The regenerative role of vitamin D receptor in joint distraction.

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July 5

2016

Osteoarthritis(OA) is one of the most common chronic afflictions in the western world. OA in the knee be very painful and debilitating. Eventually it will lead to the need for a total knee arthroplasty which is costly, painful and has a high revision rate. However with the recently discovered distraction method (in which the joint is stretched by an external frame secured by pins in the bone) improvement can be seen for up to 5 years. However the mechanisms by which the cartilage is repaired in this method are unclear. Previous research compared mRNA expression of 62 gene Markers of 4 OA dogs and 4 distracted dogs. One of the genes that is much higher expressed in distraction compared with OA was Vitamin D receptor (VDR). In this paper, these cartilage samples were immunohistochemically stained for VDR and the mRNA expression pattern of 25-OH D-α-Hydroxylase and 25-OH D-24-hydroxylse in all knee tissues were assessed. It was found that 25-OH D-α-Hydroxylase in the tibia is more expressed in distracted donors whereas in the femur and in subchondral bone it is more expressed in OA donors. 25-OH D-24-hydroxylse in cartilage and subchondral bone is more expressed in the OA donors than in the distracted ones. The assessment of the stained cartilage samples confirmed the VDR mRNA pattern, showing that distracted donors were more positive than samples of the OA donors. There was however a big variability between donors. Indeed, the positive chondrocytes interestingly followed a pattern of distribution where in normal positive donors they can be found on the sides of the superficial zone. In highly positive donors the positive cells can be seen in the whole superficial zone, sometimes even stretching into the middle zone, but never in the deep zone.

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Introduction

Osteoarthritis(OA) is one of the most common chronic afflictions present in the western world[2]. A majority of individuals over the age of 65 have radiographic and/or clinical evidence of OA[1]. Age seems to be the major risk factor[1]. During aging, several changes in the cartilage can be observed. Softening of the articular surface and decreases in the tensile strength and stiffness of the matrix are among these[1]. Most of them can be attributed to changes in the components of the extracellular matrix or ECM(secreted by the chondrocytes), as their capacity to remodel and repair the cartilage diminishes with age[1]. Another big risk factor is obesity, since obese women have nearly four times the risk of developing OA as non-obese women[1]. In men the risk is nearly five times greater[1]. Other risk factors include: female gender, pre-existing early OA, race/ethnicity, genetics, nutrition, smoking and injuries/trauma's to the joint[1,2].

OA is most clearly characterized by the degeneration of the cartilage[1], although an intraarticular inflammation with synovitis and changes in the subchondral bone can also be seen[1]. As such, it is important to know that OA is not a disease limited to the cartilage[2]. The synovium, joint capsules, ligaments, periarticular and subchondral bone and even the muscles acting across the joint are also affected[1,2].

OA occurs most frequently in hands, feet, knees and spine, but knee OA is most likely to lead to disability, and almost 95% of the total knee and hip replacements are the result of symptomatic OA[2]. Although OA is not considered a typically inflammatory disease, symptoms include joint swelling, pain and stiffness[1]. Advanced OA can also cause pain in rest, sometime enough to wake the patient while sleeping[2]. The pain is usually described as a deep aching and is poorly localized[2]. Radiation of the pain away from the joint is also possible[2]. As mentioned before, severe knee OA in humans can result in the need for a total knee replacement[3]. In humans total knee replacement is also an option, but it is not frequently used for dogs because it is not that debilitating to live without one limb[4].

The normal composition of cartilage

The knee is a synovial joint and it's articular surface is lined with cartilage. Cartilage is essential for providing lubrication to the surface of the joint allowing for painless and smooth movement[2]. Thereby, it minimizes the stress on the underlying subchondral bone[2]. The only cell type present in articular cartilage is the chondrocyte[2]. These cells lay widely dispersed within the self-produced ECM, lacking blood vessels, nerves or lymphatic vessels[2].

The ECM contains a framework of molecules which retain fluid[2]. In fact up to 80% of the wet weight of cartilage is tissue fluid[2]. Tissue fluid is water containing gases, small proteins, metabolites and a high concentration of cations[2]. Some of them(around 30%) are located within the collagen's intrafibrillar space as a gel or in the intracellular space(very low percentage), even though most is contained in the pores of the matrix[2]. Collagen is the most abundant macromolecule in the ECM(60%), which is almost all collagen type II[2]. Collagen I, IV, V, VI, IX, X and XI are also present but in much lower quantities and serve to stabilize the collagen II[2]. The second-largest group are proteoglycans, among which aggrecan is the main one[2]. It interacts with hyaluronic acid and link proteins to form large proteoglycan aggregates called Glycosaminoglycans(GAG's)[2]. As such proteoglycans are secured in within the matrix and provide cartilage with its osmotic properties[2]. The smaller ones (decorin, biglycan and fibromodulin)do not contribute directly to the mechanical properties of cartilage but interact with several types of collagen[2].

Within the cartilage itself a distinction can be made between different "zones"[5]. There are 3 zones, the superficial or tangential, the middle (also called "transitional" zone) and the deep(also called "radial" zone)(figure 1)[2,5]. Some others consider calcified zone as well and other groupings are sometimes made[2,5]. The superficial zone is in contact with the synovial fluid and is the thinnest of the zones[2]. It contains a relatively high number of chondrocytes and a lot of collagen packed parallel to the surface(figure 1)[2]. This organization gives cartilage its tensile stiffness and allow it to resist the sheer, tensile and compressive forces generated by locomotion[2]. The middle or transitional zone contains thicker collagen fibrils and proteoglycans and provides resistance to compressive forces[2]. The deep or radial zone contains most of the proteoglycans and the lowest water concentration, this zone is gives the greatest resistance to the compressive forces[2]. At the end of this zone the "tidemark" can be found (this will appear as a basophilic line in histological sections), which separates the deep zone from the calcified zone[2]. The calcified zone links the cartilage to the underlying bone and is also permeable to small-molecule transport[2]. As such it is not really considered cartilage and therefore not included in the segmentation of the zones.

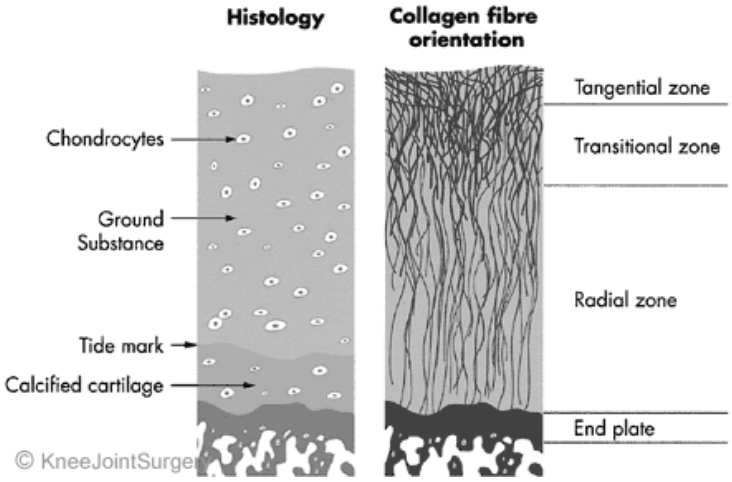

Figure 1: Zoning and orientation of collagen in articular cartilage[12].

