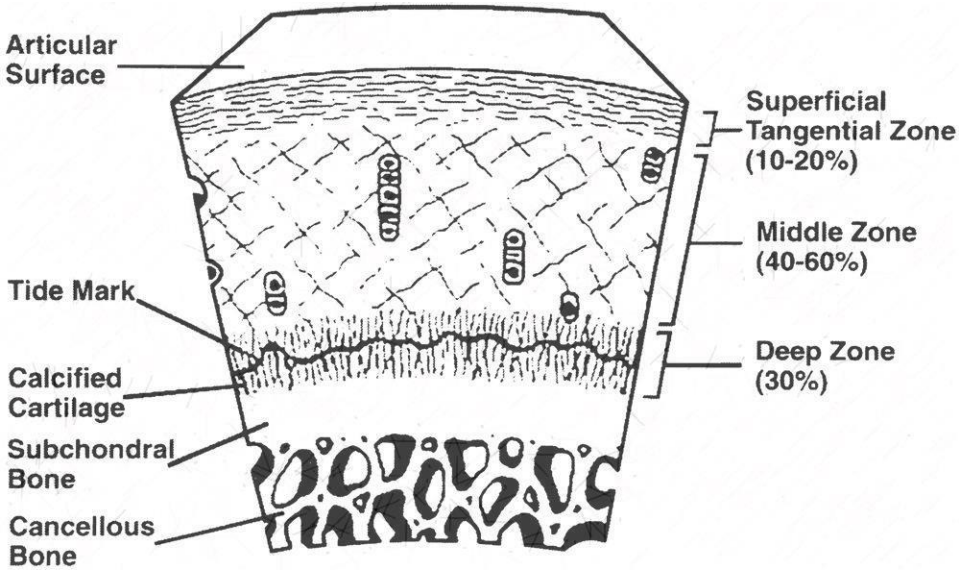
Comparison of cartilage between CD and NCD breeds by researching the Wnt/β-catenin pathway



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Abstract

Osteoarthritis (OA), or degenerative joint disease, is a condition of joints and includes degeneration of articular cartilage, degeneration of extracellular matrix and osteophyte formation. It affects approximately 20% of the dogs over 1 year of age. Despite this prevalence, only symptomatic and no curative therapies are available. It is suggested that activation of the Wnt/β-catenin pathway plays a role in the development of OA. Activation of this pathway shifts the differentiation of mesenchymal progenitor cells towards osteoblasts which can lead to osteophyte formation. Increased Wnt/β-catenin pathway activity also results in an increase of MMP13 gene expression, which accelerates breakdown of ECM and degeneration of articular cartilage. These are both important features of OA. On the other hand, inhibition of the Wnt/β-catenin pathway leads to differentiation into chondrocytes which blocks cartilage degeneration and prevents osteophyte formation. Contradictory, an inhibition of the Wnt/β-catenin pathway could also induce OA by apoptosis of articular chondrocytes and cartilage destruction. The aim of this study is to determine the differences between chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds in healthy cartilage by researching the Wnt/β-catenin pathway.

Quantitative reverse transcriptase-PCR (RT-qPCR) was performed with cDNA obtained from healthy cartilage of CD and NCD breeds. Gene expression of DKK3, Cav1, CCND1 and Axin2 were measured and compared. Additionally, immunohistochemistry was performed in order to measure the amount and location of β-catenin-positive cells in healthy cartilage of both CD and NCD breeds.

All measured genes were expressed in higher levels in healthy cartilage of CD breeds compared to healthy cartilage of NCD breeds. The higher gene expression of Axin2 and CCND1 suggest an active Wnt/β-catenin pathway in healthy cartilage of CD breeds because both genes are target genes of this pathway. This is substantiated by the upregulation of Cav1 because of its stimulating effects on the Wnt/β-catenin pathway. Upregulation of DKK3 in combination with our results suggest that it is an activator of the Wnt/β-catenin pathway in articular cartilage.

In the β-catenin staining, a trend of more positive cells in CD cartilage compared to NCD cartilage was seen, but no statistical significant differences were found between CD and NCD breeds.

Basal gene expression of the measured genes is higher in healthy cartilage of CD breeds than in healthy cartilage of NCD breeds. This suggests that the Wnt/β-catenin pathway might be more active in CD breeds, but without the development of OA. Thus NCD breeds might be more susceptible for the development of OA because an increased activity of the Wnt/β-catenin pathway could result in a shift in the differentiation of mesenchymal progenitor cells towards osteoblasts. Furthermore, the increased activity of the Wnt/β-catenin pathway could lead to an increased MMP13 gene expression which results in accelerated extracellular matrix degeneration.

Basal activity of the Wnt/β-catenin pathway might differ between the healthy cartilage of CD and NCD breeds. This could have an influence on the development of cartilage diseases as OA and the effect of therapies of these diseases. Further research is needed to clarify more differences between CD and NCD breeds on the level of cartilage.

Introduction

Development of limbs and joints

The forelimb and hindlimb first appear as a bud which grows out from the ventrolateral surface of the body of the embryo. This bud is a mass of mesenchyme covered by ectoderm. Mesenchyme is the loose embryonic connective tissue and differentiates into cells that form skeletal tissues, muscles, tendons, fasciae and blood vessels. The ectodermis becomes the epidermis and its derivatives.[1,2]

The limb bud grows, and the proximal part obtains a columnar form while the free distal part expands and flattens to form a foot plate.[1,2] In the proximal part of the bud, creases are formed so segments of the limb can be recognized. An axial condensation of mesoderm produces a denser core, this is the first indication of the future limb skeleton. A definite proximodistal gradient of differentiation occurs in early stages of development.[1]

The next step in development of the limb is the local transformation of mesoderm to create cartilaginous models in the pattern of adult bones. The differentiated mesoderm remains covered by a thin layer of unmodified mesoderm, the perichondrium. Between cartilages where joints will develop, dense mesoderm remains too. These cartilage models grow by interstitial growth to maintain the general form.[1,2]

Afterwards, cartilage is replaced by bone tissue which involves two processes, shown in **Figure 1**. One process contains the bone deposition on the cartilage by the perichondrium around the middle of the shaft and the perichondrium becomes periosteum.[1,2] The other process contains degenerative changes of the cartilage of the shaft. The cells hypertrophy and occupy enlarged spaces in the matrix after which the cells die. During this, the matrix becomes saturated with calcium salts. The periosteum pushes a connective tissue sprout to invade the dead cartilage. This sprout is cellular and well vascularized, and facilitated by the spongy texture of the dead cartilage. Three groups of cells are carried inwards: chondroclasts that have the capacity to engulf and remove calcified matrix, osteoblasts that have the capacity to deposit bone on the surviving framework and lastly, marrow precursor cells.[1-4]

The centers of the epiphyses are invaded by similar sprouts from the perichondrium, these are second ossification centers.[1,2,4] This central bone formation is not preceded by bone formation at the shaft. In canines, the ossification of the epiphysis completes after birth.[1,4]

The original cartilaginous model is now only present as articular cartilage and as two growth plate cartilages. These plates are responsible for length growth of the long bones. Eventually, these plates will be replaced by bone as well. When this is completed, longitudinal growth is no longer possible.[1-4]

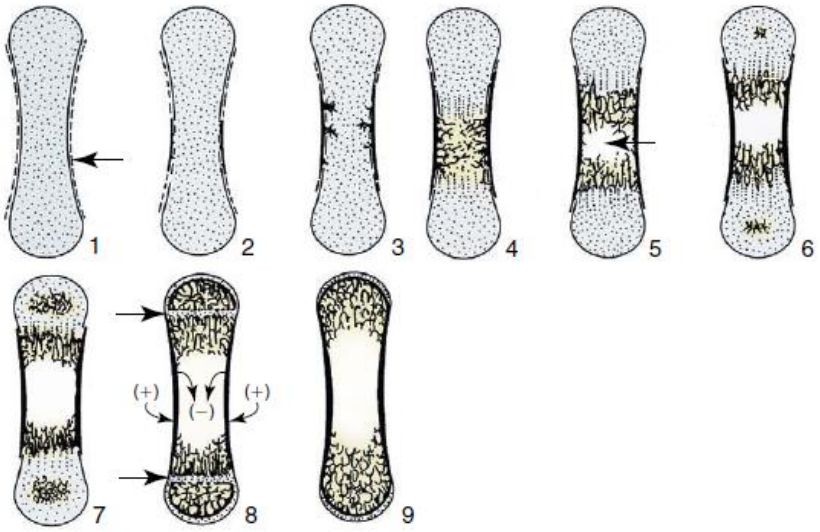

Figure 1. Development of a long bone, schematic. 1, cartilage model with perichondrium (arrow); 2, bone deposition by the perichondrium; 3, 4, connective tissue sprout replacing cartilage with bone; 5, beginning of medullary cavity (arrow); 6, 7, bone formation in extremities; 8, narrow cartilages plates (arrows) and circumferential growth by removal (-) and addition (+) of compact bone; 9, mature bone consisting of articular cartilage, spongy bone, and compact bone and the growth plate cartilages have disappeared. Reprinted from: K.M. Dyce et al., 2010.[1]

The dense mesoderm that is left between the two cartilaginous models differentiate into joint tissues, shown in **Figure 2**. A single synovial cavity is formed by apoptosis and coalescing of spaces that develop in the mesoderm. This synovial cavity is bounded by articular cartilage and synovial membrane. Articular cartilage is probably developed by delayed chondrification of the mesoderm that borders the cartilaginous models. The synovial membrane is formed by a transformation of mesoderm bordering the space. Periarticular ligaments and the fibrous part of the capsule develop from more peripheral mesoderm.[1,2]

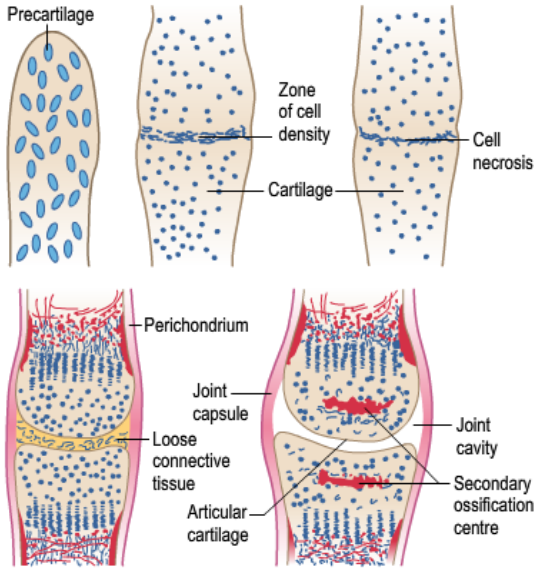

Figure 2. Formation of joints of the limbs. Reprinted from: B.M. Carlson, 2004.[5]

Joint anatomy

Synovial joints allow movement of two surfaces over each other. This is facilitated by articular cartilage and synovial fluid. A joint capsule surrounds the joint, the inner layer of this capsule is the synovial membrane which produces synovial fluid and the outer layer is a fibrous layer that extends from the periosteum and contributes to the joint stability.[3,4,6]

The forelimb contains three major joints: the shoulder joint, the elbow joint and the carpal joint. The shoulder joint (**Figure 3**) is the link between the scapula and the humerus. It enables movement in sagittal direction, but some rotation, abduction, adduction and supination are possible too. The shoulder joint does not have pericapsular ligaments, but its capsule is fused with some tendons of the surrounding muscles. Especially the tendons of both the subscapularis and the infraspinatus muscles brace the joint.[1]

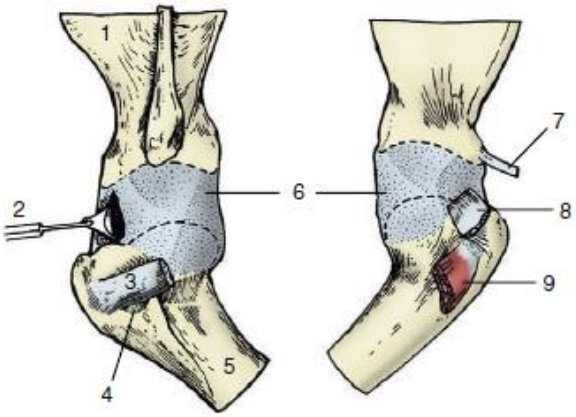

Figure 3. Anatomy of the left shoulder joint. 1, scapula; 2, joint capsule opened to expose biceps tendon; 3, tendon of infraspinatus; 4, infraspinatus bursa; 5, humerus; 6, joint capsule; 7, tendon of coracobrachialis; 8, tendon of subscapularis; 9, biceps tendon emerging from intertubercular groove. Reprinted from: K.M. Dyce et al., 2010.[1]

The elbow joint (**Figure 4**) actually consists of two kinds of joints: a hinge joint between the humerus and radius and ulna, and a pivot joint between the radius and ulna. This results in both movement in sagittal direction and some rotation.[1] The joint capsule is quite large and consist of three pouches: craniolaterally beneath the common digital extensor, craniomedally beneath the biceps and caudally between the lateral epicondyle and the olecranon. The caudal part of the joint capsule is important as a tensor and prevents it from being nipped between the bones.[1,7] The medial and lateral collateral ligaments are the strongest ligaments, but this is logical in a hinge joint. In dogs, an additional ligament is present: the annular ligament.[1] This ligament inserts on the cranial part of the medial coronoid process and completes the ring in which the head of the radius rotates.[7]

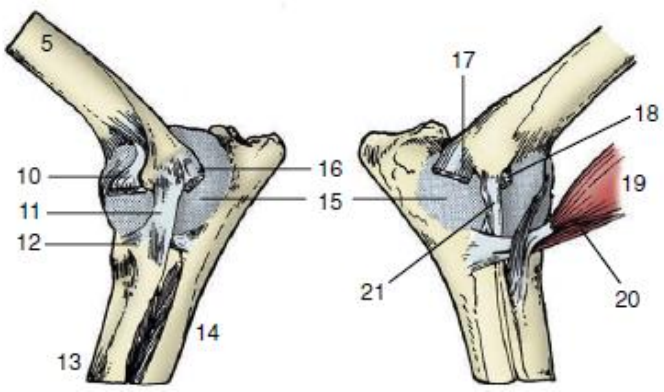

Figure 4. Anatomy of the left elbow joint. 5, humerus; 10, stump of extensor carpi radialis and tubercular groove; 11, lateral collateral ligament; 12, annular ligament of radius; 13, radius; 14, ulna; 15, joint capsule; 16, stump of ulnaris lateralis; 17, common stump of carpal and digital flexors; 18, stump of pronator teres; 19, biceps; 20, brachialis; 21, medial collateral ligament. Reprinted from: K.M. Dyce et al., 2010.[1]

The carpal joint includes a distal radioulnar joint and three other levels of articulation: antebrachiocarpal, midcarpal and carpometacarpal.[1] The antebrachiocarpal and radioulnar joint have the same joint cavity, and the midcarpal and carpometacarpal joint are interconnected. Movement in a sagittal direction is possible in both the antebrachiocarpal and midcarpal joint, but in the carpometacarpal joint no movement is allowed. A little abduction and adduction are possible because of the relative weakness of the medial and lateral collateral ligaments.[1,7]

The hindlimb contains three major joints, which is similar to the forelimb. These joints are the hip joint, the stifle joint and the tarsal joint. The hip joint is formed between the acetabulum and femoral head which are connected by the intracapsular ligament of the head of the femur. The walls of the articular cavity are formed by an articular labrum, the transverse acetabular ligament and a synovial membrane that is supported by a fibrous covering. No peripheral ligaments to limit movement are present. The hip joint is a spheroidal joint so movement in a sagittal direction, abduction and adduction are possible.[1,7]

The stifle joint (**Figure 5**) consists of one synovial cavity containing several joints: the femorotibial joint, the femoropatellar joint, the proximal tibiofibular joint, joints between the femur and paired sesamoids in the gastrocnemius muscle, and the joint between the tibia and the sesamoid in the popliteus tendon.[1,8] The femorotibial joint contain two menisci that compensate for the incongruence of the articular surfaces. Each meniscus is secured by ligaments between its caudal and cranial extremities and the central nonarticular area of the proximal extremity of the tibia. The lateral meniscus is also secured caudally to the intercondylar fossa of the femur.[1] An additional ligament, the intermeniscal ligament, secures both menisci to each other.[9]

Four ligaments extend between the lower leg and the femur: the medial collateral ligament which is attached to the medial femoral epicondyle and the proximal part of the tibia, the lateral collateral ligament which is attached to the lateral femoral epicondyle and the head of the fibula, and two cruciate ligaments that run centrally.[1]

