated in small lobuli. In NCD dogs >7 years of age, which represents old age in dogs, Hansen (1952) stated that it was very likely to find degenerated IVDs in these breed types, although this degeneration does not take place in all IVDs, as in the CD breeds. Hansen termed the changes taking place in the NP of NCD dogs *fibroid metamorphosis* since he noted that the process was characterized by collagenization and that the cells in the degenerated NPs of these dogs adopted a fibrocyte-like morphology (Hansen, 1952).

Degenerative changes also occur in the AF, and follow somewhat similar patterns in both breed types, although the degenerative changes are commonly more severe in the CD than in the NCD breeds (Hansen, 1952). Hansen described these degenerative changes taking place both within and between the lamellae, the latter being most frequently observed, with foci of granular masses seen in the interlammelar tissue (Hansen, 1952). These degenerative changes cause a loosening of the connection between the lamellae, leading to disorganization and, in some cases, ruptures of the lamellae. In CD breeds, degeneration of the AF usually takes place after severe degeneration of the NP, whereas this can appear concurrent with, or even before degeneration of the NP in NCD breeds. (Hansen, 1952)

As mentioned above, Hansen (1952) described the histopathological changes taking place during IVD degeneration in the CD dogs as chondroid whereas the degenerative changes taking place in the IVDs of NCD dogs were described as fibroid. Although stating clear differences in the pathological processes he also described many similarities in the histopathological appearances of IVDs of the two different dog types, and he stated that in the later stages of degeneration, the histologic distinctions between the two dog types became unapparent, a statement which is also supported by Royal et al. (2009). It is thus still questionable if there are significant histopathological differences between the IVDs of CD and NCD dogs during the process of degeneration. A recent study by Kranenburg et al. (2013) on the histopathological appearance of surgically removed IVD tissue, showed only notochordal cells and chondrocyte-like cells and no presence of fibrocyte-like cells in the NP of CD nor in NCD dogs with varying degrees of degeneration, except in cases with the presence of granulation tissue. An other study on sagitally transected spines from both dog types, came to the same conclusion (Bergknut et al., 2013a). This conflicts with Hansen's thesis of a fibrous type of metaplasia in the NCD dogs.

Macroscopic grading of intervertebral disc degeneration

For the assessment of gross morphological changes in human lumbar IVDs, Thompson et al. (1990) developed a five category grading scheme, which was recently validated for use in dogs by Bergknut et al. (2011). This grading scheme takes into account the degenerative changes in the NP, the AF, the endplates and the margins of the vertebral bodies, where grade I represents a healthy IVD and grade V represents end-stage degeneration. The grading scheme is presented in Table 1.

Histological grading of intervertebral disc degeneration

To evaluate the degenerative changes in canine IVDs, the histological changes are often considered to be the "gold standard" (Stigen and Kolbjornsen, 2007, Seiler et al., 2003). To grade such degenerative changes, Boos et al. (2002) have developed a histological grading scheme for midsagittal sections of human lumbar IVDs. Although there are several similarities between human and canine IVD degeneration, there are also some differences, the most important being that the endplates in canine IVDs are significantly thinner and the subchondral bone is significantly thicker than in human IVDs (Bergknut et al., 2012b). Since the grading scheme according to Boos et al. (2002) has a considerable focus on the vertebral endplates, that scheme is not regarded suitable for grading of canine IVDs (Bergknut et al., 2013a). Therefore, Bergknut et al. (2013a) have modified and validated this histological grading scheme for the use in canine IVD degeneration in both CD and NCD breeds. The scheme takes into account the histomorphology of the AF, the NP, the endplates, the subchondral bone and the margins of the vertebral epiphyses. Also cellular changes (chondrocyte metaplasia of AF, chondrocyte proliferation of NP and presence of notochordal cells in NP) and changes in the intercellular matrix of the NP are represented in the scheme, which includes a total of 9 different parameters (Table 2). This study also took into account the correlation between gross morphology (represented by Thompson grades) and histological alterations and thereby came to the conclusion that there is a significant correlation between increasing Thompson grades and increasing total histological scores. This correlation with increasing Thompson grades was also significant for all individual histological parameters. The high reproducibility and the strong correlation with the two gold standards (macroscopic grading by the Thompson grading scheme and glycosaminoglycan content, which was measured in a Farndale assay) regarded this new histological grading scheme to be a reliable and objective method for classification of IVD degeneration in both CD and NCD dog breeds. (Bergknut et al., 2013a)

Table 1. *Gross morphology grading scheme for canine intervertebral disc degeneration according to Thompson et al. (1990) and Bergknut et al. (2011)*

Thompson grade	**Nucleus pulposus**	**Annulus fibrosus**	**End plates**	**Vertebral bodies**
1	Bulging gel	Discrete fibrous lamellae	Hyaline; uniform thickness	Rounded margins
2	White fibrous tissue peripherally	Mucinous material between lamellae	Irregular thickness	Pointed margins
3	Consolidated fibrous tissue	Extensive mucinous infiltration; loss of annular-nuclear demarcation	Focal defects in cartilage	Early chondrophytes or osteophytes at margins
4	Horizontal (vertical) clefts parallel to end plate	Focal disruptions	Fibrocartilage extending from subchondral bone; irregularity and focal sclerosis in subchondral bone	Osteophytes < 2 mm

5	Clefts extend through nucleus and annulus	Diffuse sclerosis	Osteophytes > 2 mm

Table 2. *Histological grading scheme for canine intervertebral disc degeneration according to Boos et al. (2002) and Bergknut et al. (2013a)*