Underneath the calcified cartilage the subchondral bone starts, the borderline between these two zones is very sharp and is called the "cement line"[2]. First the subchondral bone plate can be found[2]. It contains small venous and arterial vessels that are directly in contact with the (calcified) cartilage, the subchondral bone plate and the trabecular bone beneath it[2]. Subchondral trabecular bone is more porous than the subchondral bone, more metabolically active and contains blood vessels, sensory nerves and bone marrow[2].

Besides the tendons, ligaments and patella, there is a structure in the knee which is important in the context of OA. This is the infrapatellar fat pad which is composed of a fibrous framework with embedded fat tissue[2]. This fat pad can be found underneath the patella and in close contact with the articular cartilage, bone and synovium[2]. Not much is known about its function but it contains large numbers of adipocytes, fibroblast, macrophages, leukocytes and other immune cells capable of producing inflammatory cytokines[2]. Also nociceptive nerve fibers are present and anterior knee pain (which is common in OA) is thought to be associated with pathology of the fat pad[2].

The joint cavity of the knee is filled with synovial fluid and surrounded by articular cartilage and fibrous capsule[2]. The inner lining of this fibrous capsule is made up by synovium[2]. The synovium is a thin layer of vascularized connective tissue with fibroblast- and macrophage-like cells within a ECM[2]. This are the cells that produce the synovial fluid. Synovial fluid provides boundary lubrication and so it reduces friction, helping to maintain the cartilage surfaces[2]. The synovium cells also produce a variety of molecules like hyaluronan (HA) and proteoglycan 4 (PRG4 also known as lubricin or superficial zone protein SZP)[2]. HA aids in regulating the viscosity and maintenance of the synovial fluid, thus playing a role in regulating the coupling between draining and input (synovial fluid an ultra-filtrate of blood plasma)[2]. Compared to blood, there are a lot less cells present in the fluid, with less than 200 leukocytes per mm^3 (in whole blood this number is 3.540-9.060)[2]. Furthermore lymphocytes,

macrophages and shed lining cells can also be found[2]. Molecules present in the fluid are cytokines and growth factors which are either pro- or anti-inflammatory[2]. Normally these are present in low concentrations but this can change drastically in the case of injury or disease e.g. OA[2]. Proteolytic and matrix-degrading enzymes (like aggrecanases ADMTS, serine and cysteine proteinases) can be found as well, being strictly regulated like in the case of the cytokines and growth factors[2,19].

The origin and course of osteoarthritis

Al the normal structures mentioned above are affected or play a role in OA. The chondrocytes in the cartilage are post-mitotic and normally don't replicate, being responsible for maintaining the ECM[2]. The cells are metabolically active and guard the homeostasis by synthesizing ECM products, ensuring a turnover of its components[2]. They respond to extracellular cues and mechanical signals (altering the ECM to the joints use)[2]. However the lack of replication means that chondrocytes in an older individual can be decades old[2]. Over time the cells have accumulated detrimental changes and the capacity to synthesize certain ECM-products and responding to anabolic stimuli decreases[2]. This is one of the reasons that age is the biggest risk factor for OA.

OA can be induced by "abnormal" loading on normal cartilage or "normal" loading on abnormal cartilage[2]. After a trauma or in the elderly, a small fibrillation or disruption at the surface of articular cartilage emerges[1,2]. If the degradation continues, the articular surface becomes irregular forming clefts extending deeper into the cartilage and eventually reaching the subchondral bone[2]. The tips of the irregularities and walls of the clefts eventually collapse causing a thinner cartilage layer and fragments of ECM[2]. These fragments such as fibronectin and collagen type II bind to Toll-like receptors (TLRs) and so initiate an immune response[2]. Other fragments like, but not restricted to, HA, fibronectin and biglycan are also ligands to TLR's[2]. TLRs are activated as part of the non-specific immune response[2]. This is why degradation of the matrix in OA mirrors a chronic injury[2]. Free fragments also come into the joint space where they come in contact with the synovium and the synovial lining cells[2]. Phagocytic cells incorporate the particles and become hypertrophic (also hyperplasia can be seen)[2]. Catabolic and pro-inflammatory mediators are produced(also as a result of binding to TLR's)[2]. They activate the chondrocytes to produce matrix metalloproteinases or MMP's which are a group of matrix-degrading enzymes[2]. These proteins are capable to break down even the large components of the ECM, also making the ECM more porous[2]. As a result of this, the cartilage is degenerating even further and more fragments are released into the joint space leading to a vicious cycle[2]. Binding to synovial TLR's also results in de production of chemokines and cytokines which effectuate cellular infiltration of lymphocytes, macrophages and granulocytes[2]. TLR activation by these pathways are for a major part responsible for synovitis in OA[2].

The downward extending cartilage degradation eventually reaches the calcified cartilage layer and the subchondral bone[2]. Here they result in microdamage and cracks which initiates bone remodeling[2]. So in the early stages of OA remodeling and loss of the subchondral bone can be seen[2]. Over time this results in reduced thickness of the subchondral bone and again more porosity[2]. Even deeper down in the trabecular bone there is also deterioration and decreased bone volume and a wider spacing of the trabecula[2]. These alterations in the bone also generate changes in the joint shape and thus loading of the joint this may probably enhance further cartilage loss[2]. Moreover, the growth of osteophytes, which are fibrous and cartilaginous bony protrusions(figure 2), and intraosseoussubchondral bone cysts (SBC's) can be

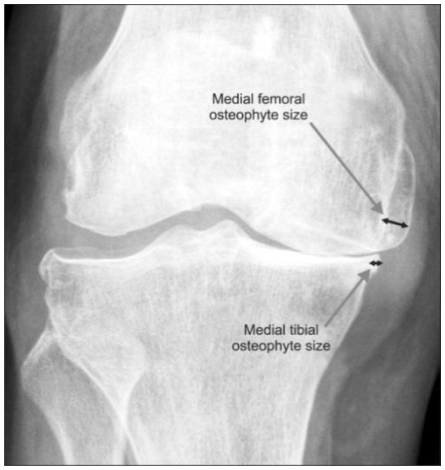

Figure 2: Osteophytes in a human knee as seen on a radiograph[6].

seen(figure 3)[2]. Their presence can restrict motion and cause pain with movement[2].

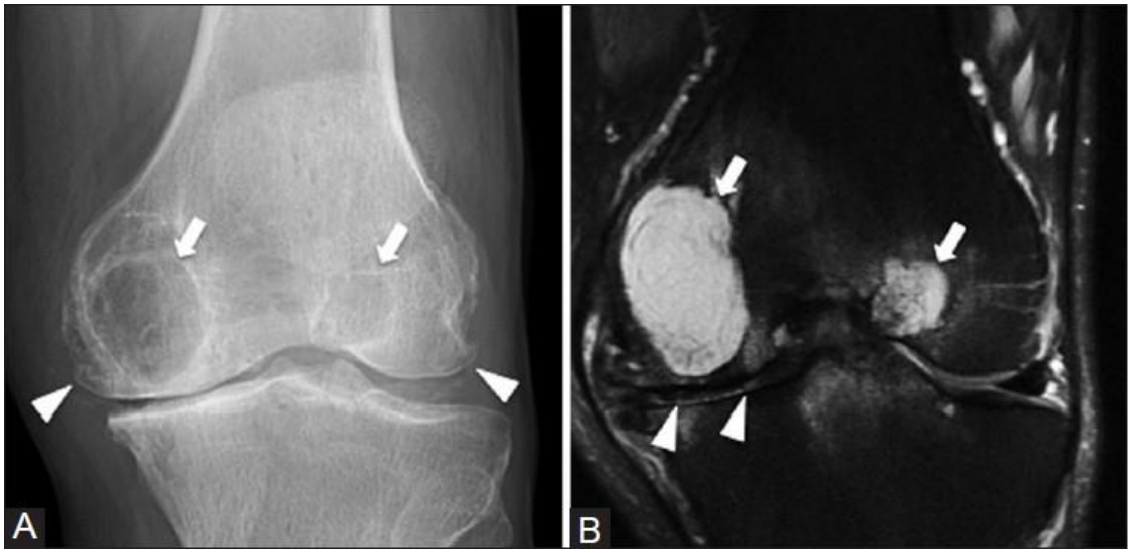

Figure 3: Subchondral bone cysts in degenerative knee osteoarthritis in human. A: as seen on a radiograph and B: as seen in MRI imaging[7].