The femoropatellar joint links the femoral trochlea and the patella. This joint is extended by the parapatellar cartilages. Three ligaments support this joint: two collateral femoropatellar ligaments that are attached to the cartilages and the femur, and one ligament runs between

the patella and the tibial tuberosity. The latter ligament represents the insertion tendon of the quadriceps femoris.[1]
The synovial membrane attaches to the peripheries of the articular surfaces and the menisci, and it covers the cruciate ligaments. This membrane forms a partition between the femorotibial joints. The femoropatellar part of the synovial cavity is extended proximally between the femur and the quadriceps. This part of the synovial cavity communicates with the two femorotibial compartments. The lesser joints, fibula and sesamoid bones are embraced by diverticula of the synovial membrane, these diverticula also extend along the tendons of the long digital flexor and popliteus muscle.[1]
Although the anatomy of the stifle joint is very complex, its movement is not. The joint acts like a hinge joint, so only movement in sagittal direction is possible. The stability of the articulation depends much on the both cruciate ligaments.[1]

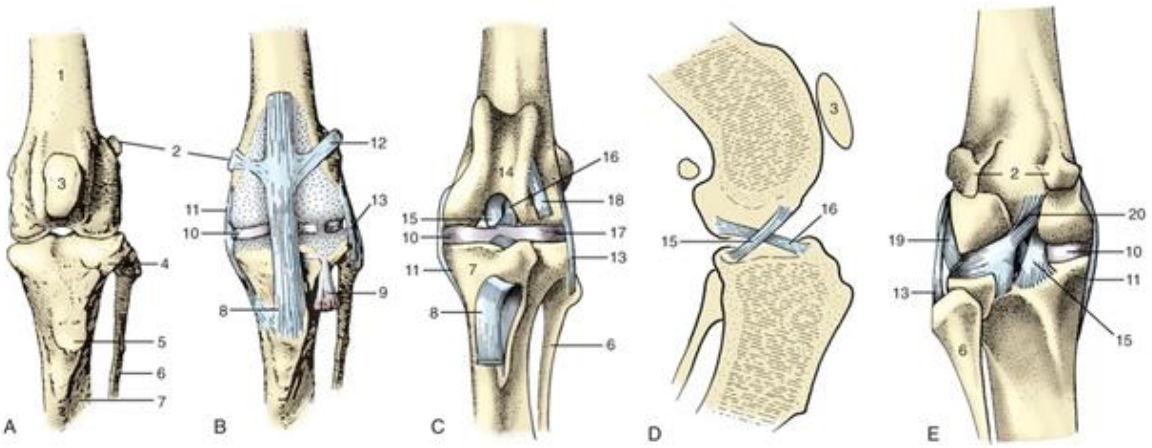

Figure 5. Anatomy of the left stifle joint. Cranial view (A-C), the extent of the joint capsule is shown in B, the patella has been removed in C, D shows the direction of the cruciate ligaments and E is a caudal view. 1, femur; 2, sesamoids in gastrocnemius; 3, patella; 4, extensor groove; 5, tibial tuberosity; 6, fibula; 7, tibia; 8, patellar ligament; 9, tendon of long digital extensor; 10, medial meniscus; 11, medial collateral ligament; 12, lateral femoropatellar ligament; 13, lateral collateral ligament; 14, trochlea; 15, caudal cruciate ligament; 16, cranial cruciate ligament; 17, lateral meniscus; 18, stump of 9; 19, popliteus tendon; 20, meniscofemoral ligament. Reprinted from: K.M. Dyce et al., 2010.[1]

The tarsal joint contains four levels of articulation, but most movement occurs at the crurotarsal level. A little flexion is also possible in the talocentral joint at its curved surfaces. The tarsal joint is also a hinge joint, so movement is possible in sagittal direction. But during movement in forward direction on flexion, the foot is deviated laterally. A lot of ligaments are present in the tarsal joint, the most important ligaments are the medial and lateral collateral ligaments. These run between the tibia (and fibula) and the proximal extremity of the metatarsus. The other ligaments are smaller and their function is to hold the tarsal bones together.[1]

Cartilage

Cartilage is tissue with a low turnover rate, thus damaged or old cells are slowly replaced with new mature cells. Because of this, cartilage does not or hardly repair or regenerate.[10] This low turnover rate is partly caused by the lack of vascular supply in cartilage so progenitor cells cannot reach the site of defect.[6,10]

In synovial joints hyaline cartilage is present (**Figure 6** (left)), this consists of chondrocytes (10%) embedded in in a matrix (90%) which is produced by themselves.[4,6,11,12] This matrix consists of several structural proteins, collagen type II is the main protein and forms a framework that receives stabilization from other collagen types and other proteins, shown in **Figure 6** (right). Because of these proteins, cartilage has tensile strength.[4,6,13,14] Compressive resistance of cartilage is provided by aggrecans, which consist of proteoglycans (PG) forming complexes with hyaluronic acid. These complexes are embedded in the framework of collagen and attract water into the cartilage.[4,6,11,13-15]

Between the calcified and uncalcified layers of the deep zone, a tidemark is present. This is a thin layer of clusters of minerals, glycoproteins and lipids. Multiple tidemarks might be present in older animals and indicate that mineralization of the cartilage was interrupted and resumed several times.[4]

Chondrocytes react to changes in its chemical and mechanical environment by regulating the architecture and biochemical composition of the cartilage strictly.[13] But this capacity is limited.[12]

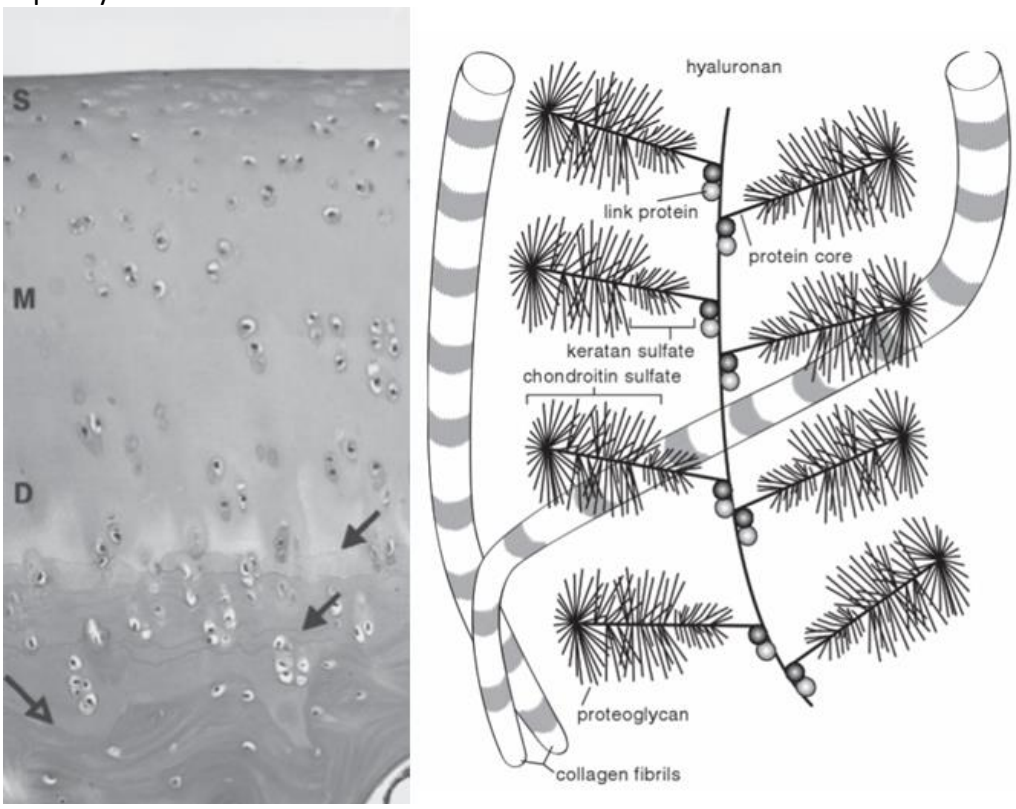

Figure 6. Left: articular cartilage zones (x290): S, superficial zone; M, middle zone; D, deep zone. Multiple tidemarks are present in the calcified cartilage region (closed arrows). The open arrow indicates the junction of articular cartilage with subchondral bone. Right: schematic view of cartilage matrix containing collagen fibrils and aggrecans, which are composed of proteoglycans bound to a hyaluronic acid chain by link proteins. Reprinted from: J.A. Eurell et al., 2006.[4]

Osteoarthritis

Pathophysiology of osteoarthritis

Osteoarthritis (OA) is also called degenerative joint disease, which is a condition of movable joints and featured by degeneration of articular cartilage. Osteophyte formation at joint

surfaces is often associated with OA and synovial inflammation could also be present.[6,12,15-18] This is the most frequently diagnosed arthropathy and affects approximately 20% of the dogs over 1 year of age.[6]

Microscopic damage can be seen before macroscopic damage: cell necrosis, degradation of intercellular matrix and exposure of superficial fibers occur microscopically, even when the surface is macroscopically intact. At first, chondrocyte necrosis is seen near the fibrillated cartilage surface and chondrocyte clusters are formed.[19] In more advanced stages, the normal cartilage structure is disturbed and the tidemark is doubled or disrupted, shown in **Figure 7**.[12,19]

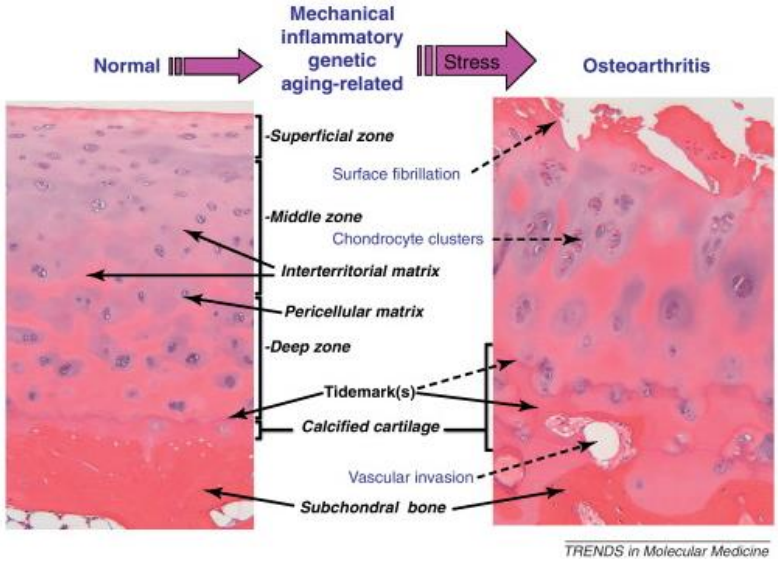

Figure 7. Major phenotypic changes by which normal cartilage tissue becomes compromised in OA. Phenotypic characteristics of OA tissue include increased stress and inflammatory signaling, proliferation of chondrocytes resulting in chondrocyte clusters, and degradation of the extracellular matrix (ECM). Reprinted from: Mary B. Goldring et al., 2012.[14]

Softening of cartilage surface is one of the first defects in OA that is macroscopically notable. If OA proceeds, a focal disruption and fissures will develop in the cartilage. This leads to fibrillation of the surface. Fissures penetrating the subchondral bone make the surface rougher and will result in detachment of the cartilage.[19]

Subchondral bone reacts to cartilage damage by forming osteosclerosis and osteophytes covered by fibrocartilage.[19,20] Osteophytes are associated with proliferation of periosteal cells at the joint margin. These cells create an osteophyte and enlarge it by differentiating into chondrocytes followed by hypertrophy and the process of endochondral ossification.[20]

Pathogenesis of subchondral cysts is not clear, but two main theories have been postulated on the pathogenesis.[21] The first theory was formulated by Freund[22], it suggested that the cysts were secondary to pathology of synovium extending into bone. This was based on the similarity of synovial fluid to the cystic fluid, abnormal cartilage over the cyst and displaced pieces of surface cartilage within the cyst. This theory is also supported by the communication between the cysts and synovial fluid, but this is not always present.[21-23] Rhaney and Lamb[23] suggested a second theory that could explain the absence of communication between subchondral cysts and the joint cavity. This theory suggests that synovial fluid intrudes the bone while this bone attempts to heal by osteoclastic resorption

of necrotic bone. The bone necrosis and microfractures are due to the impact between opposing surfaces of bone without its protective cartilage. This theory is supported by presence of osteoclasts in the disrupted bone and metaplastic cartilage.[21,23]

The bone necrosis and microfractures also cause an inflammatory response of the synovium. This inflammation will induce further cartilage damage by catabolic enzymes produced by chondrocytes which results in loss of cartilage PG and hyaluronic acid.[11,15,18,19] Breakdown of existing collagen and PG, and inhibition of chondrocyte synthesis of matrix substances are supported by inflammatory substances and influx of leukocytes.[11,24] Matrix metalloproteinase (MMP) 13 is involved in the degradation of cartilage.[25] It degrades both collagen type II and proteoglycan which are present in high amounts in normal cartilage.[26] MMP13 is critical for OA progression because inhibition of MMP13 decelerates OA progression and global knockout of MMP13 could prevent cartilage erosion in mice.[25,27]

Other biochemical changes that occur during OA are: increase of water content, production of other types of collagen by chondrocytes (collagen type I, III and X), and because PG are lost near the articular surface, the PG synthesis increases.[19,28]
Eventually, catabolic processes excel the anabolic processes and the cartilage regeneration becomes inefficient.[11,24]

Symptoms and diagnosis of osteoarthritis

The main symptom of OA in dogs is lameness, this can either be acute after exercise or more gradually.[15,16,18] This lameness is worsened by rest, cold damp conditions, obesity and prolonged exercise, but it usually reduces during a few minutes of activity.[16]

The diagnosis of OA can be clinically made based on the symptoms, but these develop when the disease is advanced and irreversible. Reduced range of motion, crepitation during motion and joint instability can be found in physical examination. Palpation of the joint may result in a visible pain reaction.[18]
The diagnosis can also be based on radiography, taking narrowing of the joint space width, joint effusion, osteophyte formation, increase of periarticular soft tissue prominence and development of subchondral osteosclerosis and subchondral bone cysts into account.[18,29,30]
It is essential to standardize the imaging method, because joint positioning has a great influence on the joint space width.[31]
MRI is another way of diagnostic imaging. This technique reveals bone abnormalities that are associated with OA.[32]
CT scanning could detect alterations in soft tissue and bone which are not detected by radiography. This is caused by the higher contrast resolution, cross-sectional display and the ability to measure specific attenuation values in CT scanning. But in veterinary medicine, this imaging method is not widely used in diagnosing OA.[33]
Ultrasonography can directly image cartilage and synovium, but this technique cannot access all joint regions because of the impermeability of bone to sound waves.[34]
Arthrocentesis could be helpful in the diagnosis of OA. Normally, synovial fluid is viscous and clear but during OA this fluid is less viscous and more cells are counted in the fluid.[29]
Another method is arthroscopy, it provides a direct view of the articular surface and makes it possible to assess injury to non-calcified structures in the stifle joint.[30] Because of the direct view, early OA can be detected and cartilage damage and synovial proliferation can be seen. A great disadvantage is its invasiveness.[18,30]

Therapy of osteoarthritis

In the medical approach of OA, alleviation and prevention of the symptoms is the main goal.[13] Obesity increases mechanical stress placed on joints so it is important to control obesity and weight loss will even improve the symptoms of OA.[6,13,18,35,36]

Swimming or walking as low-impact exercise is important in the treatment of OA because it could strengthen the joint supporting structures, improve the well-being of the patient and stimulate endorphin-release.[18,35] This release is beneficial because of both its anti-inflammatory effect and its actions as a pain neuromodulator.[37,38] The amount of daily exercise must be individualised for each patient and needs to be adjusted to symptoms of inflammation and pain.[35] Usually, exercise is started with multiple short walks on a leash and then gradually increased until clinical signs are present.[39] As OA worsens, exercise should be reduced because of the cartilage degeneration.[36]

Steroidal or nonsteroidal anti-inflammatory drugs ((N)SAIDs) are frequently used in therapy of OA. However, these only control the inflammation and signs of pain but do not tackle the pathophysiological process.[6,11,16,18,19,28,36] NSAIDs inhibit the cyclo-oxygenase (COX) which convert arachidonic acid into inflammatory substances.[36,40] Preferably, selective COX II-blockers are used, because COX I produces cytoprotective PG.[28,41] Because of its gastrointestinal side effects like ulcerations, NSAIDs should only be used as long as OA induced lameness is present. When OA signs are well controlled, administration of NSAIDs should be stopped. Examples of NSAIDs are: aspirin, phenylbutazone, carprofen, piroxicam, meloxicam, ibuprofen and tenidap.[6,18,28]