Morphology of annulus fibrosus (AF)	
0	Well-organized, half ring-shaped, collagen lamellae
1	Mild disorganized; some loss of half ring-shaped structure, most lamellar layer, still distinguishable (<25%)
2	Moderately disorganized; partly ruptured AF, loss of half ring-shaped structure (25-75%)
3	Completely ruptured AF; no or few distinguishable half ring-shaped collagen lamellae (>75%)
Chondrocyte metaplasia of AF	
0	No chondrocyte morphology, just spindle-shaped fibroblasts
1	Mild chondrocyte proliferation (i.e., limited to inner most AF layers)
2	Moderate chondrocyte proliferation (i.e., chondroid cells in up to half of the AF)
3	Marked chondrocyte proliferation (i.e., chondroid cells up to outer layers of the AF)
Tears and cleft formation	
0	Absent
1	Rarely present
2	Present in intermediate amounts
3	Abundantly present
4	Scar/tissue defects
Chondrocyte proliferation of nucleus pulposus	
0	No proliferation
1	Increased chondrocyte-like cell density
2	Connection of two chondrocytes
3	Small size clones (i.e., several chondrocytes group together, i.e. 2-7 cells)
4	Moderate size clones (i.e., > 8 cells)
5	Huge clones (i.e., >15 cells)
6	Scar/tissue defects

Presence of notochordal cells in nucleus pulposus

0 Abundantly present (>50%)

1 Present (1-50%)

2 Absent

Matrix staining of nucleus pulposus with Alcian Blue/Picrosirius Red staining

0 Blue stain dominates

1 Mixture of blue and red staining

2 Red stain dominates

Endplate morphology

0 Regular thickness; homogeneous structure

1 Slightly irregular thickness

2 Moderately irregular thickness

3 Severely irregular thickness with interruption of the endplate

New bone formation

0 Absent

1 Minor new bone formation

2 Moderate amounts of new bone formation

3 Abundant new bone formation; tendency towards bridging/complete bridging

Subchondral bone sclerosis

0 No sclerosis (< 2 x the thickness of the dorsal vertebral cortex)

1 Mild sclerosis (2-4 x the thickness of the dorsal vertebral cortex)

2 Moderate sclerosis (> 4 x the thickness of the dorsal vertebral cortex)

3 Severe subchondral bone irregularities

MATERIALS AND METHODS

Animals and sample preparations

For this histological study IVDs were collected from randomly selected fresh (<48h) canine cadaveric spines. The dogs were patients and research animals that had died or were euthanized for reasons unrelated to this study, at the Faculty of Veteriary Medicine, Utrecht University, the Netherlands, and subsequently donated for research. Owner consent was given to collect tissue for research purposes post mortem from the privately owned dogs and ethical approval from the Faculty of Veterinary Medicine was granted to collect tissue post mortem from the research dogs.

The spinal segments were cut midsagitally using a belt saw, cooled with water to prevent burning of the cut surface. Information of each IVD (breed, age and IVD number) was registered and the cut spinal surfaces were photographed for macroscopic grading. Subsequently, each IVD was individually fixated in 4% buffered formalin and decalcified in EDTA. After decalcification, the IVDs were embedded in paraffin, sliced with a microtome and stained with Hematoxilin and Eosin (H&E) and Alcian Blue/Picrosirius Red. H&E staining was used to evaluate the morphological changes in the IVD as well as determining the type and number of cells. Alcian blue/Picrosirius red staining was used to evaluate the changes in the composition of the extracellular matrix, where Alcian blue stains proteoglycans light blue and Picrosirius red stains collagen red, with a higher affinity to collagen type I. Histological slides from seven IVDs from CD dogs and seven IVDs from NCD dogs from each of the Thompson grades I, II and III respectively were selected for this study. In total 84 IVD samples were used since 2 samples were collected from each IVD represented in the study (i.e. 42 for H&E and 42 for Alcian Blue/Picrosirius Red staining). The samples originated from 42 different IVDs from 33 different dogs (16 CD and 17 NCD), i.e. some of the dogs contributed with more than one IVD.

Histological grading

For histological grading, the modified Boos grading scheme was used (Bergknut et al., 2013a). All (in total 84) histological slides were blinded and subsequently graded histologically according to the 9 individual parameters in this scheme. Furthermore, the additional parameter, “presence of fibrocyte-like cells in nucleus pulposus” was evaluated separately, since this parameter is not included in the 9 parameter grading scheme, but is of interest regarding Hansen’s statement that the NP of NCD dogs undergoes a fibroid metaplasia. The histological grading was performed by the student (Tove Hansen) and the results were subsequently validated by a veterinary Resident in neurology (Niklas Bergknut) and a veterinary pathologist (Guy Grinwis).

Statistical analysis

Normal distribution for the histological values was verified through Q-Q plots. A two-way ANOVA was performed in IBM SPSS 20.0 to evaluate whether there were statistical differences between CD and NCD dogs in the three different Thompson grades overall and also for the individual histological parameters. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

Total histological scores

Total scores, including all histological parameters in Boos grading scheme, show a statistically significant difference between CD and NCD dogs in Thompson grade I, where CD dogs have a higher mean histological scoring, i.e. more histopathological degenerative changes, than NCD dogs (Table 3 and Figure 1). In Thompson grade II and III there are no significant differences between the two breed types regarding total histological scores.

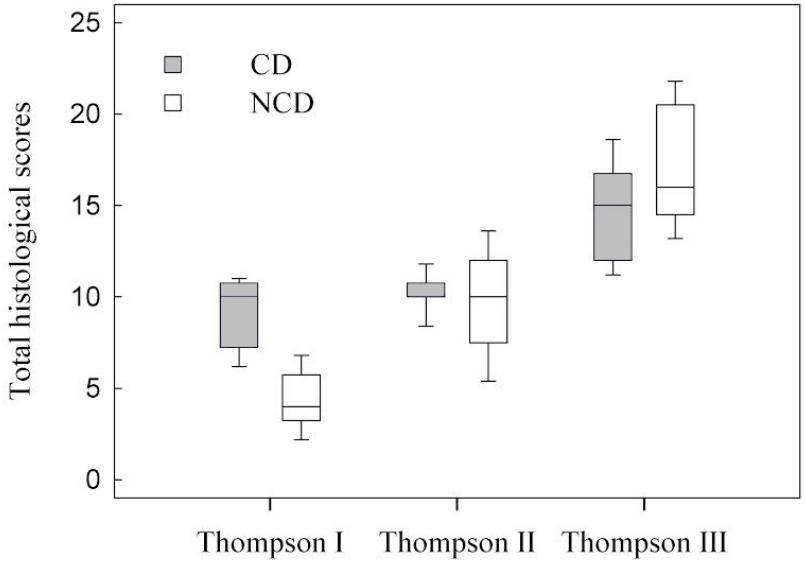

Figure 1. *Total histological scores for chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs in Thompson grade I, II and III.*