As the OA progresses further, the ECM (the chondrocytes biochemical environment) changes, and several mediators are released by the chondrocytes. It gives a boost of anabolic activity and proliferation, predominantly in the upper cartilage zones in an attempt to restore the ECM[2]. Normal chondrocytes don't replicate but in OA chondrocytes in a later stage can be found in clusters[2]. The mechanisms by which this feature commences is still unclear[2]. The clustered chondrocytes start to produce products that are normally found in chondrocytes undergoing terminal differentiation (like in the process of endochondral ossification)and are clearly hypertrophic[2]. Mineralization follows and the chondrocytes are replaced with bone tissue which further decreases the articular thickness[2]. The vascular zone containing bloods vessels, sensory nerves and osteoblasts/–clasts reach the deep cartilage and progress to the surface[2].

In late OA, in contrast with the early OA a thickening of the subchondral bone is seen as well as an increase in density[2]. The trabecular bone shows a decreased trabecular separation and bone marrow spacing, and an increased thickness[2]. The subchondral bone is now described as sclerotic[2]. The chondrocytes are unable to fix the damage and the normal activity and homeostasis is lost[2]. There is a decline in proliferative and anabolic activity[2]. Which leads to more tissue destruction and eventually chondrocyte cell death[2]. By this stage the whole joint is severely damaged.

Treating osteoarthritis by the distraction method

OA can be treated in several ways. Pharmacologically it can be handled with nonsteroidal anti-inflammatory medications. Intra-articular injections are also possible with corticosteroids, hyaluronic acid (HA) or a plasma-rich protein(PRP)[2]. However these options don't run out surgery, and in most cases a total knee replacement/arthroplasty(TKA) is required[2,3]. The number of TKA's is increasing also due to a higher rate of obesity and an aging population[3]. This also means that the number of revisions for the TKA is going up as it only last for about 10 to 15 years depending on use and loading[3,8]. 44% Of all total knee revisions are remarkably preformed in patients younger than 65 years of age[3]. Besides a lot of discomfort for the patient, this is also very costly so new methods would be beneficial for both sides[8].

Over the past years such a new method was developed: the knee distraction. In this treatment, an external fixation frame (with coiled springs) is applied to bone with pins that are secured into the tibia and the femur (figure 4)[3]. The cartilage surfaces are then gradually separated to a certain extent (+-5 mm) for a given period of time (for humans 6-8 weeks)[1]. In human, using various imaging and biochemical markers to determine structural repair, in a group of 20 end stage OA patients after one year, there were still positive clinical benefits and signs of cartilage repair[3]. In a group of patients, longer follow ups were conducted and after 2 years VAS-pain, OARSI-scores, radiographic analysis and biomarkers analysis were conducted[3]. It was also proven that the benefits were present in the long term (at least 5 years) [1,3]. In a canine OA model treated with distraction, macroscopic joint damage, collagen damage, synovial inflammation and proteoglycan loss were significantly less than in dogs with just OA[1]. However the mechanisms why and how the cartilage improves are still not found[1,3].

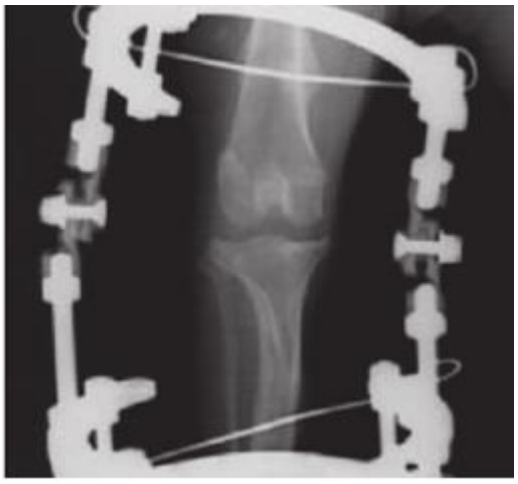

Figure 4: Knee Distraction frame and pins in a human knee[9].

A previously conducted study by Miranda-Bedate et al. further looked into the possible mechanisms. They induced OA (with a groove model) in 8 dogs. After 10 weeks 4 of the animals received treatment by knee distraction (D-group) while the others served as disease controls(OA). After 4 weeks of distraction, the dogs were euthanized and joint tissues including fat pad, synovium, meniscus, cartilage and bone were recovered. qPCR analysis was performed on these tissues for 62 regenerative gene markers. Typical markers of OA were present in the cartilage of the OA group, like matrix remodeling genes (MMP9, MMP13 an ADAMTS5), ECM components (COL1, COL2 and ACAN), inflammation (COX2, PTGES1 and ptgeS2) and hypertrophy (COLX, SMOH, VEGFA and VDR)(figure 5). The presence of these markers, together with a higher OARSI scoring in cartilage, validated the applied canine OA-model (figure 5). Principal component analysis (PCA) was conducted to see which genes could be playing an important role. One of those was the Vitamin D Receptor(VDR). As a marker for hypertrophy, it was expected to be upregulated in the OA group but, interestingly, was significantly more expressed in the D group.