SAIDs play a minor role in the treatment of OA. Glucocorticoids induce the production of lipocortin which has an anti-inflammatory effect by inhibiting phospholipase A_2.[6,42,43] Corticosteroids inhibit chondrocyte metabolism and alter cartilage matrix, this results in decreased synthesis of proteoglycan and collagen.[6,18] These medicines are related with many side effects and therefore its use should be avoided.[6,18,36] Besides systemic administration of SAIDS, intra-articular administration is also possible. Multiple injections at short intervals results in a high intra-articular concentration of SAIDs which could have a catabolic effect, while lower sustained concentration of SAIDs intra-articular could have a protective effect.[44] Intra-articular administration of SAIDs is, just like systemic administration, associated with many side effects. These include post-injection pain and swelling which are both self-limited, but also more severe side effects were observed.[44-46] In general, SAIDs only play a role when other therapies have failed, but it should only be used shortly and discontinued if possible.[28]

In contrast to the medicines mentioned above, polysulphated glycosaminoglycans and related agents may treat the pathophysiological process.[6,47] These substances have three major mechanisms of action. First, chondrocytes are stimulated to produce more proteoglycans and cartilage matrix synthesis is stimulated too. More hyaluronic acid is produced by synovial fibroblasts, this increases viscosity of synovial fluid and improves joint lubrication.[48] Second, inhibition of MMPs, complement, hyaluronidase and other harmful enzymes released from leukocytes. As a result of this inhibition, cartilage degeneration is prevented.[48] Third, these agents have antithrombotic, fibrinolytic and anticytokine effects so blood flow and perfusion of both joint tissues and subchondral bone is increased.[48] The one important adverse effect of polysulphated glycosaminoglycans and related agents is their heparin-like action.[6,47]

Some alternative therapies of OA have been reported.[28] Omega-3 and omega-6 fatty acids could be used, because its derivatives are less pro-inflammatory than other lipid derivatives.[6,49-51] Green-lipped mussel extract could be used in the management of OA

because of its ant-inflammatory effects and it contains glycosaminoglycans.[52,53] Doxycycline could be beneficial because it inhibits MMP activity by chelating divalent cations.[28,54] Acupuncture therapy is also used in the management of OA.[55,56]

Surgical possibilities in dogs include arthrodesis, arthroplasty, prosthetic joint replacement and arthroscopy.[18,57,58] Arthrodesis causes an irreversible osseous fusion and unloads the contralateral joint, so it benefits the overall function. In the affected limb, acceptable limb function is established.[18,59] It is important to create the right angle during the fusion, otherwise the limb fails to bear weight and protraction is complicated.[58,59] Arthrodesis is associated with a high complication rate, for example infection, failure of fusion, implant failure, incorrect arthrodesis angle, lameness, OA in other joints and fracture.[59-62]

During arthroplasty, the joint is reshaped and its function is restored. Different arthroplasty techniques have been described in veterinary patients, for example femoral head and neck ostectomy and glenoid cavity resection.[18,58] In the stifle joint, cartilage flaps that might encroach degenerated menisci are removed by curettage because this could cause further cartilage degeneration.[6,19,63] Vascular ingrowth from the subchondral bone can be stimulated by drilling of the subchondral bone plate. This is a prerequisite for the regenerative process.[64-66] The joint is flushed repeatedly before closure to remove small fragments.[63] Pseudoarthrosis is formed and acceptable joint function might be established by continuous passive motion in the immediate postoperative care.[18,58,64,67,68]

Joint replacement is possible for the elbow, hip and stifle joint, but the results vary tremendously.[18] For total hip replacement, cemented and cementless implants are available. The cementless implants are preferred because they are much less inclined to loosen with time. But before total hip replacement is performed, obese patients should lose weight because this can delay or eliminate the need of replacement and it decreases the risks of complications.[69]

Arthroscopy is used in both diagnostic evaluation and in therapy of OA. Several procedures can be performed during arthroscopy: debridement, lavage of the stifle joint and partial synovectomy when synovial hypertrophy is present. Arthroscopy causes limited trauma so the worsening of inflammation and degenerative changes is minimized.[58]

A new promising chirurgical therapy of OA in humans is joint distraction.[70] This could delay the need for an arthrodesis procedure.[57,71] During joint distraction cartilage repairs, the joint space width becomes bigger, inflammation reduces and PG turnover changes. The positive clinical effects of this method have not been explained yet, but it has been hypothesized that it depends on the absence of mechanical stress in combination with intermittent fluid pressure and flow.[57,70]

Osteoarthritis in experimental animals

Induction of osteoarthritis

Several animal models in different species are used to investigate OA. These models can be divided in spontaneous and induced OA. Spontaneous OA can be subdivided in naturally occurring and genetic models. Induced OA can be subdivided in chemical and surgical models. In chemical models, modifying factors are injected intra-articular or noxious agents are administered systemically. Using surgical models is most common, these procedures either alter the stress on the joint or alter the load bearing so joint instability is created.[72]

Mostly, the stifle joint is used for studying OA. The following models are used to induce OA in dogs surgically in this joint: partial or total meniscectomy, transection of

anterior or posterior cruciate ligament (ACLT or PCLT), articular groove, transarticular impact, abrasion and valgus osteotomy.[8,72]

Partial and total meniscectomy

In partial and total meniscectomy in dogs, the medial part of the meniscus is transected because dogs usually load the medial aspect of their stifle. That is why dogs develop more severe lesions with medial partial meniscectomy than with lateral partial meniscectomy.[72] In order to develop moderate osteoarthritic changes, these studies should last 1-3 months or more.[73] In both the procedures of partial and total meniscectomy, a transverse incision is made parallel to the medial joint line. In line with the skin, the capsule and synovium are incised too.[74] The medial collateral ligament could be incised in line with the skin[74], however, a more recent study reported that the collateral ligament is not damaged during this procedure.[72] The joint is incised above the menisci in order to prevent cartilage and meniscus damage. With this incision, the medial joint structures are well visualized. In case of total meniscectomy, the meniscus is excised at its periphery. When partial meniscectomy is performed, the peripheral rim is left intact and one- to two-third of the width of the medial meniscus is excised. Articular cartilage is not damaged in both procedures.[74]

Anterior cruciate ligament transection

The ACLT model is most frequently used to induce OA and is performed either by incision, blind lance or arthroscopically.[72] During the ACLT procedure by incision, a small incision of less than 2 cm is made close to the ligamentum patellae so the cruciate ligament is visible. Without damaging other joint structures, the cruciate ligament is lifted and transected.[70] The completeness of ACLT can be verified by a positive anterior drawer test.[70,72] As a result of this procedure, permanent joint instability is established which triggers the development of OA.[75]

Canine articular groove model

The canine groove model contains damaging the medial and lateral femoral condyles. The stifle joint is approached through a 3 cm incision medial from the patella to visualize the femoral condyles.[76] A 1.5 mm diameter Kirschner-wire is used to make grooves into the cartilage, this wire is bent 90° at 0.5 mm from the tip so the grooves do not go deeper than 0.5 mm. Ten longitudinal and diagonal grooves are made on weight-bearing parts of the condyles and the subchondral bone is not touched.[75,77,78] By this procedure, no joint instability is caused and the damage to articular cartilage of the femoral condyles triggers the development of OA.[77,79,80] Advantages of this procedure are the restricted inflammation that is caused and no lasting trigger for OA development.[79]

Transarticular impact model

The transarticular impact model is originally used in the automotive industry to investigate mechanisms and effects of 'dashboard injuries'.[81-83] Nowadays, this model is often used to induce damage to the subchondral bone without initial cartilage injury.[84]. It results in subchondral bone injury, edema, microfractures and bleeding. This damage only affects the cartilage when forces are high enough.[72,85] By using this model, the role of subchondral bone damage in OA pathogenesis can be investigated.[84,85] The dog is laid in lateral recumbency. The hip is abducted and 90° flexed and the tibia was held in 100° of flexion in the stifle joint. The thigh was positioned in a frame to secure the lower extremity rigidly. After the right position of the dog is accomplished, weight is dropped onto the patella to create the transarticular force. This force is transduced to the lateral part of the trochlea femoris. A

drop-tower construction is used with a drop weight of 2.1 kg and a force-transmitting rod-tip with a diameter that is adapted to the patella of the dog (1.9 cm), a load cell (KistlerR Swiss Type 5001) and a force tranducer (KistlerR Quarz). An oscilloscope is used to record the transmitted forces (0.5 ms/div. and 0.5 V/DN (1V/1000 N)). To verify if the impact is perpendicular, radiographs are taken in sagittal and axial direction in pre-impact state.[84,85]

Abrasion

Abrasion involves the removal of superficial areas of bone from the articular joint until the bleeding cortical bone is present. An ulcer of 5 mm^2 is made to expose the underlying bone and surface vessels are exposed by trimming 1 mm of the surface bone without expressing red bone. Blood is observed and streams from scattered areas of the abraded surface. Formation of new cartilage is stimulated by the exposure of cancellous subchondral bone.[86]

Valgus osteotomy

During valgus osteotomy, a skin incision is made from above the tibial tuberosity and it extends downward along the tibial shaft. By this incision, the upper 3/4 on the medial site and the upper 1/3 on the lateral side are exposed. A special saw guide is designed and used to achieve an exact osteotomy angle of 30°. This is performed 10-20 mm distal to the growth plate and in direction of the tibial plateau. Another skin incision is made to excise 5 mm of the fibula at its upper 1/3. In order to fix the osteotomy, an oblique T shaped AO plate with five holes is used. This plate is bended to the right angle before the operation and the proximal horn of the plate is cut shorter to fit the shape of the bone. The T shaped plate is fixed with screws to the medial aspect of the tibia.[87]

Evaluation of osteoarthritis

In the past, the Mankin or modified Mankin scoring systems were used to determine the presence or absence of OA and to estimate the extent and severity.

The Mankin scoring system combines scores assessing structure (max 6 points), cellular abnormalities (max 3 points), matrix staining (max 4 points) and tight mark integrity (max 1 point). A total score of 0 points implies normal cartilage and a total score of 14 points implies the most severe cartilage lesions. The specific scores are shown in **Table 1**. In order to score the tissue, safranin O fast green staining is used.[19]

Benchmark	Score
Cartilage structure	
- Normal	0
- Surface irregularities	1
- Pannus and surface irregularities	2
- Clefts to transitional zone	3
- Clefts to radial zone	4
- Clefts to calcified zone	5
- Complete disorganisation	6
Cartilage cells	
- Normal	0
- Pyknosis, lipid degeneration, hypercellularity	1
- Clusters	2
- Hypocellularity	3

Matrix staining	
- Normal	0
- Slight reduction	1
- Moderate reduction	2
- Severe reduction	3
- No staining	4
Tide mark integrity	
- Intact	0
- Destruction	1

Table 1. Mankin scoring system for OA.[19]

The minimum score in the modified Mankin score is 0 points, which is similar to the minimum score in the Mankin scoring system, but the maximum score is different: 13 and 14 points respectively (**Table 2**). This is not the only difference between these scoring systems. General remarks are given in the modified Mankin score: cartilage at the edge of a section is not representative because of processing artefacts, incidental cartilage changes are irrelevant, and disorganization should be present in at least 25% of the section.[19]

Benchmark	Score
Cartilage structure	
- Normal	0
- Irregular surface including fissures to the radial layer	1
- Pannus	2
- Superficial cartilage layers absent (at least 6)	3
- Slight disorganisation (cellular rows absent, some small superficial clusters)	4
- Fissures into calcified layer	5
- Disorganisation (chaotic structure, clusters and osteoclast activity)	6
Cartilage cells	
- Normal	0
- Hypercellularity, including small superficial clusters	1
- Clusters	2
- Hypocellularity	3
Matrix staining	
- Normal/slight reduction	0
- Staining reduced in radial layer	1
- Reduced in inter-territorial matrix	2
- Only present in pericellular matrix	3
- Absent	4

Table 2. Modified Mankin scoring system for OA.[19]

In the (modified) Mankin scoring system the structural, cellular and staining abnormalities of OA cartilage are assessed, but this methods have several limitations.[8,19] These include: only assessing the pathology of the articular cartilage and not the pathology of other tissues as synovium, no global assessment of the joint, no weighting of the scores along the importance in OA, no standardized methodology for collecting samples, and no statistical analysation for validation to clinical or functional outcome.[8]

This led to the development of another method, the OARSI scoring system. This scoring system is standardized, repeatable and comparable among studies, and it includes several instructions for containing and scoring the macroscopic and microscopic samples. This scoring system assesses the pathology of articular cartilage, menisci and synovium. Each

kind of tissue has a different grading or scoring table and the scores are weighted along their importance in OA.[8]

The macroscopic cartilage score (**Table 3**) should be assigned to all the weight bearing surfaces: medial femoral condyle, lateral femoral condyle, medial tibial plateau, lateral tibial plateau. For both the macroscopic cartilage score and the macroscopic synovium score, multiple independent observers score the tissue. This means that high-resolution photographs of the cartilage are necessary.[8]

Benchmark	Score
Macroscopic scoring of cartilage	
- Smooth surface	0
- Slightly fibrillated/roughened surface	1
- Fibrillated surface with focal partial thickness lesions	2
- Deep lesions with surrounding damage	3
- Large areas of severe damage	4
Macroscopic scoring of synovium	
- Normal – opal white, semitranslucent, smooth, with sparse well defined blood vessels	0
- Slight – focal involvement, slight discoloration, visible proliferation/fimbriation/thickening, notable increase in vascularity	1
- Mild – diffuse involvement, slight discoloration, visible proliferation/fimbriation/thickening, notable increase in vascularity	2
- Moderate – diffuse involvement, severe discoloration, consistent notable proliferation/fimbriation/thickening, moderate vascularity	3
- Marked – diffuse involvement, severe discoloration, consistent and marked proliferation/fimbriation/thickening, diffuse hypervascularity	4
- Severe – diffuse involvement, severe discoloration, consistent and severe proliferation/fimbriation/thickening, thickening to the point of fibrosis, severe hypervascularity	5

Table 3. Macroscopic scoring of cartilage and synovium in the OARSI scoring system.[8]

A minimum of two blinded scorers with independent evaluations are needed to asses microscopic scores of the cartilage (**Table 4**), they should evaluate three or four sections at least. The cartilage tissue should be stained with Hematoxylin and eosin to criticize the general architecture and cell features and a Toluidine blue or Safranin O fast green staining is needed to stain the proteoglycans. In order to visualize the collagen, a Picrosirius red with or without polarized light microscopy is recommended. To asses microscopic score of synovium (**Table 4**), at least three sections from medial, axial and lateral compartments of each joint should be scored by at least two blinded observers. These sections should be stained with Hematoxylin and eosin. Each section should be evaluated for each category. When a section includes local or multi-focal areas of pathology, the scores should be added to derive a total score for that section.[8]

Tissue and benchmarks	Area of None	Section Local (1/3)	affected Multi-focal (2/3)	Global (>2/3)
Cartilage structure				
A. Normal volume, smooth surface with all zones intact	0	0	0	0
B. Surface undulations including fissures in surface/upper zone and/or pannus tissue formation on surface	0	1	2	3
C. Fissures to mid zone and/or erosion of surface/upper zone	0	2	4	6
D. Fissures that extend to deep zone and/or erosion through mid zone	0	3	6	9
E. Full thickness loss of cartilage	0	4	8	12
Chondrocyte pathology				
A. Normal	0	0	0	0
B. Loss of cells in the surface zone or relative increased density with occasional superficial clusters	0	1	2	3
C. Small cell clusters (2-4 cells/cluster) predominate	0	2	4	6
D. Large cell clusters (≥5 cells/cluster) predominate	0	3	6	9
E. Cell loss (necrosis/apoptosis) predominates	0	4	8	12
Proteoglycan staining				
A. Normal	0	0	0	0
B. Decreased proteoglycan content in surface/upper zone	0	1	2	3
C. Decreased proteoglycan content into the mid zone	0	2	4	6
D. Decreased proteoglycan content into the deep zone	0	3	6	9
E. Full depth decrease in proteoglycan content	0	4	8	12
Collagen integrity				
A. Normal	0	0	0	0
B. Loss of integrity of surface/upper zone	0	1	2	3
C. Loss of integrity of surface/upper and mid zones	0	2	4	6
D. Loss of integrity of surface/upper, mid, and deep zones	0	3	6	9
Tidemark				
A. Intact and distinct	0	0	0	0
B. Not consistent or distinct (loss and/or duplication)	0	1	2	3
C. Loss of tidemark which is crossed by blood vessels	0	2	4	6
Subchondral bone plate				
A. Intact with normal thickness (<300 microns)	0	0	0	0
B. Mild increase in thickness (>300-<450 µm)				
C. Moderate increase in thickness (450-<750 µm)	0	1	2	3
D. Marked increase in thickness (>750 µm), subchondral pseudocysts, and/or marrow fibrosis	0 0	2 3	4 6	6 9