Table 3. *Means with standard errors (±) and p-values from the two-way Anova for total histological scores for chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs in Thompson grade I, II and III*

	Thompson I	**Thompson II**	**Thompson III**
CD mean score	9.0 ±0.75	10.1 ±0.45	14.6 ±1.13
NCD mean score	4.4 ±0.64	9.9 ±1.17	17.3 ±1.32
p-value	0.002	0.835	0.054

Histological scores in individual parameters

Mean scores in each of the 9 individual histological parameters showed significant differences between the two breed types in the parameters “chondrocyte proliferation of nucleus pulposus” and “presence of notochordal cells in nucleus pulposus” in Thompson grade I and II (Table 4). CD dogs got higher mean scores than NCD dogs in both parameters (Figure 2 and 3) in both Thompson grades.

In Thompson grade II, there was a significant difference between CD and NCD dogs in the parameter “morphology of annulus fibrosus” (Table 4 and Figure 4). NCD dogs had higher mean scores than CD dogs in this parameter.

In Thompson grade III, there was a significant difference between the two breed types in the parameter “new bone formation”, where NCD dogs had higher mean scores than CD dogs (Table 4 and Figure 5).

In the remaining histological parameters there were no significant differences between the two breed types (Table 4 and Appendix).

Table 4. *Means ± SE and p-values from the two-way ANOVA for chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs in each histological parameter in Thompson grade I, II and III. AF=annulus fibrosus, NP=nucleus pulposus, AB/PR=alcian blue/picrosirius red staining*

	Thompson I			**Thompson II**			**Thompson III**		
Parameter	**CD**	**NCD**	**p-value**	**CD**	**NCD**	**p-value**	**CD**	**NCD**	**p-value**
Morphology of AF	0.57±0.38	0.14±0.14	0.207	0.57±0.38	1.29±0.29	0.039	1.71±0.18	2.14±0.34	0.207
Chondrocytes AF	2.43±0.30	2.43±0.37	1.000	2.71±0.29	2.43±0.37	0.499	2.71±0.29	3.00±0.00	0.499
Tears and clefts	0.00±0.00	0.29±0.29	0.451	0.00±0.00	0.57±0.30	0.136	1.86±0.34	2.57±0.37	0.065
Chondrocytes NP	2.57±0.37	0.86±0.14	0.001	3.14±0.26	2.00±0.44	0.025	3.00±0.38	3.14±0.40	0.772
Notochordal cells NP	1.43±0.37	0.00±0.00	0.000	2.00±0.00	1.29±0.29	0.012	2.00±0.00	2.00±0.00	1.000
Staining NP (AB/PR)	0.29±0.18	0.00±0.00	0.118	0.00±0.00	0.14±0.14	0.428	0.14±0.14	0.14±0.14	1.000
Endplate morphology	0.71±0.29	0.14±0.14	0.206	1.00±0.22	1.42±0.30	0.341	2.00±0.44	1.86±0.40	0.750
New bone	0.29±0.18	0.00±0.00	0.377	0,14±0.14	0.21±0.18	0.657	0.29±0.18	1.43±0.43	0.001
Subchondral sclerosis	0.71±0.18	0.57±0.38	0.718	0.57±0.38	0.43±0.38	0.718	0.86±0.46	1.00±0.31	0.718

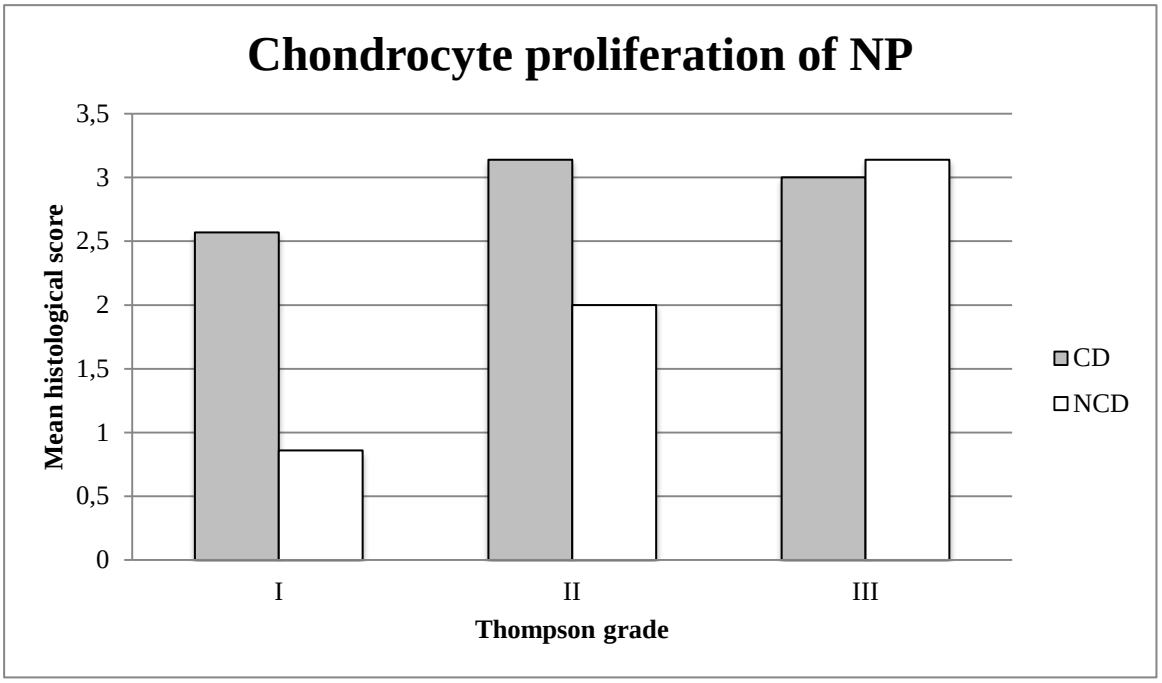

Figure 2. *Histological scores for the parameter "chondrocyte proliferation of nucleus pulposus" in Thompson grade I, II and III. NP=nucleus pulposus, CD=chondrodystrophic, NCD=non-chondrodystrophic.*