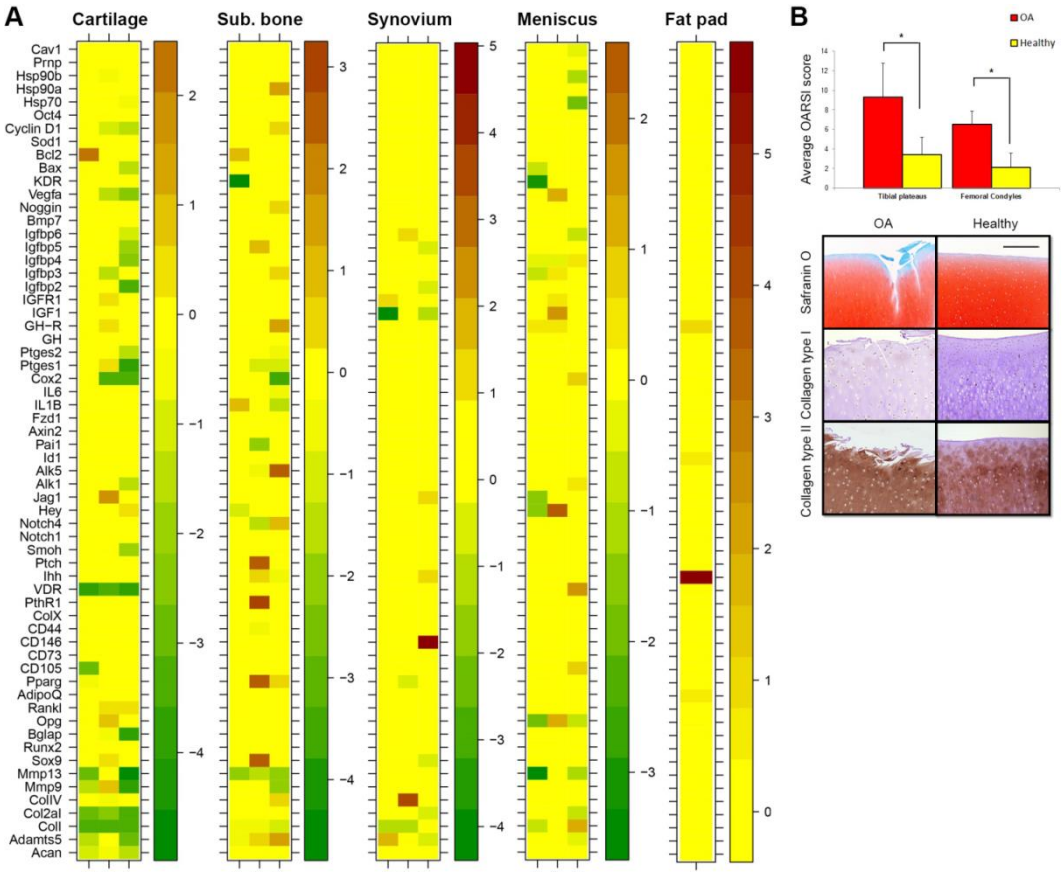

Figure 5 A: Gene expression profile (ΔCq values; upregulated genes are represented in green while downregulated in red) of the different tissues studied in OA vas Healthy.
B: OARSI score from OA and healthy cartilage with corresponding Safranin-O/fast Green staining. As expected, higher scoring was obtained for OA than the healthy situation. Collagen type I and II are also inclued with no apparent differences between OA and healthy knee joints. (Miranda-Bedate et al. unpublished data).

Vitamin D

The component that binds to the VDR is 1,25-dihydroxyvitamin D (from now on referred to as $1,25(OH)_2$ vit D), which regulates the synthesis of specific proteins for the maintenance of the calcium homeostasis[10]. Exposure to ultraviolet light (sunlight) in the skin converts 7-dehydrocholesterol into vitamin D_3, a prohormone[10]. When there is not enough exposure to sunlight, vitamin D_2 or vitamin D_3 are absorbed from diet in the small intestines[10]. In the liver this circulating vitamin D is hydroxylated to form 25-hydroxyvitamin D (25(OH) vit D)[10]. This metabolite is the major circulating form of vitamin D[10]. 1-α-hydroxylation of 25(OH) vit D (which mostly occurs in the kidney) forms the earlier mentioned steroid hormone $1,25(OH)_2$ vit D which can bind to VDR[10]. Parathyroid hormone (PTH) is the primary stimulus for $1,25(OH)_2$ vit D, by the increase of 1-α-hydroxylation activity[10]. The parathyroid gland secretes PTH when the concentration of the calcium in the plasma is getting low[10]. The reactivity to $1,25(OH)_2$ vit D depends on the number of VDR's present in the tissue, by which the higher the number of VDR's the higher the biological response[10]. The binding of $1,25(OH)_2$ vit D to a VDR results in a specific DNA binding which provokes the induction or suppression of specific messenger RNA's[10]. The classic target tissues of $1,25(OH)_2$ vit D are bone, intestine and kidney[10]. The full extent of vit D is still not fully determined and it has a wide range of effect on different cell types[12]. Even more so, the complex of a VDR and $1,25(OH)_2$ vit D can bind to 2000 to 8000 different vitamin D response elements in the DNA again depending on cell type[12].

In the intestine 1,25(OH)$_2$ vit D stimulates the synthesis of calcium-binding protein which will facilitate the transportation of calcium from the luminal side of the enterocytes (calcium enters the enterocyte by a concentration gradient) to the basolateral membrane[10]. Then the calcium is exported out of the enterocyte into the extracellular fluid[10].

24-and 25-1-α-Hydroxylases

As mentioned before, the enzyme that converts 25(OH) vit D to 1,25(OH)$_2$ vit D is a 1-α-hydroxylase, 25-OHD-1-α-hydroxylase, from now on mentioned as 25OH-αhydroxylase. This is a mitochondrial cytochrome P450 enzyme that is under regulation of PTH, calcium, phosphorus and 1,25(OH)$_2$ vit D itself and is mostly located in the kidney[10,15]. 25(OH)vit D is also converted into 24,25-dihydroxyvitamin D (24,25(OH)$_2$ vit D) by 25-OHD-24-hydroxylase (24-hydroxylase), a hormone which role is not yet clear[15]. This is however, one of the degrading steps of 25(OH) vit D[16]. It can also degrade 1,25(OH)$_2$ vit D, so 24-hydroxylase initiates for both compounds their degradation to excretory metabolites[16]. 24-Hydroxylase activity is present in almost all target tissues[16]. As such, in joints (bone) 25OH-α- and 24-hydroxylase activity is reported[17].

The role of vitamin D and VDR in bone and cartilage

Bone is highly dependent on 1,25(OH)$_2$ vit D not only for normal growth but also for remodeling[10]. Vit D deficiency results in osteomalacia in adults, where the osteoid (the unmineralized matrix made by osteoblasts which is present prior to bone maturation) cannot be mineralized[10]. Young animals will develop into rickets. In this case not only the osteoid but also the cartilaginous matrix at the growth plates is not be mineralized[10]. Defective mineralization of bone and cartilage is a tell-tale sign of vitamin D deficiency[10]. This derives primary from decreased plasma calcium and phosphorus concentrations[10]. Moreover, there is evidence that 1,25(OH)$_2$ vit D plays a role in the production of bone matrix proteins and that it plays a direct role in the repair of bone fractures[10].This also indicates that 1,25(OH)$_2$ vit D has a role in bone formation[10]. Notably 24,25-dihydroxyvitamin D (24,25-(OH)$_2$ vit D) can be involved in the mineralization process[10]. If PTH is present 1,25(OH)$_2$ vit D importantly participates in bone resorption and it has stimulatory effects on osteoclasts[10]. By resorption of the bone low blood calcium levels can be elevated by the calcium obtained from the degradation of the mineralized bone[10,12].

Differentiation of mesenchymal stem cells into osteoclasts can be stimulated by vitamin D[12]. Osteoblast, mainly the immature ones, express VDR[12]. The primary function of osteoblasts is mineralization and bone formation[12]. Evidence suggests that OA osteoblasts show increased vitamin D induced bone formation activity which is consistent with the findings of osteophyte formation and subchondral sclerosis[12]. The expression of vascular endothelial growth factor (*VEGF*) is also influenced by vitamin D in OA osteoblasts[12]. As stated before in previous work, PCA correspondingly retrieved *VEGF* to be upregulated in canine OA knee compared to the healthy controls. *VEGF* is a strong angiogenic cytokine which results in angiogenesis and the extension of the vascular network in the joint[12]. The growth of the blood vessels is associated with increased pain (extension sensory nerves) and also with the loss of the structural integrity of the cartilage[12]. As mentioned, osteoclast are responsible for the resorption of bone[12]. High doses of vitamin D have been shown to increase bone resorption although VDR expression is low, so it has been suggested that vitamin D acts directly on this cells[12]. Evidence is however conflicting[12].