Synovial changes				
Lining cell characteristics				
A. 1-2 layers of cells	0	0	0	0
B. 3-6 layers of cells	0	1	2	3
C. >6 layers of cells	0	2	4	6
Lining characteristics				
A. No villous hyperplasia	0	0	0	0
B. Short villi	0	1	2	3
C. Finger-like hyperplasia	0	2	4	6
Cell infiltration characteristics				
A. No cellular infiltration	0	0	0	0
B. Mild to moderate inflammatory cell infiltrates including small lymphoid follicles	0	1	2	3
C. Marked, diffuse inflammatory cell infiltrates including large lymphoid follicles	0	2	4	6

Table 4. Microscopic scoring of cartilage, subchondral bone and synovium in the OARSI scoring system.[8]

Macroscopic scores of the menisci are assessed by scoring three zones per meniscus: anterior, middle and posterior. Per meniscus, a total score of max 12 points is derived (**Table 5**). Three sections from each zone of each meniscus are scored by at least two blinded observers. These sections are stained with the same staining as the cartilage sections. The scores from each section are added to derive the total score of each meniscus.[8]

Tissue and benchmarks	Area of Anterior 1/3	Section Middle 1/3	affected Posterior 1/3
Macroscopic scoring of meniscal pathology			
A. None	0	0	0
B. Fibrillation only	1	1	1
C. Incomplete tear or tears	2	2	2
D. Complete tear or tears	3	3	3
E. Complete disruption of structure (maceration of tissue)	4	4	4
Microscopic scoring of meniscal pathology			
Tissue Architecture – Tissue loss			
- Normal	0	0	0
- Minimal disruption	1	1	1
- Moderate disruption with loss of tissue	2	2	2
- Complete loss of tissue architecture, >50% loss	3	3	3
Cell and Matrix (PG and Collagen) Content and Morphology			
- Normal	0	0	0
- Minimal alterations in cell and matrix content and morphology	1	1	1
- Moderate alterations in cell and matrix content and morphology	2	2	2
- Severe loss/disruption of cells, PG, and collagen	3	3	3

Proliferative response			
- None	0	0	0
- Minimal proliferation of cells at synovial-meniscal junction	1	1	1
- Proliferation of cells at synovial junction and extending into tissue or along surfaces	2	2	2
- Marked proliferation of cells involving majority of remaining tissue	3	3	3

Table 5. Macroscopic and microscopic scoring of meniscal pathology in the OARSI scoring system.[8]

Chondrodystrophic and non-chondrodystrophic dog breeds

Dog breeds can be divided in chondrodystrophic (CD) and non-chondrodystrophic breeds (NCD), shown in **Figure 8**. Examples of CD breeds are: (miniature) Dachshund, Basset Hound, French and English Bulldog, Shi Tzu, miniature Schnauzer, Pekingese, Beagle, Lhasa Apso, Bichon Frisé, Tibetan Spaniel, Pembroke Welsh Corgi, and the American Cocker Spaniel.[88-97] This division is based on a different process of endochondral ossification.[98]

The process of endochondral ossification is disturbed in CD breeds.[88,91,99,100] The growth plates of long bones calcify early in development, which results in disproportionally short curved limbs.[88,91,94,100] Because of that, chondrodystrophy is also called short-limbed or disproportional dwarfism.[94] It is not only associated with short limbs, but also with premature degeneration and calcification of intervertebral discs.[99]

Figure 8. Examples of CD (Pembroke Welsh Corgi, Basset Hound, and Dachshund) and NCD breeds (Collie, Whippet, German Shepherd dog). Reprinted from: H.G. Parker et al., 2009.[94]

Two fibroblast growth factor 4 (FGF4) insertions on two different chromosomes can explain chondrodystrophy in different breeds. Two single-nucleotide polymorphisms on chromosome 18 (CFA18) are associated with chondrodystrophy in domestic dogs, these are discovered by single-marker analysis.[94] This FGF4 insertions induce atypical expression of the FGF4 transcript in chondrocytes, which leads to abnormal activation of fibroblast growth factor receptors.[94]

The CFA18 insertion only explains chondrodystrophy in breeds as Basset Hound, Pembroke Welsh Corgi and Dachshund. Chondrodystrophy in other breeds, such as the American Cocker Spaniel, Beagle, and French Bulldog is explained by a second FGF4 insertion. This is an insertion on chromosome 12 (CFA12) and is not only related with short legs but also with the predisposition to intervertebral disc disease Hansen type I. A combination of the CFA18 and the CFA12 insertion leads to an even greater decrease in height.[99]

Wnt pathway

Wnt pathways regulate chondrocyte development, and joint and digit formation.[101-104] These pathways begin with Wnt protein binding to its receptor, which is encoded by Frizzled (Fzd) genes.[105] The pathway that is described the most, is the β-catenin pathway or canonical Wnt signaling, shown in **Figure 9** (left).[106,107] This pathway influences the mesenchymal progenitor cell differentiation into osteoblasts or chondrocytes. Increased Wnt/β-catenin signaling results in differentiation into osteoblasts, this leads to osteophyte formation.[108,109] Increase of the Wnt/β-catenin pathway activity also results in increased MMP13 gene expression, which accelerates breakdown of ECM and degeneration of articular cartilage.[110-112] These are both important features of OA.[6,12,14-18] On the other hand, inhibition of the Wnt/β-catenin pathway leads to differentiation into chondrocytes which prevents osteophyte formation and blocks cartilage degradation.[102,109] Contradictory, it is reported that an inhibition of the Wnt/β-catenin signaling could also induce OA by apoptosis of articular chondrocytes and cartilage destruction.[113]

Normally, β-catenin in cytosol is phosphorylated by the degradation complex, this phosphorylated β-catenin has a short half-life. When Wnt binds to the Fzd/LRP coreceptor complex, the degradation complex disintegrates and binds to the intracellular part of the Fzd/LRP coreceptor complex. This results in an accumulation of β-catenin in the cytosol. The β-catenin translocates to the nucleus and interacts with TCF/LEF which results in transcription of target genes.[106,114,115] The degradation complex consists of glycogen synthase kinase 3β (GSK 3β), axin, the β-catenin transportation complex APC and CK-1.[115] One of the proteins induced by activation of the Wnt/β-catenin pathway is cyclin D1 (CCND1), but this protein is also induced by several other pathways.[116] Another target gene of the Wnt/β-catenin pathway is Axin2 and the produced protein is also known as conductin.[117,118] Axin2 binds to the degradation complex, promotes phosphorylation of β-catenin and thereby, it supports the degradation of β-catenin. So Axin2 is a negative regulator of the Wnt/β-catenin pathway.[117] Axin2 also increases the number of degradation complexes, this results in a higher capacity to degrade β-catenin.[118]

Another Wnt pathway is known as the noncanonical or calcium pathway, shown in **Figure 9** (right). Binding of Wnt to its receptor triggers intracellular Ca^{2+} release and activation of protein kinase C.[119,120] This pathway stimulates β-catenin degradation or prevents β-catenin from translocation to the nucleus, thus this pathway antagonizes the β-catenin pathway.[121,122]

Which pathway is activated by Wnt is regulated by competition for the Dishevelled protein, a component of both pathways.[120,121,123,124]

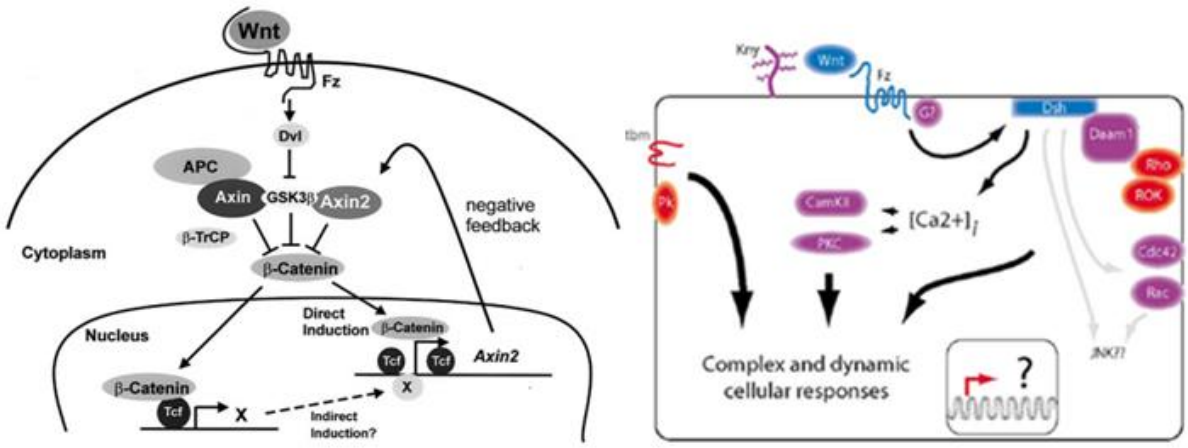

Figure 9. Left: the Canonical β-catenin Pathway and the role of Axin2. Reprinted from: Eek-hoon Jho et al., 2002.[117] Right: the vertebral Noncanonical Signaling. Reprinted from: Michael T. Veeman et al., 2003.[120]

It is suggested that an altered activity of the Wnt/β-catenin pathway plays a role in the development of OA.[125] During OA, proinflammatory stimuli are present which lead to production of proteolytic enzymes by chondrocytes.[126] In this process, the Wnt/β-catenin pathway is involved as elevated levels of β-catenin in chondrocytes within areas of degenerated cartilage have been found.[114,127] Increased levels of β-catenin might lead to higher levels of COX-2 in articular chondrocytes, but its mechanism is unknown.[127] Another indication for the involvement of the Wnt/β-catenin pathway in OA is the increased amount of Wnt-1-induced signaling protein 1 (Wisp) in synovium and cartilage in experimentally induced OA models.[128] Wisp1 is one of the target genes of the Wnt/β-catenin pathway and stimulates differentiation of mesenchymal progenitor cells into osteoblasts and it suppresses the differentiation into chondrocytes.[129] Caveolin 1 (Cav1) also plays a role in the pathogenesis of OA due to the Cav1-induced downregulation of articular chondrocytes.[130] Cav1 influences the Wnt/β-catenin pathway in several ways. Cav1 expression leads to the accumulation of β-catenin in caveolae membranes causing inhibition of both β-catenin degradation and transcription of Wnt/β-catenin target genes. But the β-catenin in the caveolae membranes can also serve as a depot in order to prevent degradation of all the β-catenin and it can be released in order to transcript the target genes.[131] Cav1 is also involved in the activation of the Wnt/β-catenin pathway in three other ways: it internalizes coreceptor LRP6[132], it inhibits the binding of β-catenin to axin which prevents β-catenin degradation by the degradation complex[132], and it activates integrin-linked kinase which inhibits GSK 3β so β-catenin is less degraded.[133]

In OA cartilage, expression of Dickkopf protein (DKK) 3 mRNA is increased and Wnt inhibitory factor (Wif) 1 expression is decreased.[134,135] Both of these mediators are extracellular Wnt antagonists, shown in **Figure 10**.[134-137] DKK3 expression is regulated by injury and inflammatory cytokines. Upregulation of this protein could be a protective mechanism to limit cartilage damage by inhibiting the Wnt/β-catenin pathway.[134] However, Nakamura et al.[138] reports that the function of DKK3 variates in different kinds of tissue and its function in cartilage is unclear. Wif1 expression is negatively correlated with the severity of OA. Reduction of Wif1 may occur early in OA and might be associated with the pathogenesis.[135]

DKK1 is an extracellular Wnt antagonist like DKK3 and Wif1, shown in **Figure 10**.[139,140] The levels of this protein are increased in OA cartilage compared to healthy cartilage[141] and

multiple studies reported that DKK1 is involved in the pathogenesis and progression of OA. However, the results are controversial.[139,141] Oh et al.[141] reported that overexpression of DKK1 in chondrocytes inhibits several components in the pathogenesis of OA. This suggests that the overexpression of DKK1 has a protective function in cartilage. Contradictory, Weng et al.[142] reported that the upregulation of DKK1 was associated with an increase in the Mankin scoring system which suggest that a decreased activity of the Wnt/β-catenin pathway can also enhance the development of OA.

Secreted, frizzled related proteins (sFRP or FRP) also have an antagonistic function, shown in **Figure 10**. These proteins have a binding site for Wnt proteins, but do not have a transmembrane region (**Figure 11**).[107,143] FRPs and Fzd compete to bind Wnt, so the Wnt pathways are partly blocked.[144] Frz-B2 is an FRP which is only found in chondrocyte clusters in OA cartilage, but not in healthy adult cartilage.[143] Another protein that has an antagonistic effect on the Wnt/β-catenin pathway is sclerostin (SOST). It inhibits this pathway through the binding to Wnt coreceptors.[145]

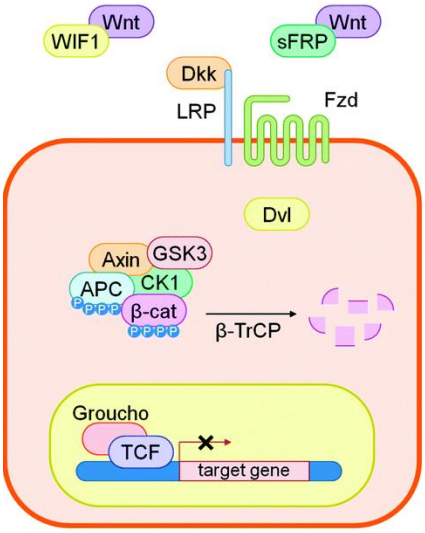

Figure 10. Influence of Wif1, sFRP and Dkk on the Wnt pathway. Reprinted from: S. Sinha, 2014.[115]

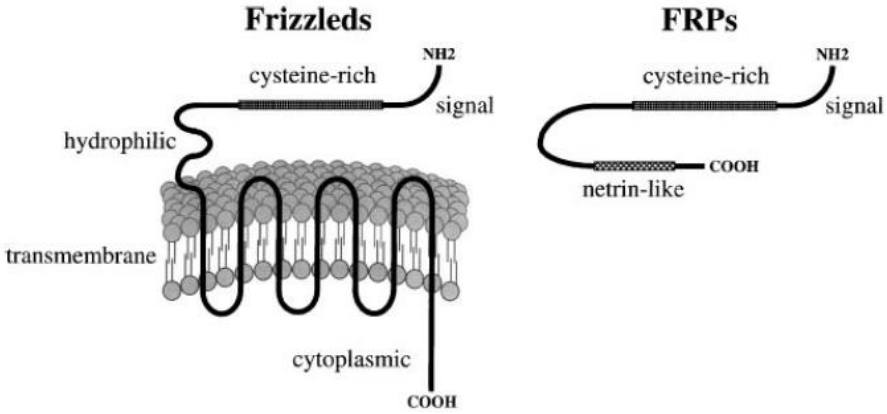

Figure 11. Structure of Frizzleds and FRPs. Both bind to Wnt protein, but FRPs do not have a transmembrane region. Reprinted from: A. Wodarz et al., 1998.[107]

Aims of the research project

Until now, no research has been done on the Wnt/β-catenin pathway in dogs on the level of cartilage. In this research, the Wnt/β-catenin pathway will be examined by quantitative reverse transcriptase-PCR (RT-qPCR) in healthy cartilage of dogs. These results will be used to determine differences in the basal activity of the Wnt/β-catenin pathway between CD and NCD breeds. Immunohistochemistry (IHC) will also be performed on cartilage samples of stifle joints of CD and NCD breeds in order to measure the amount of cells containing β-catenin and the location of β-catenin (within the cytoplasm or nucleus). Understanding the pathogenesis of OA and the differences in the development of OA between CD and NCD breeds, can be useful in the development of new therapies for OA.

An adjustment is made in this research project. In the research proposal, a comparison of OARSI scores between CD and NCD breeds was described. This comparison is not performed because the data of previous studies were not available in time. IHC is performed to replace this part of the research.

The following hypotheses have been formed:

H_0 = There is no significant difference in gene expression of the Wnt/β-catenin pathway between the CD and NCD breeds and there is no significant difference in the amount of cells containing β-catenin in cartilage of stifle joints between CD and NCD breeds found with IHC.

H_1 = There is a significant difference in gene expression of the Wnt/β-catenin pathway between the CD and NCD breeds and there is a significant difference in the amount of cells containing β-catenin in cartilage of stifle joints between CD and NCD breeds found with IHC.

Materials and methods

RT-qPCR

Tissue collection, culturing explants of canine cartilage and joint capsule tissue, RNA isolation and obtaining cDNA was performed by a predecessor.