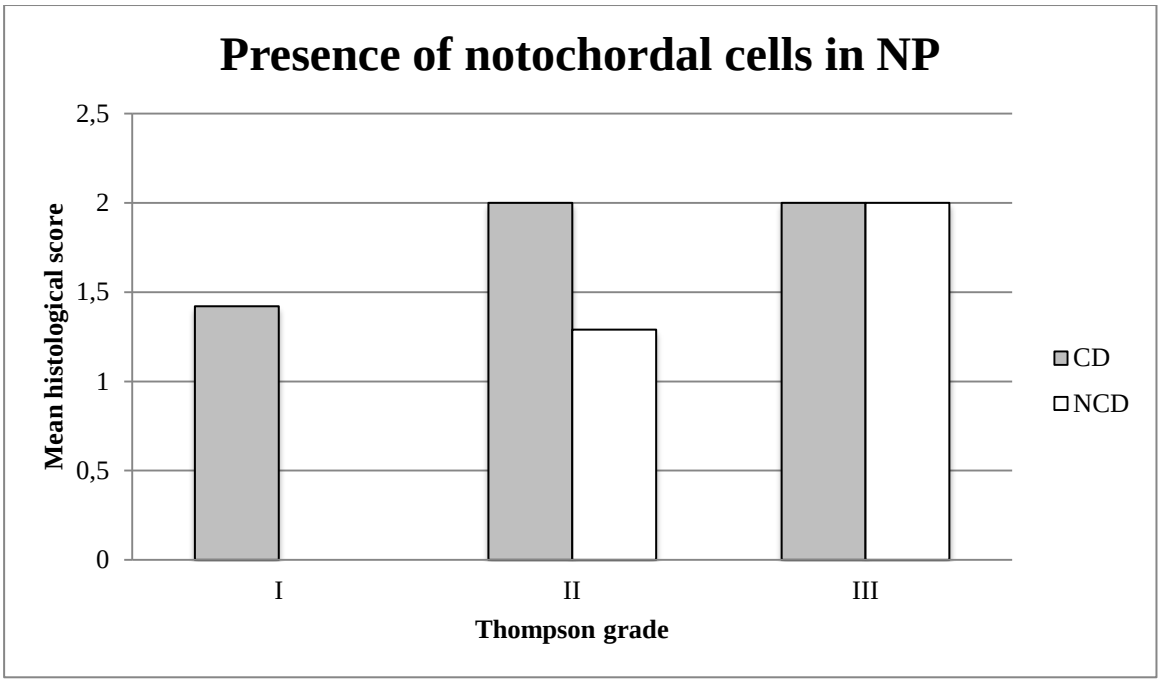

Figure 3. *Histological scores for the parameter "presence of notochordal cells in nucleus pulposus" in Thompson grade I, II and III. NP=nucleus pulposus, CD=chondrodystrophic, NCD=non-chondrodystrophic.*

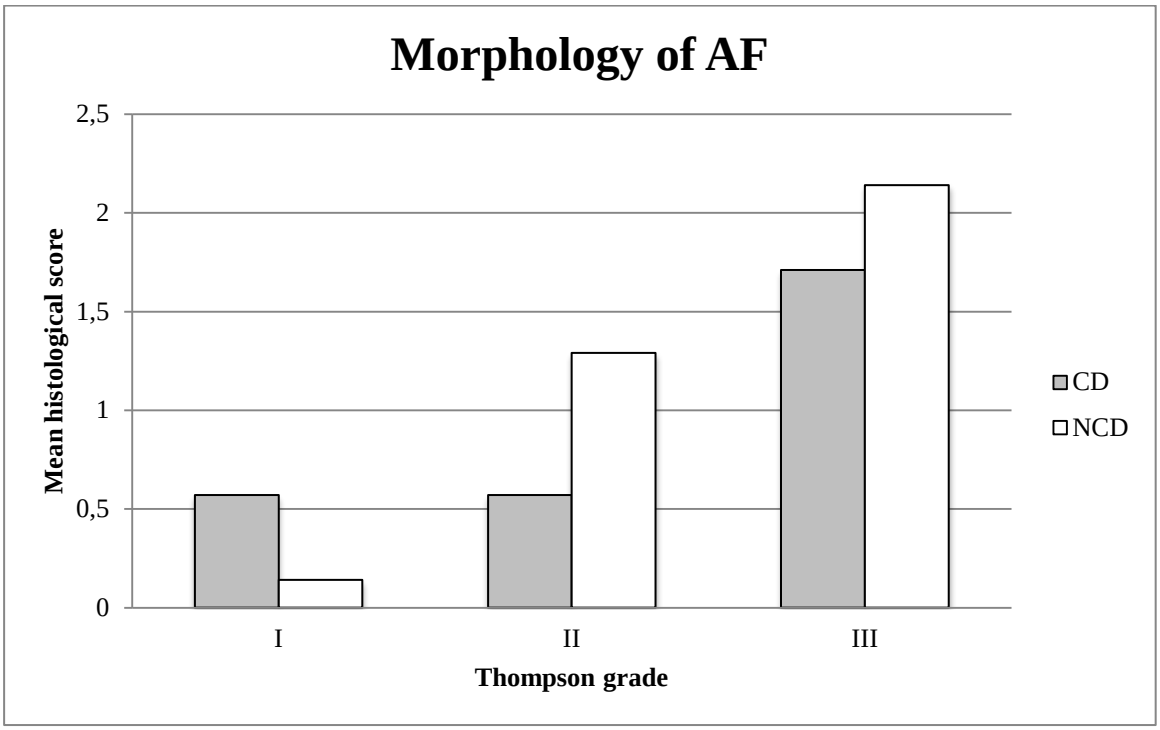

Figure 4. *Histological scores for the parameter "morphology of annulus fibrosus" in Thompson grade I, II and III. AF=annulus fibrosus, CD=chondrodystrophic, NCD=non-chondrodystrophic.*