In OA chondrocytes the balance in the maintenance of the ECM is disturbed[12]. Catabolic processes are bigger than the anabolic processes and the cartilage is degraded[2,12]. Hypertrophic and proliferation chondrocytes (as can be observed in OA) have been proven to express more VDR than in healthy chondrocytes[12,13]. VDR expression is also associated with the expression of the earlier mentioned MMP's which degrade bone and cartilage[12]. By VDR signaling, chondrocytes also regulate oesteoclastogenesis, leading to the induction of *RANKL* expression(membrane protein of the TNF

superfamily)[12]. So vitamin D is involved in the hypertrophy and impaired mineralization in OA chondrocytes[12]. It appears that vitamin D has negative effects on OA cartilage but studies strongly contradict each other[12]. Some studies show that in vitro exposure to vitamin D inhibits the hypertrophic cell differentiation (characteristic for OA)[5], and some others have shown that more exposure to vitamin D seems to have a positive result on OA decreasing pain and improving function in vivo [5,14]. So it's safe to say that the exact role and mechanisms of vitamin D and the VDR in OA chondrocytes are not yet completely understood.

Aim of the study

The research conducted and described in this paper will further look into the VDR and will be primarily be focused on cartilage. qPCR analysis of the enzymes 24OH hydroxylase and 25OH-α hydroxylase will be done comparing the OA group, D group and healthy contralateral canine groups. Also immunohistochemical staining of cartilage tissue of the donors was preformed to elucidate the role of VDR in knee distraction, OA and cartilage as a whole.

Materials and Methods

Animal model

Osteoarthritis was induced unilaterally according to the canine 'Groove model'(≥1.5-year old dogs), a well-validated and accepted model of OA, scratching the femoral side[19]. After 10 weeks distraction of the knee joint was applied (figure 6).

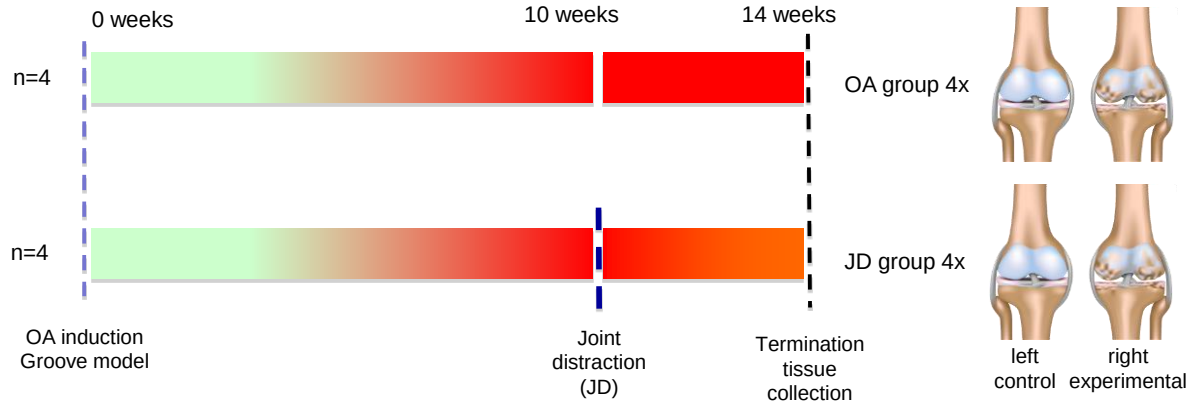

Figure 6: Schematic representation of the in vivo experiment conducted in 8 skeletally mature Mongrel dogs. Over the course of 10 weeks osteoarthritis was developed after application of the Groove model. Thereafter, distraction of the knee joint was applied in n=4 dogs over the course of 4 weeks.

Collection of material post-mortem

After 8 weeks of distraction, the animals were euthanatized, and joints were macroscopically scored and imaged. Immediately thereafter, joint cartilage from medial and lateral tibial plateaus and medial and lateral femoral condyles were collected.

RNA extraction

Total RNA (including miRNA) from cartilage was extracted (miRCURY RNA Isolation Kit (Exiqon)) after the samples have submitted to a short Tissuelyser cycle (Qiagen). The rest of the protocol was performed according manufacturer's instructions with an additional DNA digestion step with DNase (Qiagen). RNA quality and quantity was measured with a Bioanalyzer (Nano-chip , Agilent Technologies). cDNA was produced using the iScriptTM cDNA Synthesis Kit (Bio-Rad) with a similar RNA input for all samples.

qRT-PCR

VDR, 24OH-hydroxylase, 25OH-αhydroxylase genes were analyzed (for primer sequences and melting temperatures see Table 1). Quantitative PCR was performed using a *CFX384 Touch™ Real-Time PCR Detection System* and *IQ SYBR Green SuperMix* (both from Biorad) according to the manufacturer's protocols. Standard curves consisted of 4-fold serial dilutions of the cDNA template. For each standard curve, the amplification efficiency was between 90% and 110%. The normalization of gene expression was performed with a battery of 15 reference genes, 7 of which were chosen using *GeNorm* program: *RPS19, SDHA, YWHAZ, TBS, RPS5, RPL13* and *HPRT*.

Gene	Sequence	**Tm (°C)**	Amplicon(bp)
Vdr	GATTCCAGGGTTCAGTGAC ATGAGGGGCTCAATCAG	53	210
24OH-hydroxylase	TTATATGCGGCTGTCACGG TTCCTCAAATCCTCTGCCC	64	173
25OH-αhydroxylase	CATTTGCCCAGCAGCAC GCGTGTTGGATACCGTGTC	63.5	192

Table 1: Characteristics of the primers used in the present work.

Statistical Analysis

The ΔCt values obtained after a *Normfirst* transfromation were statistically analyzed (*RStudio*, http://www.rstudio.com/, Foundation for Open Access Statistics) for the 3 comparison groups (Distraction knee vs Healthy knee, OA knee vs Healthy knee and Distraction knee vs OA). Two statistical models were employed: a *Linear Mixed Model* for normally distributed data and a *CoxProportional Hazard Model* for normally and non-normally distributed data, using the canine donor and the different anatomical knee locations (medial/lateral compartment, tibial plateaus and femoral condyles) as random effects. For the normally distributed data, the chosen model for the statistical analysis of each parameter is the one which retrieves the lower AIC (*AkaikeInformation Criterion*) value. Results were subjected to corrections for multiple testing (False Discovery Rate (FDR)).

Stainings

Specific immunostaining was performed for VDR, using up and running protocols and proper isotype negative control antibodies. Briefly, sections were deparaffinized and subsequently rehydrated in a descending ethanol series. Afterwards, antigen retrieval was used to localize the VDR (citrate buffer 10 mM, pH 6, for 60 min at 70°C), followed by endogenous peroxidase blocking (*Dual Endogenous enzyme block* (S2003; Dako), 30 min). Before adding the primary antibody, serum block was performed using *normal goat serum* (G9023, sigma) at a 1:100 dilution for 30 min. The sections were incubated with primary antibody overnight at 4°C (primary monoclonal rat anti-human VDR (MAB710, 1:25 dilution; Thermo Fisher Scientific). Thereafter, secondary antibody was applied for 30 min at RT (*Goat anti-Rat IgG*, HRP conjugate, AP136P, Millipore). The staining was visualized with the DAB substrate chromogen system (Dako). For the counterstaining *Hematoxiline QC* (H3404, Vector) was used.