Tissue collection

Cartilage from the stifle joint of healthy CD (n=7) and NCD (n=8) donors was collected, donor information (both RT-qPCR and IHC) is listed in **Table 6**. These dogs were euthanized in unrelated studies. From donor 401 onwards, only tissue from the femur epicondyle, optionally with the addition of tissue from the tibial plateau, were harvested separately. This distinction was made because adult articular cartilage has noticeable biochemical and biomechanical characteristics based on differences in topographical loading within the joint. Because of this, the experimental set up used tissues from the same location in a matched manner. Obtained tissue was collected in 50 mL tubes with 25 mL hgDMEM (high glucose, GlutaMAX, pyruvate (Invitrogen, 31966)) + 2% Penicillin/Streptomycin (p/s, PAA laboratories, P11-010). After washing with hgDMEM + 1% p/s, the tissue rested overnight at 37°C in a Petri dish (353803, Corning, Durham USA) with 25 mL hgDMEM + 1% p/s.

	Breed	NCD/ CD	Gender (M/F)	Weight (kg)	Age (months)	Details	Experiment
120	German Shepherd	NCD	M	26	122	Cartilage from whole joint. Clinical signs of hip OA, on section no signs of OA in hip	RT-qPCR
161		CD				Tibia/femur	IHC
181		NCD				Hip	IHC
256		CD				Tibia	IHC
257	Foxhound	NCD	F	25	29	Cartilage from whole joint. Received antibiotics systemically for infection	RT-qPCR IHC
401	Foxhound	NCD	F	23	20	Only cartilage from medial femur epicondyle or tibia plateau	RT-qPCR IHC
447	Foxhound	NCD	F	22	22	Cartilage from whole joint. Received antibiotics systemically for infection	RT-qPCR
459	Mixed Breed	NCD	F	24		Cartilage from whole joint	RT-qPCR
482	Mixed Breed	NCD	F	24		Cartilage from whole joint	RT-qPCR IHC
692	Foxhound	NCD	F	28	19	Only cartilage from medial femur epicondyle or tibia plateau	RT-qPCR IHC
721	Mixed breed	NCD	F	21	21	Tibia/femur	IHC
733		CD				Tibia/femur	IHC
804		CD					RT-qPCR
808	Beagle/ Bedlington cross	CD	F	14.9	49		RT-qPCR
809	Beagle/ Bedlington cross	CD	F	15.9	49		RT-qPCR
840	Mixed Breed	NCD	F	24			RT-qPCR
862	Beagle	CD	M	10	48	Femur, tibia in paraffin block	IHC
936	Beagle/ Bedlington cross	CD			26	Only cartilage from the femur epicondyle	RT-qPCR

3516	Mixed breed	NCD	F	22	19	Tibia, femur in paraffin block	IHC
5053	Beagle	CD	F	12.4	22	Only cartilage from medial femur epicondyle or tibia plateau	RT-qPCR IHC
5222	Beagle	CD	F	10.5	21	Only cartilage from medial femur epicondyle or tibia plateau	RT-qPCR IHC
5550	Beagle	CD	F	12.4	22	Only cartilage from medial femur epicondyle or tibia plateau. Appeared to have thicker cartilage macroscopically	RT-qPCR IHC

Table 6. Donor information RT- qPCR and IHC.

Ex vivo explant culture of canine cartilage and joint capsule tissue

Cartilage was cut into pieces of 10.4 ± 4.9 mg per cartilage explant (wet weight). From the tibia one cartilage explant from each donor was cultured in each well of a non-adherent 96-well plate (Corning Costar, 7007). From the medial femur epicondyle two cartilage explants from each donor were cultured per well in a 24-well plate (662160, CELLSTAR® Greiner Bio-one, Alphen aan de Rijn, the Netherlands). Both well-plates were cultured for seven days in chondrogenic culture medium: hgDMEM, 10 µL/mL ITS+ premix (Corning 354352), 2 µL/mL L-Proline (Sigma, P5607), 10 µL/mL p/s, 5 µL/mL fungizone (15290-018, Invitrogen), 5 µL/mL ascorbic acid 2-phosphate (Sigma, A8960), and 10 µL/mL bovine serum albumin (BSA) (Sigma, A9418). Adhesion of the cartilage explants to the 24-well plates was prevented by coating the wells with an agarose coating (V3121, Promega Corporation, Madison, USA).

RNA isolation and obtaining cDNA

Explants were collected after seven days of culturing, snap frozen in liquid nitrogen and crushed by use of a hammer and anvil for at least three times. Afterwards, the sample was collected and 600 µL of Lysis solution (17209, Exiqon, Woburn, USA) was added. A miRCURYTM RNA Isolation Kit (300110, Exiqon, Woburn, USA) was used for RNA isolation according to the manufacturer's protocol. To assure no DNA was left behind, an on-column DNase step (RNAse free DNase Set, 79254, Qiagen, Valencia, USA) was performed. Quality of the isolated RNA was tested with the RNA nanodrop analyser. Subsequently, first-strand cDNA synthesis was achieved using the iScript™ cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's protocol.

RT- qPCR

RT-qPCR was performed using IQT™ SYBR Green Supermix Kit (Bio-Rad, Veenendaal, the Netherlands) and the CFX384 Connect™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). Six reference genes were chosen to normalize for the quantitative target gene expression, which was determined by the Normfirst method. The primers that were used are shown in **Table 7** and **Table 8**.

The mean Ct-values of the reference genes normalized the mean Ct-values of the genes of interest (GOI): $\Delta Ct = Ct_{mean\ ref} - Ct_{GOI}$. After that, $E^{\Delta Ct}$ were calculated which indicates the efficiency of amplification from the GOI/reference gene. E were calculated from the slope of the standard curve: $E = 10^{-1/slope}$.

Gen	Forward sequence	Reverse sequence	T_m (°C)
GAPDH	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	58
YWHAZ	CGAAGTTGCTGCTGGTGA	TTGCATTTCCTTTTTGCTGA	58
RPL-13	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	61
RPS-19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	63
HNRPH	CTCACTATGATCCACCACG	TAGCCTCCATAACCTCCAC	61.2
SRPR	GCTTCAGGATCTGGACTGC	GTTCCCTTGGTAGCACTGG	61.2

Table 7. Reference genes.

Gen	Forward sequence	Reverse sequence	T_m (°C)
DKK1	GGCAACGACCACAGCACC	CGCAGTCTGATGATCGGAGACAG	66
DKK3	CATCCAGTCCAGTGCTCTCA	GGGCCAGGATTGTAAGTGAA	58
Wisp1	CATCCGTCTACACATCAAGGAAGG	CAGCACCTGTCGTCCGTG	67,5
Axin2	GGACAAATGCGTGGATACCT	TGCTTGGAGACAATGCTGTT	60
SOST	TCTCTTGCTCTGTGTCTCG	TACTCGGATGCGTCTTTGG	59
Wnt5a	TGCCACTTGTATCAGGACCA	GCTGCCTATCTGCATGACC	61
Wnt7b	AACACGCACCAGTACACCAA	CACTTGCAGGTGAAGACCTC	60
IL-10	CCCGGGCTGAGAACCACGAC	AAATGCGCTCTTCACCTGCTCCAC	63
Cav1	CGCACACCAAGGAAATCG	AAATCAATCTTGACCACGTCG	60
CCND1	ACTACCTGGACCGCT	CGGATGGAGTTGTCA	58

Table 8. Target genes.

Statistics

Ratio of gene expression was compared between CD and NCD breeds. To compare these two groups, a Cox proportional hazard regression model was used (donor as random effect). Effect sizes (**Table 9**) were calculated to take the differences between the donors in one group into account.

P < 0.05 was considered statistically significant, however, in combination with small effect sizes the biological relevance is questionable. Medium to very large effect size in combination with P < 0.05 was considered substantive significant.

Cliff's delta	Effect size
≤ 0.28	Small
0.28 < effect size ≤ 0.43	Medium
0.43 < effect size ≤ 0.7	Large
> 0.7	Very large

Table 9. Effect sizes.

Immunohistochemistry

Tissue collection and the histological process before staining was performed by a predecessor.

Tissue collection

Cartilage from the stifle joint of healthy CD (n=7) and NCD (n=7) donors was collected, donor information is listed before in **Table 6**. A subset of the donors were the same as used in RT-qPCR. All dogs were euthanized in unrelated studies. Cartilage was scraped with a scalpel and fixed in formalin for further histological processing.

B-catenin staining

The collected tissues were deparaffinized by dipping it in xylene twice for 5 minutes. Subsequently, the collected tissues were dipped 5 minutes in 96%, 80%, 70% and 60% EtOH. Lastly, the tissues were dipped in demi water for 5 minutes. Hereafter, the slides were cooked in 0.01 M citrate buffer (pH 6.0) at 70°C for 30 minutes and cooled down for 20 minutes at room temperature. The samples were blocked with 0.3% H_2O_2 for 10 minutes and washed twice with PBS/0.025% Triton X for 5 minutes. Subsequently, the samples were blocked in 10% normal goat serum in PBS/0.025% Triton X for 30 minutes and incubated over night with the primary antibody rabbit anti-β-catenin (Abcam, ab6302) in 1:1000 dilution at 4°C. This antibody was diluted in PBS-BSA 1%. A negative control was performed with PBS-BSA 1% minus a primary antibody.

Next day, the slides were washed three times with PBS/0.025% Triton X for 5 minutes and incubated for 30 minutes at room temperature with the secondary antibody conjugated with HRP: EnVision HRP anti-Rabbit (Dako, K4003). After this, the slides were washed twice with PBS for 5 minutes. The samples were incubated with DAB (Dako) for 5 minutes and washed twice with demi-water for 5 minutes. Hematoxylin was used to counterstain the samples for 40 seconds and subsequently, the slides were washed with running tap-water for 10 minutes.

Lastly, the slides were paraffinized by dipping the slides in 70%, 80%, 96% and 100% EtOH for 5 minutes. The dipping in 96% EtOH was done twice before continuing in the 100% EtOH. This dipping series was finished by dipping the tissues twice in xylene for 5 minutes. This protocol was ended by covering the slides with a coverslip and permanent mounting medium.

Scoring

The samples were photographed and in the 1:1000 dilution the total amount of cells, the percentage of positive cells over total amount of cells for each layer, and the percentage of total positive cells were calculated.

Statistics

The percentage of positive cells in each layer was compared between CD and NCD breeds. To compare these two groups, the ANOVA test (donor as random effect) was used for the comparison in the percentage of positive cells in the superficial and deep layer, and for the total amount of cells in the articular cartilage. The cox proportional hazard regression test (donor as random effect) was used to compare the percentage of positive cells in the intermediate layer and to compare the overall percentage of positive cells in the articular cartilage between CD and NCD dogs. $P < 0.05$ was considered statistically significant.

Results

RT-qPCR

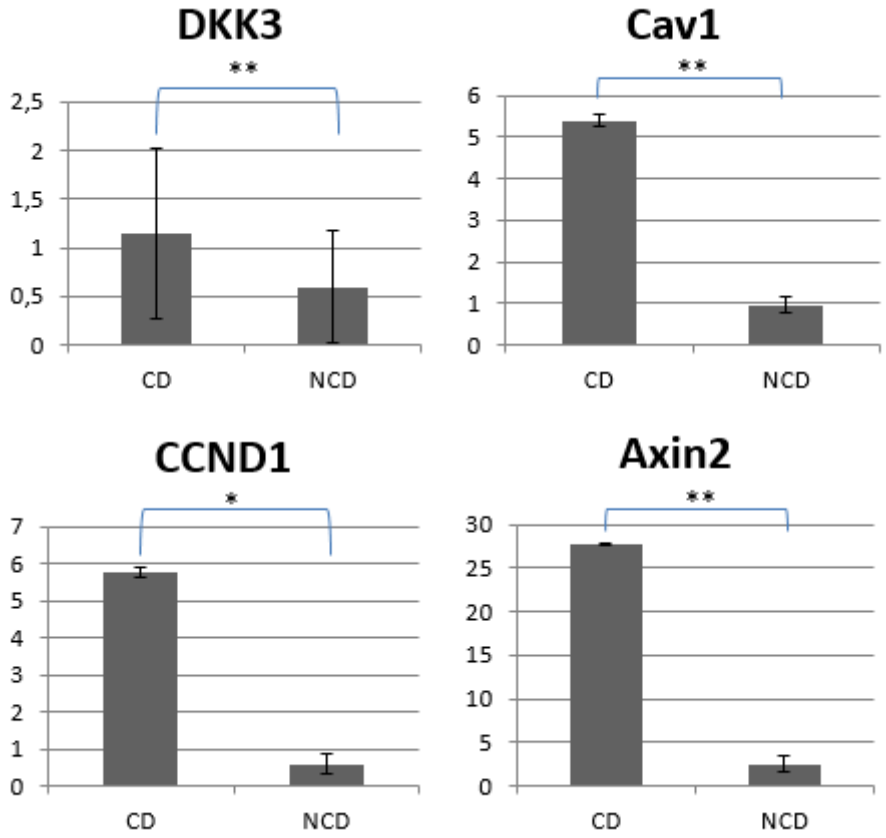

Figure 12. Ratio of gene expression in NCD and CD breeds. Upper left: DKK3; Upper right: Cav1; Lower left: CCND1; Lower right: Axin2. *: P < 0.05, **: P < 0.0001.

The ratio of gene expression in cartilage is compared between CD and NCD breeds. In CD breeds, the expression of DKK3 was higher than in NCD breeds: 1.14 ± 0.88 and 0.60 ± 0.58, respectively (**Figure 12** (upper left)). This difference is considered substantive significant (P < 0.0001 and very large effect size). Cav1 gene expression was higher in CD breeds than in NCD breeds (**Figure 12** (upper right)): 5.40 ± 0.14 and 0.97 ± 0.19, respectively. This difference is considered statistically significant (P < 0.0001), but its biological relevance is questionable (small effect size). The expression of CCND1 in CD breeds was higher than in NCD breeds (**Figure 12** (lower left)): 5.76 ± 0.13 and 0.59 ± 0.27, respectively. This difference is considered statistically significant (P < 0.05), but its biological relevance is questionable (small effect size). Axin2 was expressed in higher levels in CD breeds than in NCD breeds (**Figure 12** (lower right)). Respectively, this ratio was 27.73 ± 0.01 and 2.54 ± 0.89, and this is considered substantive significant (P < 0.0001 and medium effect size).
RT-qPCR was also performed in order to measure the values of DKK1, Wisp1, SOST, IL-10, Wnt-5a and Wnt-7b, but expression of these genes was too low to detect.

Immunohistochemistry

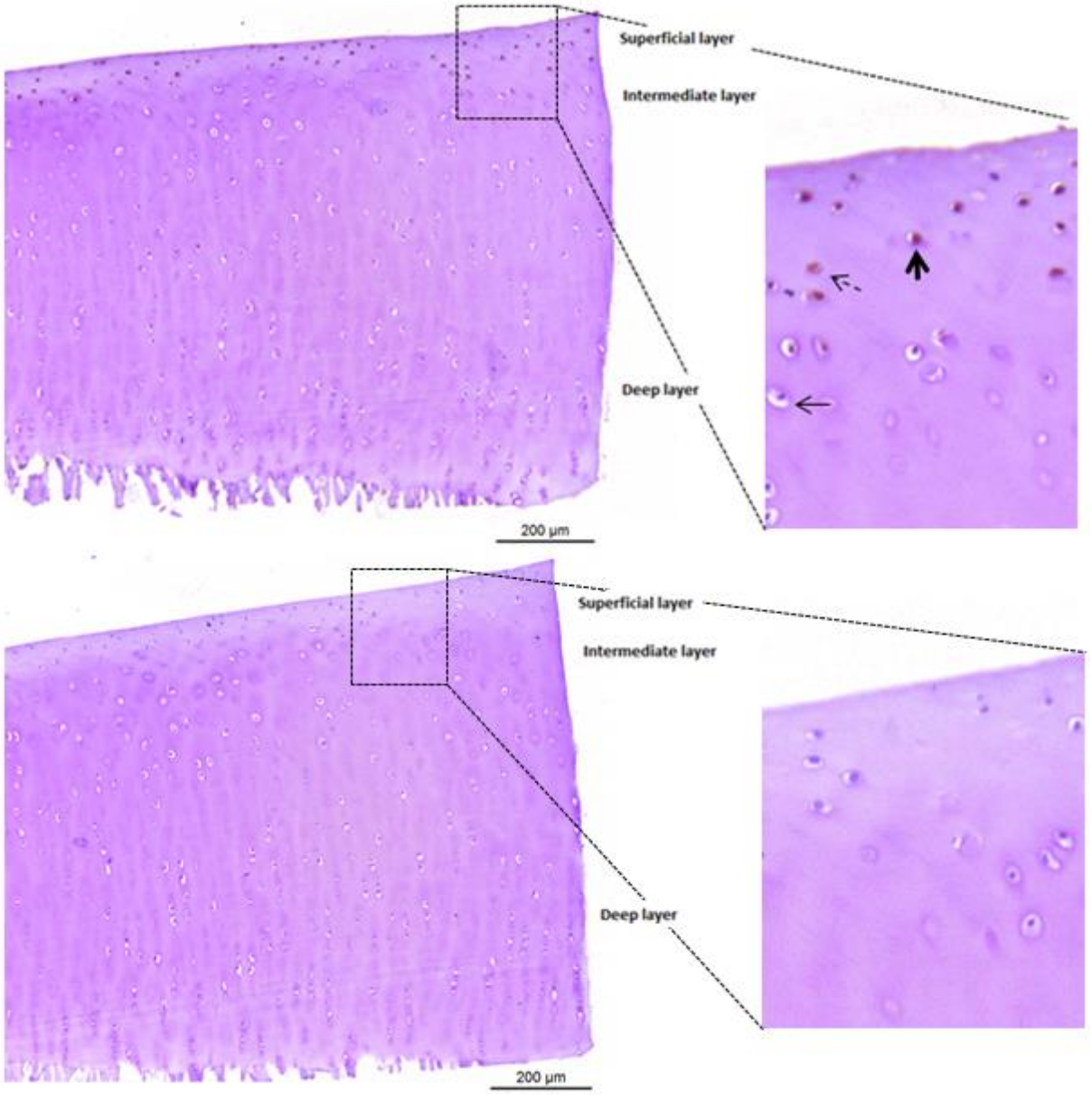

Figure 13. Upper: example of the β-catenin staining of 1:1000 dilution. Normal arrow: cell without staining; Dashed arrow: cytoplasm staining; Bold arrow: nucleus staining. Lower: example of the negative control.