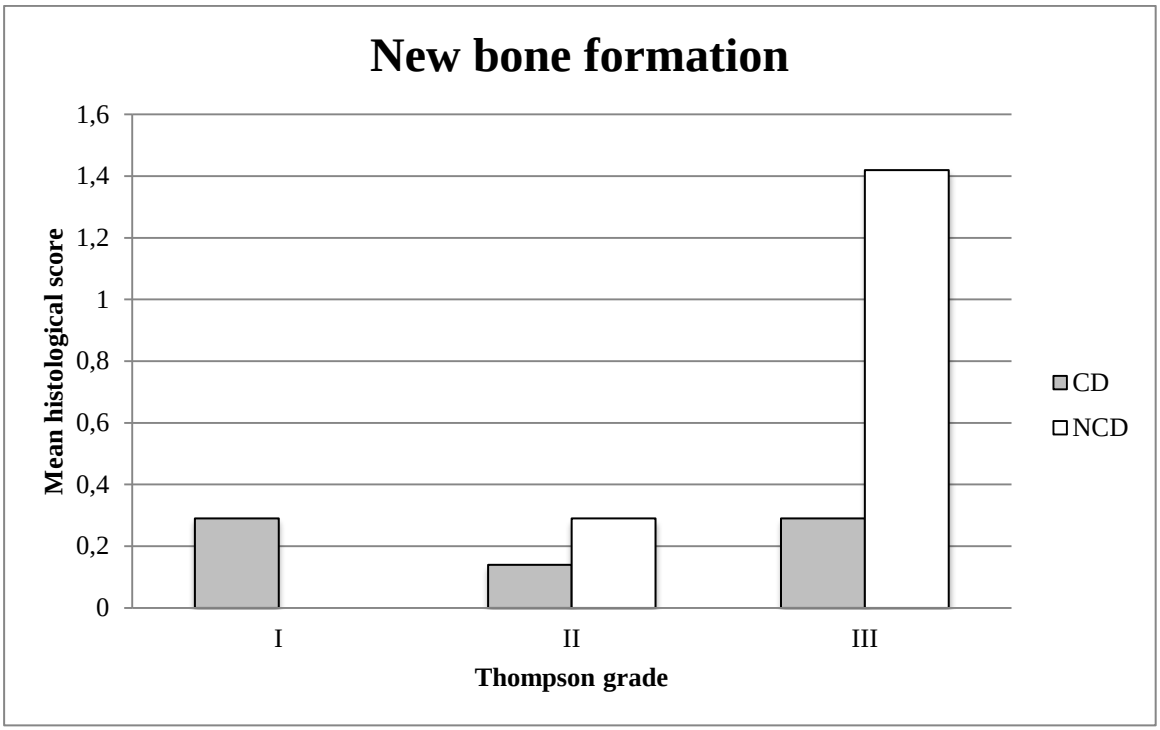

Figure 5. *Histological scores for the parameter "new bone formation" in Thompson grade I, II and III. CD=chondrodystrophic, NCD=non-chondrodystrophic.*

Scores for presence of fibrocyte-like cells in NP were registered separately, since this parameter is not included in the 9 parameter grading scheme, but is of interest regarding Hansen's statement, that the NP of NCD dogs undergoes a fibroid metamorphosis. No fibrocyte-like cells were found in the NP of any of the two dog types in any of the three Thompson grades investigated in this study (Table 5).

Table 5. *Mean fibrocyte-like cell counts in the nucleus pulposus (NP) of chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs in Thompson grade I, II and III*

	Thompson I		**Thompson II**		**Thompson III**	
	CD	**NCD**	**CD**	**NCD**	**CD**	**NCD**
Fibrocyte-like cells in NP	0	0	0	0	0	0

Figures of the remaining 5 individual histological parameters are displayed in the appendix (Figures 7-11). The histological scores in these parameters show no significant differences between CD and NCD dogs in any of the three Thompson grades.

DISCUSSION

The results of this study showed that there was a significant difference in the total histological scores (all parameters summarized) between CD and NCD dogs in Thompson grade I. CD dogs had higher mean histological scores, i.e. more histopathological degenerative changes were observed in the IVDs of CD dogs than in the NCD dogs in the lowest macroscopical grade of degeneration (Table 3 and Figure 1). The parameters contributing the most to this difference was "chondrocyte proliferation of nucleus pulposus" and "presence of notochordal cells in nucleus pulposus" (Table 4). CD dogs had significantly higher scores in the parameter "chondrocyte proliferation of nucleus pulposus", i.e. the notochordal cells have been replaced by chondrocyte-like cells to a higher extent in the CD dogs than in the NCD dogs in Thompson grade I. In the parameter "presence of notochordal cells in nucleus pulposus", CD dogs also had significantly higher scores than NCD dogs, i.e. the CD dogs had less remaining notochordal cells in the NP than NCD dogs belonging to Thompson grade I. The same applied for both parameters in Thompson grade II. These results correspond well with Hansens statement, that the degeneration process starts in the central portion of the IVD (the NP) in CD dogs and that the changes in the NP of CD dogs appear during early stage and with great rapidity compared to the changes in the NP of NCD dogs that was described as that of a slow maturation process (Hansen, 1952).