Results

qRT-PCR

qRT-PCR was focused on the cartilage. In previous research bij Miranda-Bedate et al. (publication in progress) a significant higher difference in expression of VDR in distraction than in OA was observed (figure 7).

25OH-αhydroxylase (one of the main activating enzymes of vitamin D) in tibial cartilage of distracted donors is significantly more expressed than in OA donors(figure 8). However in the femoral cartilage 25OH-αhydroxylase is more expressed in the OA donors. In subchondral bone some significant values were also found, where 25OH-αhydroxylase is more expressed in the OA group (figure 8).

24-OH hydroxylase (one of the main degrading enzymes of vitamin D) in femoral cartilage from the medial side is significantly more expressed in the OA group than in the distracted group(figure 9A) Although not significant in other cartilage locations there is also a tendency to be more expressed in the OA group (data not shown). In tibial subchondral bone, there is also significantly more expression of 24-OH hydroxylase in the OA group compared to the distracted group(figure 9B). The rest of the data in subchondral bone shows the same tendency although not significant (data not shown).

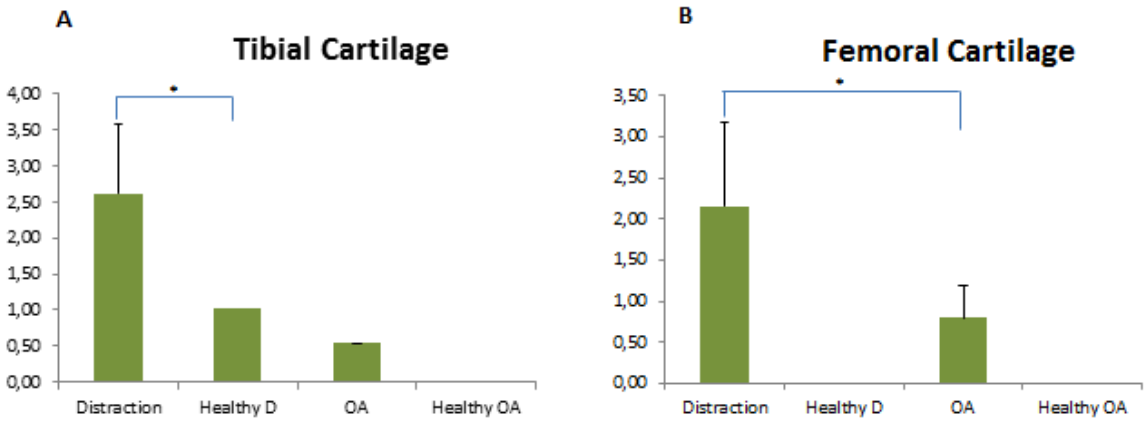

Figure 7 Fold changes of VDR mRNA expression in different regions of cartilage. Statistical significant differences between groups are marked with an asterisk. A: Tibial medial and lateral sides combined. B: Femoral cartilage medial and lateral sides combined.

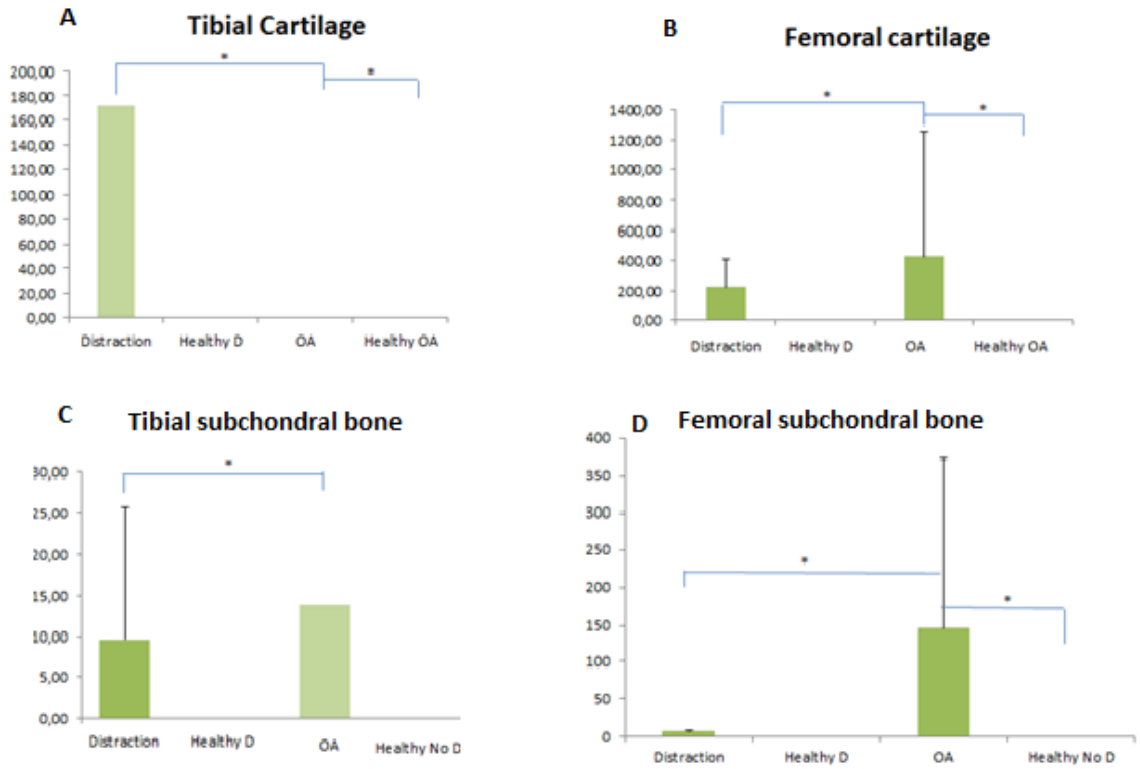

Figure 8: Fold changes of 25OH-αhydroxylase mRNA expression present in different regions of cartilage and subchondral bone. Statistical significant differences between groups are marked with an asterisk. A & B Tibial and Femoral cartilage medial and lateral sides combined. C: Tibial subchondral bone medial and lateral sides combined. D: Femoral subchondral bone medial side.

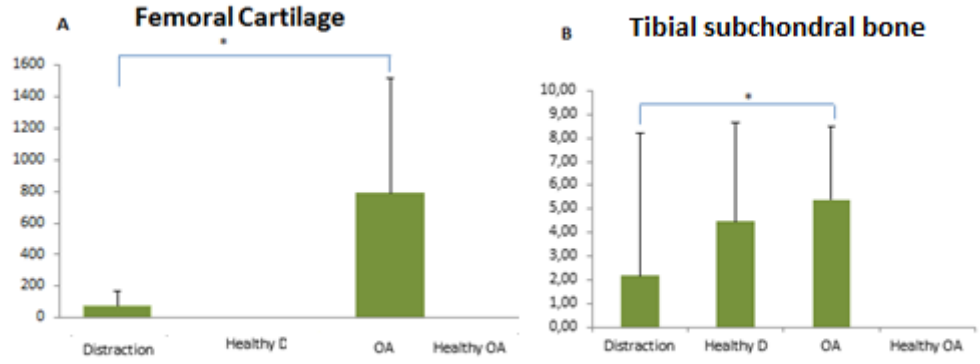

Figure 9: Fold changes of 24OH-hydroxylase mRNA expression present in cartilage and subchondral bone. Statistical significant differences between groups are marked with an asterisk. A: Femoral cartilage medial side. B: Tibial subchondral bone lateral and medial sides combined.