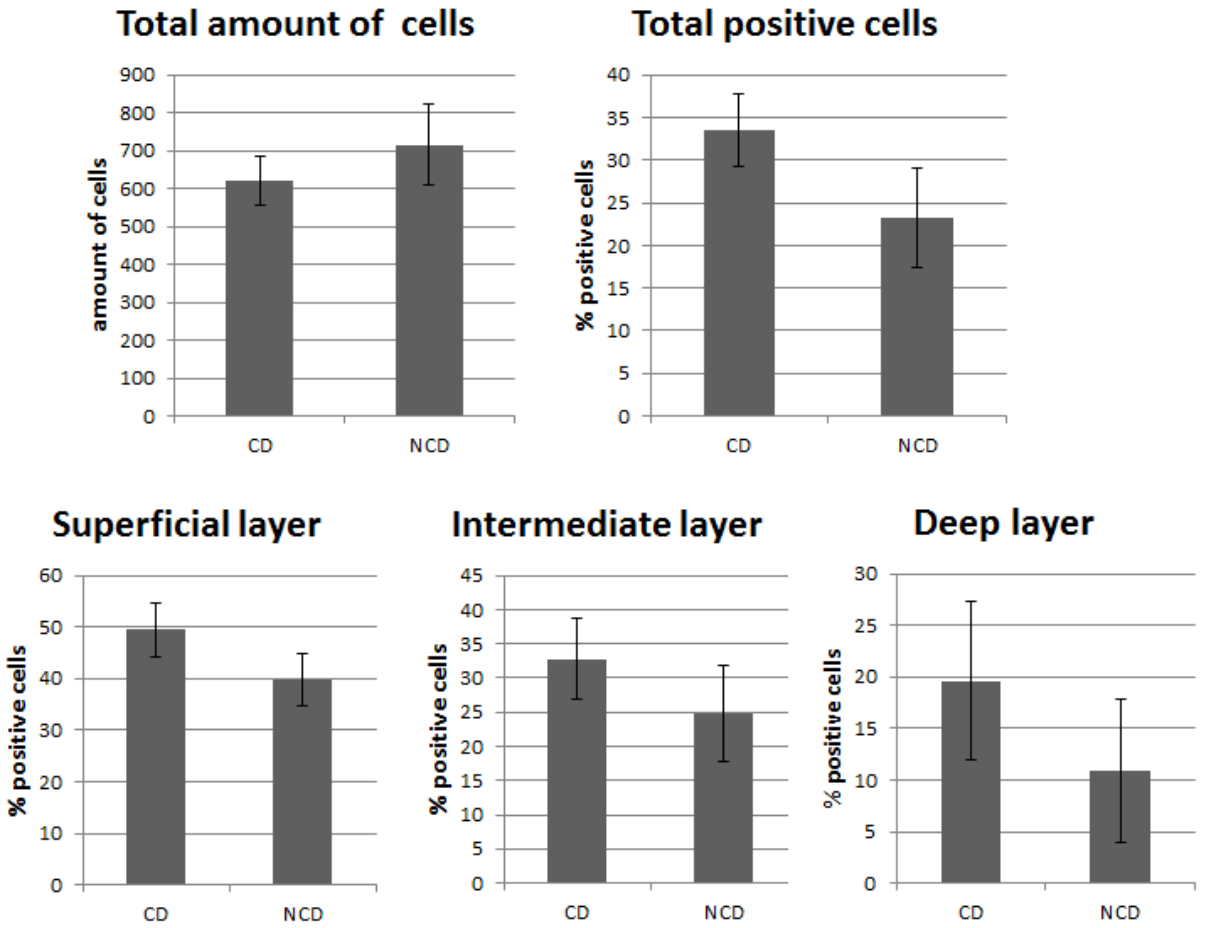

Figure 14. Total amount of cells in the cartilage and the percentages of positive cells in the β-catenin staining in CD and NCD breeds. Upper left: total amount of cells; Upper right: percentage of the total positive cells; Lower left: percentage of positive cells in the superficial layer; Lower middle: percentage of positive cells in the intermediate layer; Lower right: percentage of positive cells in the deep layer.

Examples of both a 1:1000 dilution and a negative control of the IHC in order to stain β-catenin are shown in **Figure 13**.

The total amount of cells was compared between CD and NCD breeds (**Figure 14**, upper left). Less cells were present in cartilage of CD breeds compared to NCD breeds, but no significant difference was found: respectively 619.58 ± 64.13 and 715.43 ± 107.30 ($P > 0.05$).

The percentage of positive cells over the amount of cells was compared between CD and NCD breeds. In CD breeds the percentage of positive cells in the superficial layers was higher compared to the NCD breeds: 49.46 ± 4.12% and 39.79 ± 5.07%, respectively (**Figure 14**, lower left). But this difference was not considered statistically significant ($P > 0.05$). In the intermediate layer, the percentage of positive cells was higher in CD breeds than in NCD breeds: 32.82 ± 5.01% and 24.82 ± 7.04%, respectively (**Figure 14**, lower middle). But this difference was not considered statistically significant ($P > 0.05$). The percentage positive cells in the deep layer was higher in CD breeds compared to NCD breeds: 19.61 ± 7.72% and 10.87 ± 6.96%, respectively (**Figure 14**, lower right). But this difference was not considered significant ($P > 0.05$). In both breeds and in all layers, the majority of the cells were positive in the cytoplasm.

The percentage of positive cells in total was also compared between CD and NCD breeds (**Figure 14**, upper right). This percentage was higher in CD breeds compared to NCD, but no significant difference was found: respectively 33.53 ± 4.19% and 23.21 ± 5.78% (P > 0.05).

Discussion

Basal gene expression and the percentages of positive cells over the total amount of cells in IHC was compared between CD and NCD breeds in healthy cartilage. In order to confirm that the cartilage of both breeds was healthy, macroscopic and histologic examination was performed.

The basal gene expression ratio of DKK3, Cav1, CCND1 and Axin2 are different between NCD and CD breeds according to the P-values. However, both Cav1 and CCND1 have a small effect size, so the biological relevance is questionable. If the same test was performed with more samples, the effect sizes could be higher thus the differences could be biological relevant. The effect size of DKK3 is very large and the effect size of Axin2 is medium, both in combination with the P-values smaller than 0.05. Thus these differences are considered substantive significant. All the genes named above were expressed in higher levels in CD breeds than in NCD breeds.

Both Axin2 and CCND1 are target genes of the Wnt/β-catenin pathway.[116-118] So the higher gene expression of Axin2 and CCND1 in healthy cartilage of CD breeds indicate that the Wnt/β-catenin pathway is more active. However, CCND1 is not a very specific target gene because it can be induced by multiple pathways.[116] Axin2 is a negative regulator of the Wnt/β-catenin pathway because it supports the degradation of β-catenin by binding to the degradation complex and promoting the phosphorylation of β-catenin.[117] On top of that, it also increases the number of degradation complexes so more β-catenin can be degraded.[118] So the Wnt/β-catenin pathway is activated in order to express Axin2, but as a result of this expression, the Wnt/β-catenin pathway is inhibited. The negative regulation of Axin2 prevents the activity of the Wnt/β-catenin pathway from becoming excessive.

Smolders et al.[146] reported that Cav1 is a crucial factor in the maintenance of notochordal cell health and physiology, and in initiation of intervertebral disc degeneration (IVDD). They also reported a difference in gene expression of Wnt/β-catenin pathway related genes in notochordal cells between CD and NCD breeds.[146] In healthy intervertebral discs, the expression of both Axin2 and Fzd1 was higher in CD breeds compared to NCD breeds.[146] This indicates that the activity of Wnt/β-catenin pathway is higher in CD breeds than in NCD breeds. In IVDD, the Cav1 gene expression was downregulated in CD breeds in the study of Smolders[146], but according to Bach et al.[147], its expression was increased in IVDD. The upregulation of Cav1 gene expression could indicate an attempt at repair[147] and the downregulation indicates a relation between the absence of Cav1 and the degeneration[146]. This suggests that Cav1 could protect intervertebral discs from degeneration by stimulation of the Wnt/β-catenin pathway. The same mechanism might play a role in cartilage degeneration. In healthy cartilage, Cav1 is expressed in higher levels in CD breeds than in NCD breeds. This protein is not induced by the Wnt/β-catenin pathway, but it influences this pathway in several ways and plays a role in IVDD. Accumulation of β-catenin in caveolae membranes is induced by Cav1 which can serve as a depot so degradation of β-catenin is prevented. When target genes of the Wnt/β-catenin pathway need to be transcribed, part of the amount of the accumulated β-catenin can be released.[131] Cav1 can stimulate the activation of Wnt/β-catenin pathway in three other ways: internalizing coreceptor LRP6[132],

inhibiting the binding of β-catenin to axin which prevents β-catenin degradation by the degradation complex[132], and activating integrin-linked kinase which inhibits GSK 3β so β-catenin is less degraded.[133] High expression levels of the Cav1 gene substantiates the indication that the Wnt/β-catenin pathway is activated.

Expression of DKK3 is also higher in the healthy cartilage of CD breeds compared to the healthy cartilage of NCD breeds. Although the function of DKK3 in cartilage is unclear[138], in combination with the other results in this experiment one of the functions of DKK3 might be the activation of the Wnt/β-catenin pathway. This is indicated by the other results that suggest an activation or stimulation of the active Wnt/β-catenin pathway. Contradictory, Snelling et al.[134] reported that DKK3 inhibits the Wnt/β-catenin pathway and upregulation of this protein could be a protective mechanism to limit cartilage damage. So DKK3 could play a role in prevention of or protection from OA. But more research is needed to fully understand the function of DKK3 in cartilage.

In previous studies, decreased levels of Wif1[135] and higher levels of Wisp1[128], DKK1[141] and DKK3[134] have been found in OA cartilage compared to healthy cartilage. Higher levels of Wisp1 have also been found in OA synovium compared to healthy synovium.[128] This indicates that the Wnt/β-catenin pathway plays a role in the pathogenesis of OA. This is substantiated by the downregulation of articular chondrocytes that is induced by Cav1.[130] Cav1 stimulates the Wnt/β-catenin pathway[131-133], this stimulation indeed results in a shift in differentiation of mesenchymal progenitor cells towards osteoblasts instead of chondrocytes.[108,109] But the increased gene expression of Cav1 and its function to activate the Wnt/β-catenin pathway could protect against OA because a decreased or absent activity of this pathway can also result in cartilage destruction and chondrocyte apoptosis.[113]

Altogether, the basal gene expression of the measured genes (DKK3, Cav1, CCND1 and Axin2) was higher in the healthy cartilage of CD breeds compared to the healthy cartilage of NCD breeds. This could mean that the Wnt/β-catenin pathway is more active in the healthy cartilage of CD breeds. But gene expression does not necessarily mean that the proteins are present. IHC, in order to stain β-catenin, was performed as a follow up of the basal gene expression measurement. Although a trend was seen of more positive chondrocytes in CD cartilage compared to NCD cartilage, no statistical significant differences were found between CD and NCD in IHC. However, IHC is a rough and semi-quantitative method to examine the presence of proteins in cells. Further experiments are needed to quantify and compare the amount of protein in the cells and to compare the activity of the Wnt/β-catenin pathway between CD and NCD breeds, see Future perspectives.

Because of the higher basal gene expression in healthy cartilage of CD breeds compared to NCD breeds, CD breeds might be less susceptible for developing OA. Apparently, the Wnt/β-catenin pathway might be more active in healthy cartilage of CD breeds and it does not induce OA in these dogs. In NCD breeds the basal gene expression of Wnt/β-catenin in healthy cartilage is lower, so the basal activity of this pathway might be lower in these breeds. An increase in the activity might be associated with the development of OA because the cartilage is not used to this activity. Increase in activity of the Wnt/β-catenin pathway leads to degeneration of ECM and cartilage[110-112], and a shift in differentiation of the mesenchymal progenitor cells towards osteoblast development.[108,109] This could lead to ECM and cartilage degeneration, and osteophyte formation, which are all associated with OA.[6,12,14-18]

Despite the differences that are found in the basal gene expression and the trend that is seen in the β-catenin staining, no statistical significant differences between CD and NCD

breeds are found in the percentages of positive chondrocytes in the β-catenin staining. However, the activity of the Wnt/β-catenin pathway in healthy cartilage could still differ between CD and NCD breeds.

A similar difference is present in human, achondroplasia or dwarfism is caused by an mutation in fibroblast growth factor receptor (FGFR) 3 which results in a gain of function.[148,149] Human suffering from achondroplasia are less susceptible to OA despite some features that are predisposed to OA development, for example bowed legs and higher rates of obesity. This suggests that the mutation in FGFR3 could protect against OA development.[150] It is reported that inhibition of GSK3-β results in activation of the Wnt/β-catenin pathway which leads to higher expression of FGF4 in tooth morphogenesis[151], because FGF4 is a downstream target of this pathway.[152] So the mutations in FGF4 and FGFR3 could partly mimic an activation of the Wnt/β-catenin pathway.

Conclusion

DKK3, Cav1, CCND1 and Axin2 mRNA is expressed in higher levels in CD breeds than in NCD breeds. In the β-catenin staining, a trend of a higher percentages of positive cells in the CD cartilage compared to the NCD cartilage was seen, but no statistical significant differences were found. However, the Wnt/β-catenin pathway activity could still differ between CD and NCD breeds.

Upregulations of the target genes Axin2 and CCND1 indicate that the Wnt/β-catenin pathway is active. This is substantiated by the upregulation of Cav1 which is an activator of this pathway. The function of DKK3 in cartilage is unclear, but in combination with our other results, it might activate the Wnt/β-catenin pathway.

Activation of the Wnt/β-catenin pathway could play a role in the pathogenesis of OA, especially in NCD breeds. CD breeds might be less susceptible to OA because this pathway is also active in healthy cartilage of these dogs. As a result of that, OA might be less present in CD breeds compared to NCD breeds, similar to the difference in human with and without dwarfism. In NCD breeds, increased activity of the Wnt/β-catenin pathway could influence the differentiation of mesenchymal progenitor cells. As a result of that, the differentiation shifts more towards osteoblast development instead of chondrocyte development. Increased activity of the Wnt/β-catenin pathway also influences the degeneration of both ECM and articular cartilage. This could lead to an OA phenotype including osteophyte formation and ECM and cartilage degeneration.

In future OA research in dogs, it may be preferred to use NCD breeds because these dogs might be more susceptible to the development of OA. It is important to determine which breed is used and to prevent switching from NCD to CD breeds and vice versa during the research. In the future, this differences between CD and NCD in OA could also lead to different therapies.