In Thompson grade II and III, NCD dogs got higher histological mean scores than CD dogs in the parameters “morphology of annulus fibrosus” and “new bone formation” respectively. The morphological changes in the AF were thus more severe in the NCD dogs than in the CD dogs in Thompson grade II. This might be in accordance with the statement by Hansen (1952), that degeneration of the AF can appear concurrent with, or even before degeneration of the NP in NCD breeds whereas the degeneration process begins in the centre and spreads out centrifugally in CD breeds. The fact that NCD dogs get higher scores in the parameter “new bone formation” in Thompson grade III might be connected with degeneration of the AF, causing instability to the spinal segments and thereby secondary inducing the bone tissue on the margins of the vertebrae to proliferate, although this is uncertain.

Regarding the total histological scores in Thompson grade II and III there are no significant differences between the two breed types. Except for the parameters mentioned above the same applies for the rest of the individual histological parameters studied, i.e. no significant differences are seen between the two breed types in any of these individual parameters. The fact that only a few differences are seen between the two breed types in Thompson grade II and III is in accordance with a study by Royal et al. on surgically removed degenerate disc material where it was found that the histologic differences between the two dog types was minimal (Royal et al., 2009).

The two different types of IVD degeneration, i.e. chondroid degeneration in CD dogs and fibroid degeneration in NCD dogs, first stated by Hansen (1952), has long been an accepted histological distinction between the two dog types. However, in the present study, there was no evidence of fibrocyte-like cells in the NP of any of the two breed types. The only cell types found in the NP of both breed types were notochordal cells and chondrocyte-like cells. This indicates that chondroid metaplasia is the process taking place during IVD degeneration in both CD and NCD breeds. Our theory is that Hansen (1952) might have interpreted apoptotic notochordal cells as fibrocyte-like cells, which is easy to understand, since apoptotic notochordal cells sometimes have a strong resemblance to fibrocytes with seemingly spindle shaped nuclei (Figure 6). It is important to stress that Hansen (1952) never stated that these cells were in fact fibrocytes since he only mentioned *“cells with a certain similarity of fibrocytes”* or *“fibrocyte-like cells”* when referring to cells in degenerated NPs of NCD dogs. Furthermore, both microscopes and staining methods have improved since the 1950’s, which facilitates more detailed and accurate assessment of histological preparations today compared with 60 years ago.

The majority of the histological findings in this study showed no significant differences between the two breed types. These results are similar to the findings in two recent studies on the histopathological appearance of surgically removed IVD tissue where it was found that the histologic differences between the two dog types were minimal (Royal et al., 2009, Kranenburg et al., 2013). These results also correspond well with what was reported in another study of IVD degeneration in sagitally transected spines, where the histopathological appearances were largely similar in the two breed types (Bergknut et al., 2013a).

Possible selection biases

The study population in this thesis consists of 16 CD and 17 NCD dogs. The NCD breeds included were: 7 Mixed breeds, 3 Kerry Beagles, 3 Foxhounds, 2 Bouvier des Flandres, 1 Flat Coated Retriever and 1 Welsh Terrier. The only CD breed included was Beagle. None of the two groups of dogs are representative for the whole canine population and it would also be desired to include other CD breeds than the Beagle. Due to this, the results of this study might not be possible to extrapolate on the whole

canine population. It is also important to stress that some of the dogs have contributed with more than one histological sample in this study. Although the histological samples, which in some cases originated from the same dog was always taken from different IVDs, this might have contributed to giving these individuals more weight in the data analysis.

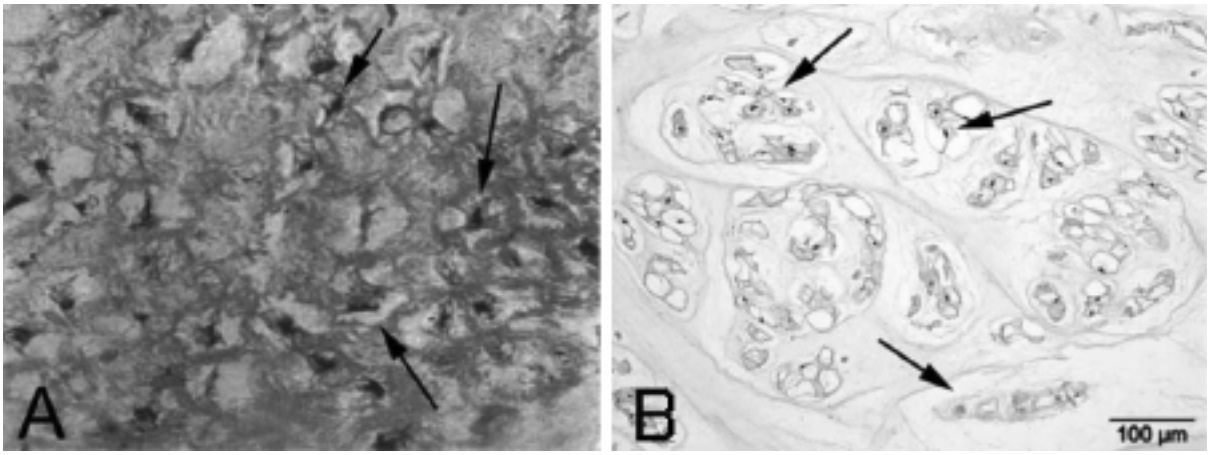

Figure 6. *(A) Original picture from the thesis by Hansen (1952) showing so-called fibroid metamorphosis of the nucleus pulposus: "Airdale terrier, 10 years old. N.p. (nucleus pulposus) from disc 9, showing still vital cells with a certain similarity of fibrocytes and an intercellular substance, rich in collagen fibers. Van Gieson 400x." (Hansen, 1952). These cells are marked by arrows in picture (A). (B) shows the nucleus pulposus of a non-chondrodystrophic dog (Hematoxilin and eosin staining), with an increase in fibrillar collagenous extracellular matrix, dividing the nucleus pulposus into islands of notochordal cells. The notochordal cells (arrows) have decreased in size and show loss of intracellular vesicles and signs of apoptosis. Note the morphological similarities between the cells in Hansen's fibroid metamorphosis (A) and the notochordal cells in picture (B). Picture B and text from Smolders et al. (2013).*