After staining, the harvested cartilage samples were assessed under the microscope. It was observed that VDR was located in the nucleus (with some cytoplasmic staining) and in general positive chondrocytes are distributed in the sides of the superficial zone of the cartilage(figure 10). Positive cells could be found further along the superficial and middle zones in the highly positive donors(figure 10 and 11).

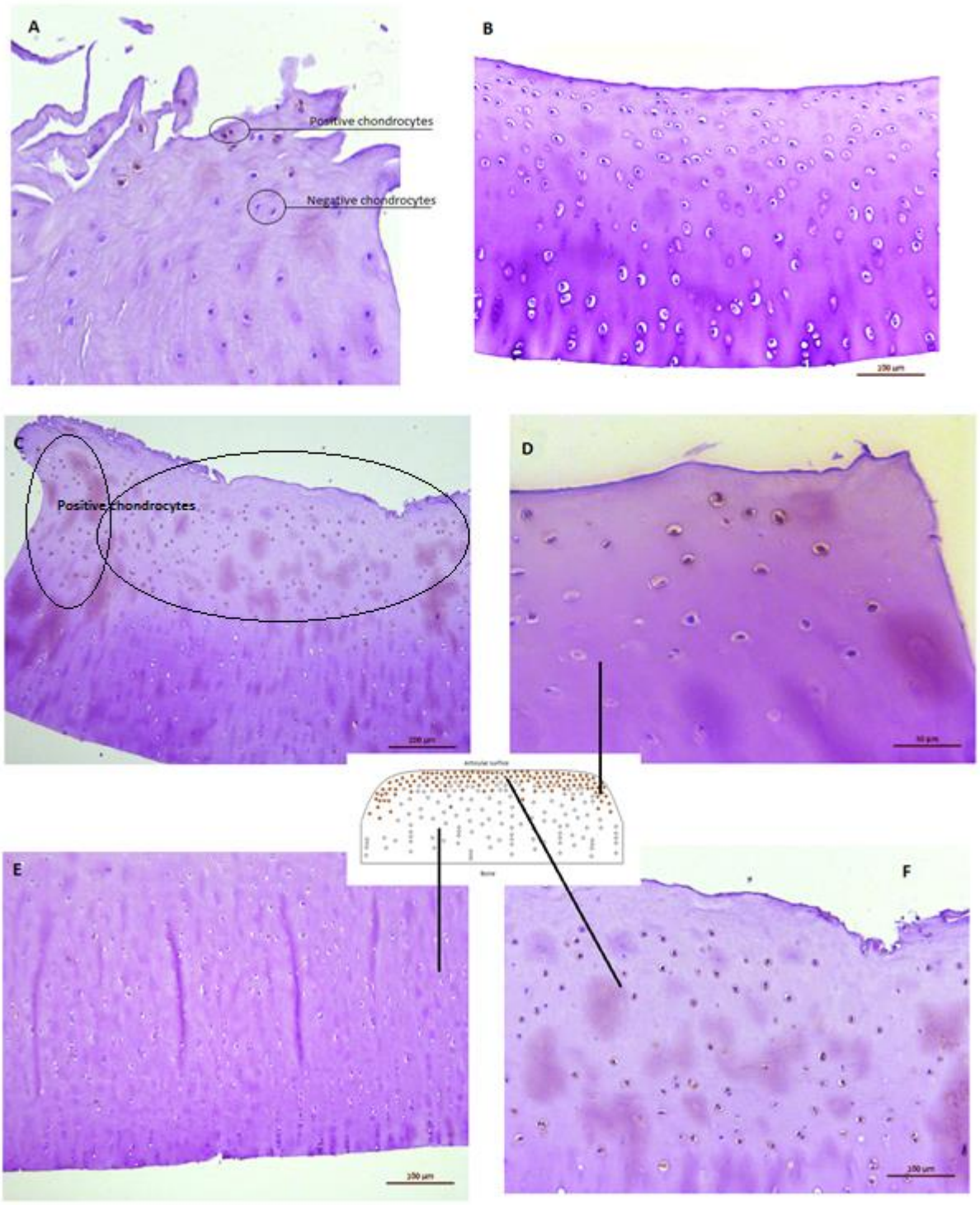

Figure 10 Photos of the observed VDR-positive chondrocytes in their samples, in the middle a schematic overview identical to figure 10A is placed. A: Typical positive and negative chondrocyte in positive sample. B: Overview of a negative sample C: Overview of a highly positve sample. D: Distribution of positive chondrocytes to the sides in a moderatly positive sample. E: Positivity does not reach the deeper zone (note there are still some positive cells in the middle zone). F: Positive chondrocytes in the middle superficial zone extending to the middle zone in a highly positive sample.

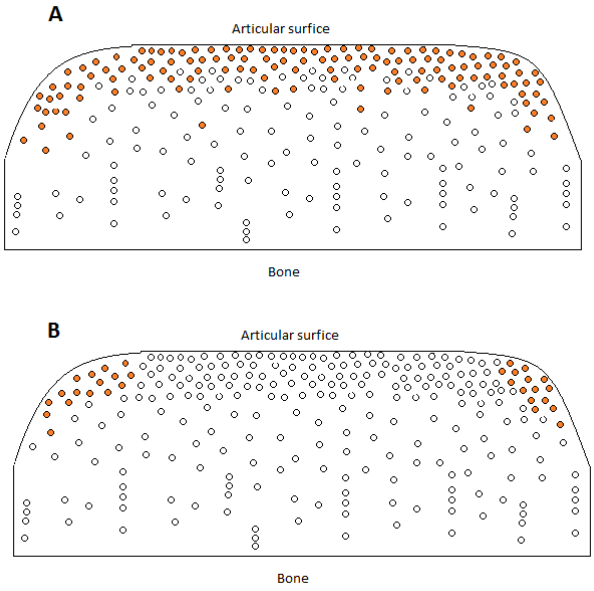

Figure 11 Schematic overview of the distribution of VDR-positive chondrocytes (orange) in a cartilage sample. A: Positive cells can be seen in the superficial zone stretching a bit into the middle zone in the highly positive donors. B: Positive cells can be seen on the sides of the superficial zone of the normal positive donors.

These data were transformed into a qualitative scoring (table 2). Negative donors are represented with a "-" (and were given the value 0). If the sample is positive a various "+" signs can be given based on the extension of positive chondrocytes along the cartilage sample in the superficial zone and middle zone, and was randomly assessed by two different researchers. "+" Has been given the value of 1 an this can be increased until "++++" which have been given the value 4. The total score of the samples of affected knees on distracted donors is 26 whereas the total of the OA donors is 5. Remarkable is that in the distracted donors the "healthy" contralateral knees have a total score of 5 (versus 0 in OA).

Furthermore numbers were grouped together in OA and distracted knees for comparative reasons. Which showed that in the tibial plateaus a higher total score was obtained (21) compared with a score of 10 in the femoral condyles.