Future perspectives

Although the basal gene expression of Wnt/β-catenin pathway related genes was higher in healthy cartilage of CD breeds compared to NCD breeds, it is not certain that this differences are also present on a protein level. Because not all mRNA is translated into protein. IHC was performed to visualize the amount of chondrocytes that were positive in the β-catenin staining. However, IHC is a rough and semi-quantitative method to demonstrate protein. The

amount of cells that contain β-catenin becomes clear, but not the amount of protein that is present in these cells. In order to quantify this amount of protein and the activity of the Wnt/β-catenin pathway, Western Blot and TOP/FOP assay could be performed as a follow-up of this study. Western Blot shows the amount of protein that is present in the cell and it can be divided into two phases. The first phase is polyacrylamide gel electrophoresis and contains the separation of the proteins based on their size, shape and charge. The smaller the protein, the faster it moves in the gel. During the second phase, the proteins are transferred on a membrane and the proteins of interest are visualized with specific antibodies.[153]

A TOP/FOP assay is used to visualize the activity of the Wnt/β-catenin pathway in cultured cells. Cells are transfected with firefly and renilla luciferase and several days later, the cells are lysed and supernatant is collected. In a luminometer, supernatant and substrate reagents of firefly and renilla luciferase are mixed and light signals are detected. Firefly luciferase is detected at 560 nm and renilla luciferase is detected at 480 nm.[154]

Usage of healthy cartilage only is another limitation of this study. In order to compare CD and NCD breeds on the level of OA, it would be better to use OA cartilage too. Repeating the RT-qPCR in OA cartilage is an interesting follow-up of this study. This way, the activity of the Wnt/β-catenin pathway in OA can be compared to the activity of this pathway in healthy cartilage. Obtaining this information could help in the development of new therapies for OA.

It is also interesting to compare the progression of OA between CD and NCD breeds. This could be done by analyzing and comparing historical data of scored OA in joints of both CD and NCD breeds by using the OARSI scoring system.

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References

1. Dyce KM, Sack WO, Wensing CJG. The locomotor apparatus. In: Dyce KM, Sack WO, Wensing CJG, eds. *Textbook of veterinary anatomy.* 4th ed. St. Louis, MO: Saunders Elsevier; 2010:32-99.
2. Sinowatz F. Musculo-skeletal system. In: Hyttel P, Sinowatz F, Vejlsted M, Betteridge K, eds. *Essentials of domestic animal embryology.* Saunders Elsevier; 2010:286-316.
3. Reece WO. Bones, joints and synovial fluid. In: Reece WO, ed. *Functional anatomy and physiology of domestic animals.* 4th ed. Ames, Iowa: Wiley-Blackwell; 2009:179-205.
4. Eurell JA, van Sickle DC. Connective and supportive tissues. In: Eurell JA, Frappier BL, eds. *Dellmann's textbook of veterinary histology.* 6th ed. Ames, Iowa: Blackwell Publishing Professional; 2006:31-60.
5. Carlson BM. *Human embryology and developmental biology.* 3rd ed. Mosby, Philadelphia, USA: ; 2004.
6. Schulz KS. General principles and techniques. In: Fossum TW, ed. *Small animal surgery.* 4th ed. St. Louis, Missouri: Elsevier; 2013:1215-1226.
7. Dyce KM, Sack WO, Wensing CJG. The forelimb of the dog and cat. In: Dyce KM, Sack WO, Wensing CJG, eds. *Textbook of veterinary anatomy.* 4th ed. St. Louis, MO: Saunders Elsevier; 2010:476-489.
8. Cook JL, Kuroki K, Visco D, Pelletier JP, Schulz L, Lafeber FPJG. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the dog. *Osteoarthritis and Cartilage*. 2010;18:S66-S79. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/j.joca.2010.04.017.
9. Dyce KM, Sack WO, Wensing CJG. The hindlimb of the dog and cat. In: Dyce KM, Sack WO, Wensing CJG, eds. *Textbook of veterinary anatomy.* 4th ed. St. Louis, MO: Saunders Elsevier; 2010:490-500.

10. Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S, Goldberg VM. Principles of cartilage repair and regeneration. *Clinical Orthopaedics and Related Research*. 1997;342:254-269.
11. Clark DM. The biochemistry of degenerative joint disease and its treatment. *Compendium on Continuing Education for the Practicing Veterinarian*. 1991;13(2):275-281, 284.
12. Goldring MB, Goldring SR. Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. *Ann N Y Acad Sci*. 2010;1192(1):230-237. doi: 10.1111/j.1749-6632.2009.05240.x.
13. Glyn-Jones S, Palmer AJR, Agricola R, et al. Osteoarthritis. *The Lancet*. 2015;386(9991):376-387. doi: http://dx.doi.org/10.1016/S0140-6736(14)60802-3.
14. Goldring MB, Marcu KB. Epigenomic and microRNA-mediated regulation in cartilage development, homeostasis, and osteoarthritis. *Trends in Molecular Medicine*. 2012;18(2):109-118.
15. May SA. Degenerative joint disease. In: Houlton J, Collinson R, eds. *Manual of small animal arthrology.* Cheltenham: Britisch Small Animal Veterinary Association; 1994:62-71.
16. Bennet D, May C. Joint diseases of dogs. In: Ettinger SJ, ed. *Textbook of veterinary internal medicine.* 4th ed. Philadelphia: W B Saunders and Co.; 1995:2053-2059.
17. Sniekers YH, Intema F, Lafeber FPJG, et al. A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models. *BMC Musculoskeletal Disorders*. 2008;9(20).
18. Schulz KS. Degenerative joint disease. In: Fossum TW, ed. *Small animal surgery.* 4th ed. St. Louis, Missouri: Elsevier; 2013:1226-1229.
19. Bulstra SK. *Histological, pathological and therapeutical aspects of osteoarthritis.* Universitaire Pers Maastricht; 1992.
20. van der Kraan PM, van den Berg WB. Osteophytes: Relevance and biology. *Osteoarthritis and Cartilage*. 2007;15(3):237-244. doi: http://dx.doi.org/10.1016/j.joca.2006.11.006.
21. Audrey HX, Abd Razak HRB, Andrew THC. The truth behind subchondral cysts in osteoarthritis of the knee. *The Open Orthopaedics Journal*. 2014;8:7-10.
22. Freund E. The pathological significance of intra-articular pressure. *Edinburgh Medical Journal*. 1940;47:192-203.
23. Rhaney K, Lamb DW. The cysts of osteoarthritis of the hip. *Bone & Joint Journal*. 1955;37B(4):663-675.
24. Shinmei M, Masuda K, Kikuchi T, Shimomura Y. The role of cytokines in chondrocyte mediated cartilage degradation. *Journal of Rheumatology*. 1989;18:32-34.
25. Wang M, Sampson ER, Jin H, et al. MMP13 is a critical target gene during the progression of osteoarthritis. *Arthritis Research and Therapy*. 2013;15:R5.
26. Shiomi T, Lemaître V, D'Armiento J, Okada Y. Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. *Pathology International*. 2010;60:447-496.
27. Little CB, Barai A, Burkhardt D, et al. Matrix metalloproteinase 13–deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. *Arthritis & Rheumatism*. 2009;60:3723-3733.
28. Vaughan-Scott T, Taylor JH. The pathophysiology and medical management of canine osteoarthritis. *Journal of the South African Veterinary Association*. 1997;68(1):21-25.
29. Innes J. Diagnosis and treatment of osteoarthritis in dogs. *In Practice*. 1995;17(3):102-109.
30. Ramírez-Flores GI, Del Angel-Caraza J, Quijano Hernández IA, Hulse DA, Beale BS, Victoria Mora JM. Correlation between osteoarthritic changes in the stifle joint in dogs and the results of orthopedic, radiographic, ultrasonographic and arthroscopic examinations. *Veterinary Research Communications*. 2017;41(2):129-137.
31. Kinds MB, Vincken KL, Hoppinga TN, et al. Influence of variation in semiflexed knee positioning during image acquisition on separate quantitative radiographic parameters of osteoarthritis, measured by knee images digital analysis. *Osteoarthritis and Cartilage*. 2012;20(9):997-1003. doi: https://doi.org/10.1016/j.joca.2012.04.016.
32. Nolte-Ernsting CCA, Adam G, Bühne M, Prescher A, Günther RW. MRI of degenerative bone marrow lesions in experimental osteoarthritis of canine knee joints. *Skeletal Radiology*. 1996;25(5):413-420.
33. Carrig CB. Diagnostic imaging of osteoarthritis. *Veterinary Clinics of North America: Small Animal Practice*. 1997;27(4):777-814. doi: https://doi.org/10.1016/S0195-5616(97)50080-5.
34. Peterty CG, Genant HK. Magnetic resonance imaging in arthritis. In: Koopman WJ, ed. *Arthritis and allied conditions: A textbook of rheumatology.* 13th ed. Baltimore, Williams & Wilkins; 1997:116.
35. Boulay JP, De Angelis M, Kincaid SA, Leeds EB. Medical therapy of osteoarthritis in dogs. *Veterinary Exchange (Supplement to the Compendium on Continuing Education for the Practising Veterinarian)*. 1995:5-19.

36. Clark DM. Current concepts in the treatment of degenerative joint disease. *Compendium of Continuing Education for the Practising Veterinarian*. 1991;13:1439-1446.
37. Martinez JH, Mondragon CE, Cespedes A. An evaluation of the anti-inflammatory effects of intra-articular synthetic β-endorphin in the canine model. *Anesthesia and Analgesia*. 1996;82:1-5.
38. Yaksh TL. Opioid receptor systems and endorphins: A review of their spinal organization. *Journal of Neurosurgery*. 1987;67:157-176.
39. Tomlinson J, McLaughlin R. Medically managing canine hip dysplasia. *Veterinary Medicine*. 1996;91:48-53.
40. Doherty M. "Chondroprotection" by non-steroidal anti-inflammatory drugs. *Annals of the Rheumatic Diseases*. 1989;4:619-621.
41. Engelhardt G, Pairet M. Meloxicam: A new NSAID with an improved safety profile through preferential inhibition of COX-2. *European Rheumatology*. 1995;24(3):272.
42. Cohn LAA. The influence of corticosteroids on host defence mechanisms. *Journal of Veterinary Internal Medicine*. 1991;5:95-104.
43. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: Molecular mechanisms. *Trends in Pharmacological Sciences*. 1993;36:340-343.
44. Pelletier J, Martel-Pelletier J. Protective effects of corticosteroids on cartilage lesions and osteophyte formation in the pond-nuki dog model of osteoarthritis. *Arthritis & Rheumatism*. 1989;32(2):181-193. doi: 10.1002/anr.1780320211.
45. Jüni P, Hari R, Rutjes AW, et al. Intra-articular corticosteroid for knee osteoarthritis. *Cochrane Database of Systematic Reviews*. 2015(10). doi: - 10.1002/14651858.CD005328.pub3.
46. Bannuru RR, Schmid CH, Kent DM, Vaysbrot EE, Wong JB, McAlindon TE. Comparative effectiveness of pharmacologic interventions for knee osteoarthritis: A systematic review and network meta-analysis. *Annals of Internal Medicine*. 2015;1621:46-54.
47. Huber M, Bill R. The use of polysulphated glycosaminoglycan in dogs. *Compendium on Continuing Education for the Practicing Veterinarian*. 1994;16:501-504.
48. Francis DJ, Read RA. Pentosan polysulfate as a treatment for osteoarthritis (degenerative joint disease) in dogs. *Australian Veterinary Practitioner*. 1993;23:104-109.
49. Campbell K. Use of fatty acid supplements in dogs. *Veterinary Dermatology*. 1993;4:167-173.
50. Miller WH, Scott DW, Wellington JR. Treatment of dogs with hip arthritis with a fatty acid supplement. *Canine Practice*. 1992;17:6-8.
51. Vaughn D, Reinhart G, Swaim S, et al. Evaluation of effects of dietary n-6 to n-3 fatty acid ratios on leukotriene B synthesis in dog skin and neutrophils. *Veterinary Dermatology*. 1994;5:163-173.
52. Croft JE. *The new zealand green-lipped mussel.* London: HarperCollins Publishers; 1995.
53. Korthauer W, De-La-Torre J. Treatment of deforming arthropathy in working dogs with "canosan", a new glycosaminoglycan preparation. *Kleintierpraxis*. 1992;37:467-478.
54. Yu L, Smith G, Brandt K, Myers SL, O'Connor B, Brandt D. Reduction of severity of canine osteoarthritis by prophylactic treatment with oral doxycyline. *Arthritis & Rheumatism*. 1992;35:1150-1159.
55. Gaw A, Chang L, Shaw L. Efficacy of acupuncture on osteoarthritic pain. *New England Journal of Medicine*. 1975;21:375.
56. Janssens LAA. Observations on acupuncture therapy of chronic osteoarthritis in dogs: A review of sixty-one cases. *Journal of Small Animal Practice*. 1986;27:825-837.
57. van Valburg AA, van Roermund PM, Lammens J, et al. Can ilizarov joint distraction delay the need for an arthrodesis of the ankle? A preliminary report. *Bone & Joint Journal*. 1995;77-B(5):720-725.
58. Cook JL, Payne JT. Surgical treatment of osteoarthritis. *Veterinary Clinics of North America: Small Animal Practice*. 1997;27(4):931-944. doi: https://doi.org/10.1016/S0195-5616(97)50087-8.
59. Dyce J. Arthrodesis in the dog . *In Practice*. 1996;18:267-279.
60. Brinker WO, Piermattei DL, Flo GL. Arthrodesis. In: *Handbook of small animal orthopedics and fracture treatment.* Philadephia: WB Saunders; 1990:339.
61. de Haan JJ, Roe SC, Lewis DD, Renberg WC, Kerwin SC, Bebchuk TN. Elbow arthrodesis in twelve dogs. *Veterinary and Comparative Orthopaedics and Traumatology*. 1996;9:115.
62. Penwick RC. Arthrodesis. *Veterinary Clinics of North America: Small Animal Practice*. 1987;17:821.
63. Schulz KS. Scapulohumeral joint luxation. In: Fossum TW, ed. *Small animal surgery.* 4th ed. St. Louis, Missouri: Elsevier; 2013:1255-1261.
64. Convery FR, Akeson WH, Keown GH. The repair of large osteochondral defects. *Clinical Orthopaedics and Related Research*. 1972;82:253-262.
65. De Palma AF, McKeever CD, Sabin DK. Process of repair of articular cartilage by histology and autoradiography with tritiated thymidine. *Clinical Orthopaedics and Related Research*. 1968;148:229.