CONCLUSIONS

CD dogs had higher total histological scores in the two lowest macroscopical grades of degeneration (Thompson grade I and II). The most prominent differences were the degenerative changes in the NP, where the original notochordal cells had been replaced by chondrocyte-like cells to a higher extent in the NP of CD dogs than in the NCD dogs. The histological changes being most conspicuous in the NCD dogs were those in the periphery, i.e. the degenerative changes in the AF and the margins of the vertebrae, where these dogs had higher histological scores than CD dogs in Thompson grade II and III respectively. No fibrocyte-like cells were found in the NP of any of the two breed types. This might lead to the conclusion that the so-called *chondroid metamorphosis* is the process taking place during IVD degeneration in both CD and NCD breeds and that the theory of a *fibroid metamorphosis* in non-chondrodystrophic breeds is inaccurate.

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APPENDIX

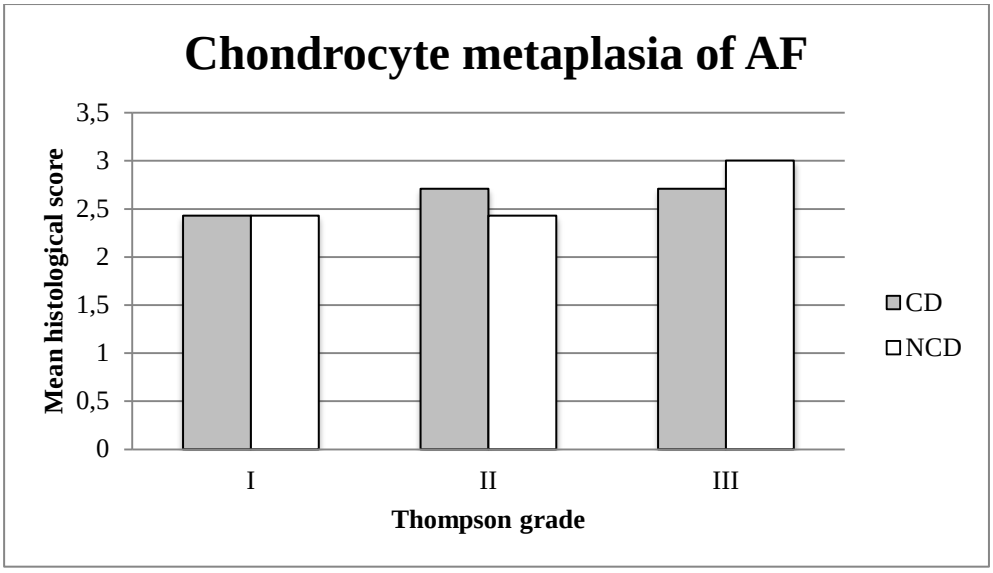

Figure 7. *Histological scores for the parameter "chondrocyte metaplasia of AF" in Thompson grade I, II and III. AF=annulus fibrosus, CD=chondrodystrophic, NCD=non-chondrodystrophic.*

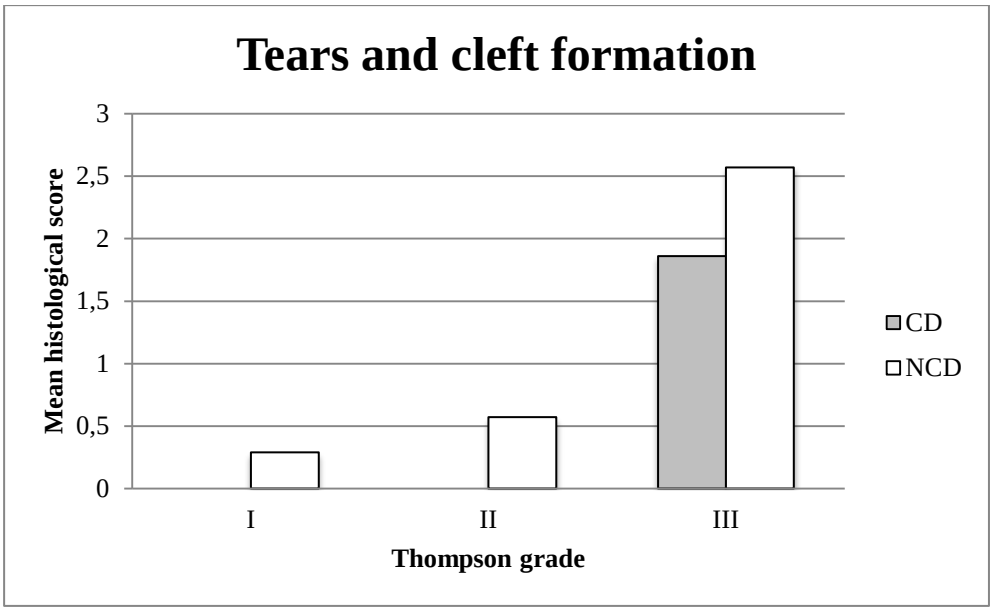

Figure 8. *Histological scores for the parameter "tears and cleft formation" in Thompson grade I, II and III. CD=chondrodystrophic, NCD=non-chondrodystrophic.*