Donor	84	85	86	87	88	89	90	91
II-1 lat.	-	+	-	+-	-	+	+-	+++
II-2 med.	+	++	-	-	-	-	+(+)	+++
II-3 lat.	-	-	-	-	-	-	-	+
II-4 med.	++	-	-	-	-	-	-	-
II-5 lat.	+	-	-	-		-	-	++
II-6 med	-	+	-	-	-	-	-	+(+)
II-7 lat.	-	-	-	-	-	-	-	-
II-8 med.	-	-	-	-	-	-	-	-

Table 2 Qualitative schematic overview of the VDR positivity degree observed for the different donors . The grey donors are those treated with distraction. White donors are OA dogs. II-1 II-2 II-5 II-6 are samples from the affected knee (OA or distraction) with "lat" meaning "lateral side" and "med" meaning "medial side". The rest are their respective healthy contralateral knees. In red, a sample that was lost.

Discussion

This research has been based on cartilage samples of OA induced dogs treated with the distraction method. They received this treatment for over 4 weeks and after that, the samples were studied. It is Confirmed that the expression of VDR in the staining is in line with the findings of the qRT-PCR. VDR is more expressed in samples of donors which had received the distraction treatment. However there is a big variability between donors, even within the same group (OA or distraction) with some donors being much more positive. Interesting is that the positive chondrocytes in the samples can be seen according a pattern. In "normal" positive donors the cells can be seen on the sides of the superficial zone. However in highly positive donors the positive chondrocytes stretched all over the superficial zone even extending into the middle zone. No positive cells were observed in the deep zone though. It is noticable that in de qualitative scoring of the VDR-positive chondrocytes positive cells were also seen in the healthy contralateral knees and, in accordance the cartilage also looked damaged. This was only seen in the distracted group. It can be suggested thatthe external frame and operation might have caused abnormal loading of the not affected knee inducing the injury.

In the mRNA expression of tibial cartilage 25OH-αhydroxylase was significantly higher in the distracted group than in the OA group. However, in the rest of the anatomical locations mRNA of 24-OH and 25OHα-hydroxylases were higher expressed in the OA group while VDR was higher in the distraction group. Bearing in mind, as explained, that the active form of vit D, $1,25(OH)_2$ vit D is synthesized by 25OH-αhydroxylase and that it is degraded into $24,25\text{-}(OH)_2$ vit D by 24 OH-hydroxylase, it seems to be a higher turnover of vit D in OA than in distraction, perhaps suggesting that more active $1,25(OH)_2$ vit D is present in distraction. Another suggestion that could be given is the following: There is a paracrine feedback loop between PTH and $1,25(OH)_2$ vit D where PTH related protein increases the expression of VDR but does not affect the expression of 24 OH-hydroxylase and 25OH-αhydroxylase[20]. This mechanism could be the responsible for the higher amount of VDR expression in distraction but not of the hydroxylases[20]. Apart from these changes in cartilage some significant differences in subchondral bone were also found. This suggests that subchondral bone is also very active in both the process of OA and distraction, although it is beyond the scope of this study to further look into it.

As stated before it is known that VDR is a marker for hypertrophy and is upregulated in OA[12]. But if the distraction method is beneficial for OA it is contradictory that VDR is upregulated by the distraction treatment as shown by Miranda-Bedate et al(unpublished results)[3]. However VDR was not the only marker that was examined.(figure 12).

Cluster 1	Cluster 2		Cluster 3		Cluster 4				
Mmp9	Acan	Bcl2	Adamts5	Notch1	ColIV	Jag1	Ptges2	Vegfa	Prnp
Mmp13	Col2a1	Hsp90a	ColI	Notch4	Bglap	Alk5	GH	KDR	Cav1
	Sox9		Runx2	Hey	AdipoQ	Id1	IGF1	Bax	
	Opg		Rankl	Alk1	CD44	Axin2	Igfbp3	Sod1	
	CD73		Pparg	Pai1	PthR1	Fzd1	Igfbp5	Cyclin D1	
	ColX		CD105	IL6	Ihh	IL1B	Igfbp6	Oct4	
	GH-R		CD146	Igfbp2	Ptch	Cox2	Bmp7	Hsp70	
	IGFR1		VDR	Igfbp4	Smoh	Ptges1	Noggin	Hsp90b	

Figure 12: Differences in mRNA expression between the distraction group and the OA group in canine knees. Green markers are upregulated in the distraction group, red are downregulated in the distraction group. Data by Miranda-Bedate et al. (unpublished results).

and endochondral ossification[22]. In this case, although *RUNX2* is significantly upregulated in the distraction group compared with the OA group, it seems however to be unable to cause hypertrophy in the chondrocytes as it lacks *SOX9*[22]. It is worth to mention that SOX9 is essential for the onset of endochondral bone formation as it is needed to maintain chondrocyte proliferation and viability in growth plates[22]. In line with this idea, *SOX9* was found to be downregulated just as *Col X* (an important marker for hypertrophy), suggesting that there is a reverse of the hypertrophy and endochondral bone formation. The balance between *OPG* an *RANKL* may also play a role in this. *OPG* appears to be involved in OA progression by increasing catabolic factors that are involved in cartilage pathophysiology and, as stated before, *RANKL* plays a role in osteoclastogenesis[12]. The balance between these two markers in chondrocytes, together with the upregulation of VDR, suggests that in distraction there is an pre-hypertrophic state[21].
In conclusion, distracted chondrocytes become pre-hypertrophic by the expression VDR and the already described balance *OPG/RANKL*. However, in contrast to OA, hypertrophy could have been reversed due to the downregulation of *SOX9*, which directly activates *Col X*, and is essential to steer the chondrocytes into a higher hypertrophic state[22]. The relationship between the expression of the VDR and all these transcriptional events could reveal the involvement of VDR in regeneration.

An in vivo study by Arden et al. has shown that administration of vitamin D (and so increasing serum levels) did not have any effect to reduce pain, stiffness or functional loss over a 3 year period in patient with OA[23]. It could mean that VDR is not the only repairing factor, but that it is essential for the regenerative process induced by distraction. Further research over other markers and how VDR is present at later moments in the treatment is necessary to unveil its true role.

Finally it is worth to mention that results could be slightly different if the length of applying the distraction method is changed. Furthermore it is not completely clear in which part of the period which processes exactly take place. Expression of certain factors could differ a lot looking at different time points. There are samples available of dogs sacrificed 25 weeks after the start of the experiment (11 weeks after applying the distraction method), hence for a better understanding of all mechanisms involved, future research should extend to those samples.

Conclusion

Assessing the cartilage samples under the microscope and giving scores for positivity, it can be concluded that VDR is more present in distracted donors in comparison with OA. This follows the VDR qRT-PCR data previously obtained from Miranda-Bedate et al. Furthermore a pattern in the distribution of positive chondrocytes is observed. Positive cells could only be seen in the superficial layer of the cartilage stretching a bit into the middle layer in very positive donors, in less positive donors the positive chondrocytes were only seen in the superficial layer to the side of the sample.

25OH-αhydroxylase in the cartilage of the tibia of distracted donors is more expressed than in OA donors. However in the femur it is more expressed in OA than in distracted donors. In subchondral bone and in the other cartilage samples (although in cartilage not significant) it can also be seen. 24-OH hydroxylase in the cartilage of the femur is significantly more expressed in the OA group than in the Distracted group. In other bone and cartilage samples (although in cartilage not significant) it can also be seen. Together, it could be suggested that a higher turnover of Vit D in OA in comparison with the distraction is taking place.

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