66. Salter RB, Simmons DF, Malcolm BW, Rumble DJ, McMichael D, Clements ND. The biological effect of continuous passive motion on the healing of full thickness defects in articular cartilage. an experimental investigation in the rabbit. *The Journal of Bone and Joint Surgery. American Volume.* 1980;62A:1232-1251.
67. Frank C, Acheson WH, Woo SL, Amiel D, Coutts RD. Physiology and therapeutic value of passive joint motion. *Clinical Orthopaedics and Related Research*. 1984;185:113-125.
68. O'Driscoll SW, Salter RB. The induction of neochondrogenesis in free intra articular periostal autografts under the influence of continuous passive motion. *The Journal of Bone and Joint Surgery. American Volume.* 1984;66-A:1248-1257.
69. Schulz KS. Hip dysplasia. In: Fossum TW, ed. *Small animal surgery.* 4th ed. St. Louis, Missouri: Elsevier; 2013:1305-1316.
70. van Valburg AA, van Roermund PM, Marijnissen ACA, et al. Joint distraction in treatment of osteoarthritis (II): Effects on cartilage in a canine model. *Osteoarthritis and Cartilage*. 2000;8:1-8.
71. van Valburg AA, van Roermund PM, Marijnissen ACA, et al. Joint distraction in treatment of osteoarthritis (I): A two years follow-up of the ankle. *Osteoarthritis and Cartilage*. 1999;7:474-479.
72. Lampropoulou-Adamidou K, Lelovas P, Karadimas EV, et al. Useful animal models for the research of osteoarthritis. *European Journal of Orthopaedic Surgery & Traumatology*. 2014;24(3):263-271.
73. Berjon JJ, Munuera L, Calvo M. Degenerative lesions in the articular cartilage after meniscectomy: Preliminary experimental study in dogs. *Journal of Trauma-Injury Infection & Critical Care*. 1991;31(3):342-350.
74. Cox JS, Nye CE, Schaefer WW, Woodstein IJ. The degenerative effects of partial and total resection of the medial meniscus in dogs' knees. *Clinical Orthopaedics and Related Research*. 1975;109:178-183.
75. Brandt KD, Myers SL, Burr D, Albrecht M. Osteoarthritic changes in canine articular cartilage, subchondral bone, and synovium fifty-four months after transection of the anterior cruciate ligament. *Arthritis & Rheumatism*. 1991;34:1560-1570.
76. Frost-Christensen LN, Mastbergen SC, Vianen ME, et al. Degeneration, inflammation, regeneration, and pain/disability in dogs following destabilization or articular cartilage grooving of the stifle joint. *Osteoarthritis and Cartilage*. 2008;16:1327-1335.
77. Marijnissen ACA, van Roermund PM, TeKoppele JM, Bijlsma JWJ, Lafeber FPJG. The canine 'groove' model, compared with the ACLT model of osteoarthritis. *Osteoarthritis and Cartilage*. 2002;10:145-155.
78. Wiegant K, Intema F, van Roermund PM, et al. Evidence of cartilage repair by joint distraction in a canine model of osteoarthritis. *Arthritis & Rheumatology*. 2015;67(2):465-474.
79. Mastbergen SC, Marijnissen AC, Vianen ME, van Roermund PM, Bijlsma JW, Lafeber FP. The canine 'groove' model of osteoarthritis is more than simply the expression of surgically applied damage. *Osteoarthritis and Cartilage*. 2006;14:39-46.
80. Marijnissen ACA, van Roermund PM, Verzijl N, TeKoppele JM, Bijlsma JWJ, Lafeber FPJG. Steady progression of osteoarthritic features in the canine groove model. *Osteoarthritis and Cartilage*. 2002;10:282-289.
81. Atkinson PJ, Haut RC. Injuries produced by blunt trauma to the human patellofemoral joint vary with flexion angle of the knee. *Journal of Orthopaedic Research*. 2001;19:827-833.
82. Viano DC, Stalnaker RL. Mechanisms of femoral fracture. *Journal of Biomechanics*. 1980;13:701-715.
83. Fildes B, Lenard J, Lane J, Vulcan P, Seyer K. Lower limb injuries to passenger car occupants. *Accident Analysis & Prevention*. 1997;29:785-791.
84. Lahm A, Uhl M, Edlich M, Erggelet C, Haberstroh J, Kreuz PC. An experimental canine model for subchondral lesions of the knee joint. *The Knee*. 2005;12(1):51-55. doi: https://doi.org/10.1016/j.knee.2004.01.005.
85. Mrosek EH, Lahm A, Erggelet C, et al. Subchondral bone trauma causes cartilage matrix degeneration: An immunohistochemical analysis in a canine model. *Osteoarthritis and Cartilage*. 2006;14(2):171-178. doi: https://doi.org/10.1016/j.joca.2005.08.004.
86. Altman RD, Kates J, Chun LE, Dean DD, Eyre D. Preliminary observations of chondral abrasion in a canine model . *Annals of the Rheumatic Diseases*. 1992;51:1056-1062.
87. Panula HE, Helminen HJ, Kiviranta I. Slowly progressive osteoarthritis after tibial valgus osteotomy in young beagle dogs. *Clinical Orthopaedics and Related Research*. 1997;343:192-202.
88. Hansen HJ. A pathologic-anatomical study on disc degeneration in dog, with special reference to the so-called enchondrosis intervertebralis. *Acta Orthopaedica Scandinavica*. 1952;11:1-117.
89. Hoerlein BF. Intervertebral disc protrusions in the dog. I. incidence and pathological lesions. *American Journal of Veterinary Research*. 1953;14(51):260-269.
90. Goggin JE, Li AS, Franti CE. Canine intervertebral disk disease: Characterization by age, sex, breed, and anatomic site of involvement. *American Journal of Veterinary Research*. 1970;31(9):1687-1692.

91. Braund KG, Ghosh P, Taylor TK, Larsen LH. Morphological studies of the canine intervertebral disc. the assignment of the beagle to the achondroplastic classification. *Research in Veterinary Science*. 1975;19(2):167-172.
92. Priester WA. Canine intervertebral disc disease – occurence by age, breed, and sex among 8117 cases. *Theriogenology*. 1976;6:293-303.
93. Olby N, Harris T, Burr J, Munana K, Sharp N, Keene B. Recovery of pelvic limb function in dogs following acute intervertebral disc herniations. *Journal of Neurotrauma*. 2004;21(1):49-59.
94. Parker HG, VonHoldt BM, Quignon P, et al. An expressed fgf4 retrogene is associated with breed-defining chondrodysplasia in domestic dogs. *Science*. 2009;325:995-998.
95. Brisson BA. Intervertebral disc disease in dogs. *Veterinary Clinics of North America: Small Animal Practice*. 2010;40(5):829-858. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/j.cvsm.2010.06.001 ".
96. Bergknut N, Egenvall A, Hagman R, et al. Incidence of intervertebral disk degeneration-related diseases and associated mortality rates in dogs. *J Am Vet Med Assoc*. 2012;240(11):1300-1309. doi: https://doi.org/10.2460/javma.240.11.1300;.
97. Kranenburg HJ, Grinwis GC, Bergknut N, et al. Intervertebral disc disease in dogs - part 2: Comparison of clinical, magnetic resonance imaging, and histological findings in 74 surgically treated dogs. *The Veterinary Journal*. 2013;195(2):164-171.
98. Smolders LA, Bergknut N, Grinwis GCM, et al. Intervertebral disc degeneration in the dog. part 2: Chondrodystrophic and non-chondrodystrophic breeds. *The Veterinary Journal*. 2013;195(3):292-299. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/j.tvjl.2012.10.011.
99. Brown EA, Dickinson PJ, Mansour T, et al. FGF4 retrogene on CFA12 is responsible for chondrodystrophy and intervertebral disc disease in dogs. *Proceedings of the National Academy of Sciences*. 2017;114(43):11476-11481. doi: 10.1073/pnas.1709082114.
100. Riser WH, Haskins ME, Jezyk PF, Patterson DF. Pseudoachondroplastic dysplasia in miniature poodles: Clinical, radiologic, and pathologic features. *Journal of the American Veterinary Medical Association*. 1980;176(4):335-341.
101. Hartmann C, Tabin CJ. Wnt-14 plays a pivot role in inducing synovial joint formation in the developing appendicular skeleton. *Cell*. 2001;104:341-351.
102. Guo X, Day TF, Jiang X, Garret-Beal L, Topol L, Yang Y. Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation. *Genes & Development*. 2004;18(19):2404-2417.
103. Chimal-Monroy J, Montero JA, Ganan Y, Macias D, Garcia-Porrero JA, Hurle JM. Comparative analysis of the expression and regulation of wnt5a, fz4, and frzb1 during digit formation and in micromass cultures. *Developmental Dynamics*. 2002;224:314-320.
104. Grotewold L, Ruther U. The wnt antagonist dickkopf-1 is regulated by bmp signaling and c-jun and modulates programmed cell death. *The EMBO Journal*. 2002;21:966-975.
105. Bhanot P, Brink M, Samos CH, et al. A new member of the frizzled family from drosophila functions as a wingless receptor. *Nature*. 1996;382:225-230.
106. Yates KE, Shortkroff S, Reish RG. Wnt influence on chondrocyte differentiation and cartilage function. *DNA and Cell Biology*. 2005;24(5):446-457.
107. Wodarz A, Nusse R. Mechanisms of wnt signaling in development. *Annual Review of Cell and Developmental Biology*. 1998;14:59-88.
108. Boyden LM, Mao J, Belsky J, et al. High bone density due to a mutation in LDL-receptor-related protein 5. *The New England Journal of Medicine*. 2002;346:1513-1521.
109. Gong Y, Slee RB, Fukai N, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell*. 2001;107(4):513-523. doi: https://doi.org/10.1016/S0092-8674(01)00571-2.
110. Corr M. Wnt-beta-catenin signaling in the pathogenesis of osteoarthritis. *Nature Clinical Practice Rheumatology*. 2008;4:550-556.
111. Zhu M, Tang D, Wu Q, et al. Activation of β-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult β-catenin conditional activation mice. *Journal of Bone and Mineral Research*. 2009;24(1):12-21. doi: 10.1359/jbmr.080901.
112. Nakashima A, Tamura M. Regulation of matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 gene expression by WNT3A and bone morphogenetic protein-2 in osteoblastic differentiation. *Frontiers in Bioscience*. 2006;11:1667-1678.
113. Zhu M, Chen M, Zuscik M, et al. Inhibition of β-catenin signaling in articular chondrocytes results in articular cartilage destruction. *Arthritis & Rheumatism*. 2008;58(7):2053-2064. doi: 10.1002/art.23614.

114. Hwang SG, Ryu JH, Kim IC, et al. Wnt-7a causes loss of differentiated phenotype and inhibits apoptosis of articular chondrocytes via different mechanisms. *The Journal of Biological Chemistry*. 2004;279:26597-26604.
115. Sinha S. Integration of prior biological knowledge and epigenetic information enhances the prediction accuracy of the bayesian wnt pathway. *Integrative Biology*. 2014;6:1034-1048.
116. KEGG T01007: 449028. http://www.genome.jp/dbget-bin/www_bget?cfa:449028+cfa:608847+cfa:611782. Updated 2017. Accessed 10/23, 2017.
117. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Molecular and Cellular Biology*. 2002;22:1172-1183.
118. Lustig B, Jerchow B, Sachs M, et al. Negative feedback loop of wnt signaling through upregulation of conductin/ axin2 in colorectal and liver tumors. *Molecular and Cellular Biology*. 2002;22:1184-1193.
119. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. The wnt/ca2+ pathway: A new vertebrate wnt signaling pathway takes shape. *Trends in Genetics*. 2000;16:279-283.
120. Veeman MT, Axelrod JD, Moon RT. A second canon: Functions and mechanisms of ß-catenin-independent wnt signaling. *Developmental Cell*. 2003;5(3):367-377. doi: http://dx.doi.org.proxy.library.uu.nl/10.1016/S1534-5807(03)00266-1.
121. Topol L, Jiang X, Choi H, Garret-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical wnt pathway by promoting gsk-3-independent beta-catenin degradation. *The Journal of Cell Biology*. 2003;162:899-908.
122. Roman-Roman S, Shi DL, Stiot V, et al. Murine frizzled-1 behaves as an antagonist of the canonical wnt/beta-catenin signaling. *The Journal of Biological Chemistry*. 2004;279:5725-5733.
123. Olson DJ, Gibo DM. Antisense wnt-5a mimics wnt-1-mediated c57mg mammary epithelial cell transformation. *Experimental Cell Research*. 1998;241:134-141.
124. Ishitani T, Kishida S, Hyodo-Miura J, et al. The tak1-nlk mitogen-activated protein kinase cascade functions in the wnt-5a/ca(2+) pathway to antagonize wnt/beta-catenin signaling. *Molecular and Cellular Biology*. 2003;23:131-139.
125. Yuasa T, Otani T, Koike T, Iwamoto M, Enomoto-Iwamoto M. Wnt/betacatenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: Its possible role in joint degeneration. *Laboratory Investigation*. 2008;88:264-274.
126. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Research and Therapy*. 2009;11:224.
127. Kim S, Im D, Kim S, et al. B-catenin regulates expression of cyclooxygenase-2 in articular chondrocytes. *Biochem Biophys Res Commun*. 2002;296(1):221-226. doi: https://doi-org.proxy.library.uu.nl/10.1016/S0006-291X(02)00824-0.
128. Blom AB, Brockbank SM, van Lent PL, et al. Involvement of the wnt signaling pathway in experimental and human osteoarthritis: Prominent role of wnt-induced signaling protein 1. *Arthritis & Rheumatism*. 2009;60(2):501-512. doi: 10.1002/art.24247.
129. French DM, Kaul RJ, D'souza AL, et al. WISP-1 is an osteoblastic regulator expressed during skeletal development and fracture repair. *The American Journal of Pathology*. 2004;165(3):855-867. doi: https://doi.org/10.1016/S0002-9440(10)63348-2.
130. Dai SM, Shan ZZ, Nakamura H, et al. Catabolic stress induces features of chondrocyte senescence through overexpression of caveolin 1: Possible involvement of caveolin 1-induced down-regulation of articular chondrocytes in the pathogenesis of osteoarthritis. *Arthritis & Rheumatism*. 2006;54:818-831.
131. Galbiati F, Volonte D, Brown AMC, et al. Caveolin-1 expression inhibits wnt/ß-catenin/lef-1 signaling by recruiting ß-catenin to caveolae membrane domains. *The Journal of Biological Chemistry*. 2000;275(30):23368-23377.
132. Yamamoto H, Komekado H, Kikuchi A. Caveolin is necessary for wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin. *Developmental Cell*. 2006;11(2):213-223. doi: https://doi.org/10.1016/j.devcel.2006.07.003.
133. Chun J, Hyun S, Kwon T, Lee EJ, Hong S, Kang SS. The subcellular localization control of integrin linked kinase 1 through its protein–protein interaction with caveolin-1. *Cellular Signalling*. 2005;17(6):751-760. doi: https://doi.org/10.1016/j.cellsig.2004.10.016.
134. Snelling SJB, Davidson RK, Swingler TE, et al. Dickkopf-3 is upregulated in osteoarthritis and has a chondroprotective role. *Osteoarthritis and Cartilage*. 2016;24:883-891.
135. Gao SH, Zeng C, Liu JJ, et al. Association between wnt inhibitory factor-1 expression levels in articular cartilage and the disease severity of patients with osteoarthritis of the knee. *Experimental and Therapeutic Medicine*. 2016;11:1405-1409.

136. Sassi N, Laadhar L, Allouche M, et al. WNT signaling and chondrocytes: From cell fate determination to osteoarthritis physiopathology. *Journal of Receptors and Signal Transduction*. 2014;34(2):73-80.
137. Ai L, Tao Q, Zhong S, et al. Inactivation of wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer. *Carcinogenesis*. 2006;27:1341-1348.
138. Nakamura REI, Hackam AS. Analysis of Dickkopf3 interactions with wnt signaling receptors. *Growth Factors*. 2010;28(4):232-242. doi: 10.3109/08977191003738832.
139. Ma L, Wu J, Jin QH. The association between parathyroid hormone 1-34 and the wnt/β-catenin signaling pathway in a rat model of osteoarthritis. *Molecular Medicine Reports*. 2017;16(6):8799-8807.
140. Niehrs C. Function and biological roles of the dickkopf family of wnt modulators. *Oncogene*. 2006;25:7469-7481.
141. Oh H, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. *Arthritis & Rheumatism*. 2012;64:2568-2578.
142. Weng L, Wang C, Ko J, Sun Y, Wang F. Control of dkk-1 ameliorates chondrocyte apoptosis, cartilage destruction, and subchondral bone deterioration in osteoarthritic knees. *Arthritis & Rheumatism*. 2010;62(5):1393-1402. doi: 10.1002/art.27357.
143. James IE, Kumar S, Barnes MR, et al. Frzb-2: A human secreted frizzled-related protein with a potential role in chondrocyte apoptosis. *Osteoarthritis and Cartilage*. 2000;8:452-463.
144. Chapman SC, Brown R, Lees L, Schoenwolf GC, Lumsden A. Expression analysis of chick wnt and frizzled genes and selected inhibitors in early chick patterning. *Developmental Dynamics*. 2004;229:668-676.
145. van Bezooijen RL, Roelen BA, Visser A, et al. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *The Journal of Experimental Medicine*. 2004;199:805-814.
146. Smolders LA, Meij BP, Onis D, et al. Gene expression profiling of early intervertebral disc degeneration reveals a down-regulation of canonical wnt signaling and caveolin-1 expression: Implications for development of regenerative strategies. *Arthritis Research and Therapy*. 2013;15(1):R23.
147. Bach FC, Zhang Y, Miranda-Bedate A, et al. Increased caveolin-1 in intervertebral disc degeneration facilitates repair. *Arthritis Research and Therapy*. 2016;18:59.
148. Rousseau F, Bonaventure J, Legeai-Mallet L, et al. Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. *Nature*. 1994;371:252-254.
149. Shiang R, Thompson LM, Zhu YZ, et al. Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell*. 1994;78:335-342.
150. Klag KA, Horton WA. Advances in treatment of achondroplasia and osteoarthritis. *Human Molecular Genetics*. 2016;25(R1):R2-R8.
151. Aurrekoetxea M, Irastorza I, García-Gallastegui P, et al. Wnt/β-catenin regulates the activity of epiprofin/Sp6, SHH, FGF, and BMP to coordinate the stages of odontogenesis. *Frontiers in Cell and Developmental Biology*. 2016;4(25).
152. Kratochwil K, Galceran J, Tontsch S, Roth W, Grosschedl R. FGF4, a direct target of LEF1 and wnt signaling, can rescue the arrest of tooth organogenesis in *Lef1−/−* mice. *Genes & Development*. 2002;16(24):3173-3185.
153. Western blotting animation - part I. https://www.youtube.com/watch?v=GJJGNOdhP8w. Updated 2015. Accessed 11/03, 2017.
154. Zhao C. Wnt reporter activity assay. *Bio-protocol*. 2014;4(14):e1183.