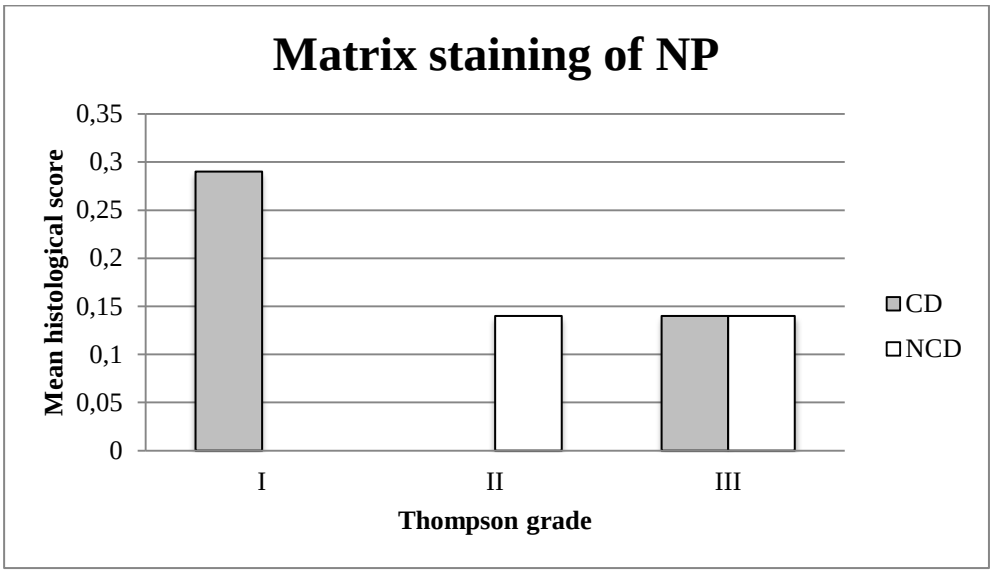

Figure 9. *Histological scores for the parameter "matrix staining of the NP with Alcian blue/Picrosirius red staining" in Thompson grade I, II and III. NP=nucleus pulposus, CD=chondrodystrophic, NCD=non-chondrodystrophic.*

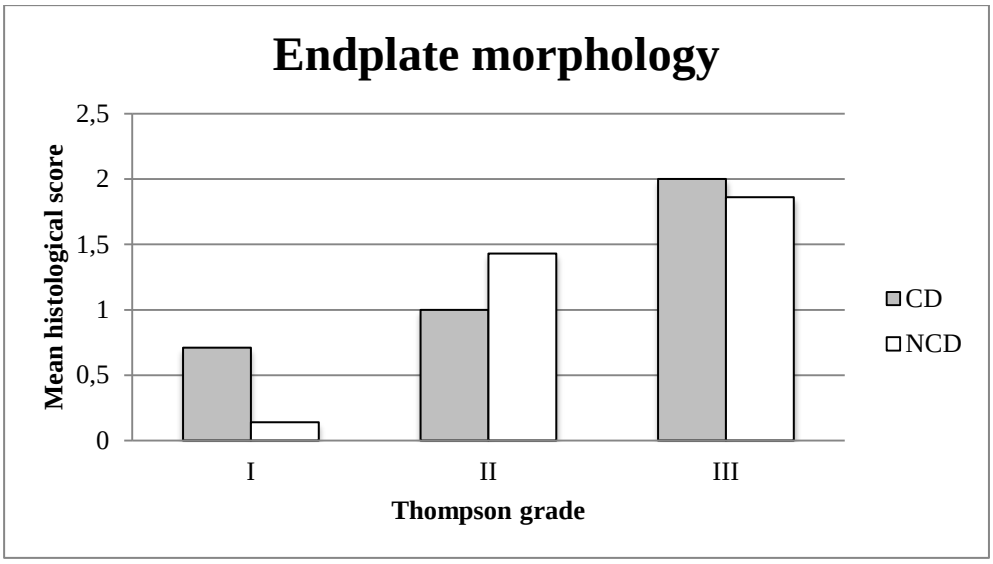

Figure 10. *Histological scores for the parameter "endplate morphology" in Thompson grade I, II and III. CD=chondrodystrophic, NCD=non-chondrodystrophic.*

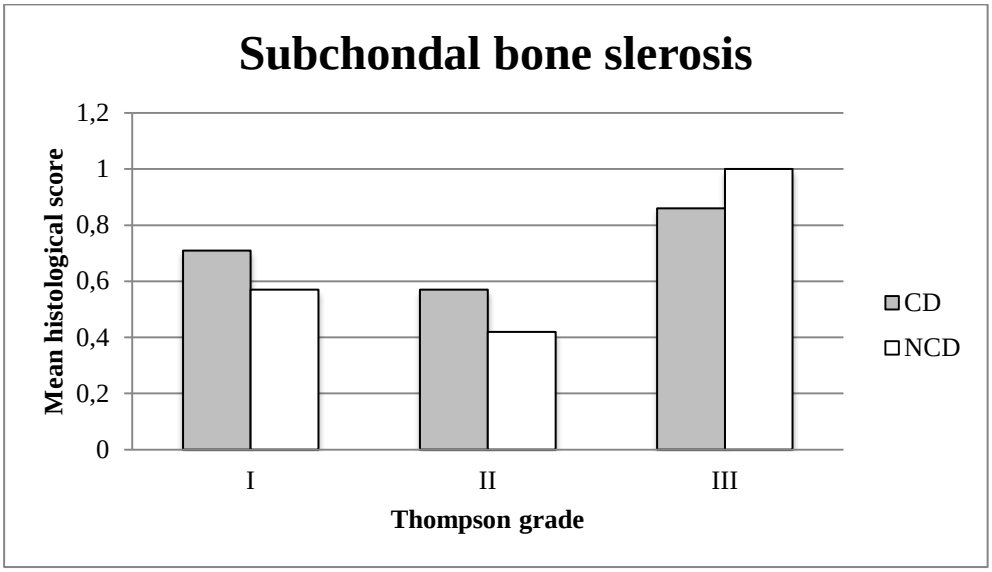

Figure 11. *Histological scores for the parameter "subchondral bone sclerosis" in Thompson grade I, II and III. CD=chondrodystrophic, NCD=non-chondrodystrophic.*