Universiteit Utrecht

An innovative view on osteoarthritis: Investigating the effect of chondrodystrophy and the continuous release of celecoxib on osteoarthritic cartilage

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General abstract

Osteoarthritis (OA) is a disease of the joint as a whole and leads to progressive degeneration. Even though OA is a frequent cause of (chronic) pain and disability among humans and canines, the pathobiology is still unclear and treatment remains symptomatic. In humans, chondrodystrophy has been related to less susceptibility for OA. In canines chondrodystrophic breeds are frequently encountered, as it is regarded a characteristic for several breeds and a distinction can be made between chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs. CD breeds express intervertebral disc degeneration differently than NCD breeds. How chondrodystrophy affects the cartilage and could influence the disease course of OA has, however, not been clarified yet.

In the first chapter, an innovative *in vitro* model for OA was tested. Canine cartilage micro-aggregates derived from NCD and CD were cultured in the presence of osteoarthritic synovial fluid. Culturing with synovial fluid proved to be challenging. Synovial fluid does seem to elevate prostaglandin E_2 (PGE_2) content in the medium, but this was the most pronounced in healthy synovial fluid. There were indications that NCD cartilage micro-aggregates were influenced in a greater extent by inflammatory conditions, as after 14 days of culturing in osteoarthritic synovial fluid the NCD micro-aggregates majorly disintegrated.

In the second chapter, the cartilage explants derived from NCD and CD donors were cultured for 21 days with or without an inflammatory stimulus and/or non-steroidal inflammatory drug (NSAID, namely celecoxib). NCD derived cartilage seemed to be more susceptible for the inflammatory stimulus (tumour necrosis factor alpha (TNF-α)). Moreover, treatment with celecoxib was only in NCD cartilage able to reduce the upregulation of PGE_2 caused by TNF-α. Moreover, indications of a difference in the activity of the Wnt pathway were revealed by gene expression levels. *AXIN2* and *DKK3* were both more expressed in CD cartilage, possibly indicating activation of the Wnt pathway in CD cartilage. Altogether, there seems to be a difference at the biomolecular and biochemical level between CD and NCD cartilage.

In the third and final chapter, the *in vitro* effects of the controlled release of celecoxib by loaded polyester amide (PEA) microspheres in a 4-week follow-up were researched. NCD derived chondrocytes were cultured in monolayers and stimulated with TNF-α. Subsequently, the monolayers were treated with two different dosages of microspheres, 10^{-4} M and 10^{-7} M, or unloaded PEA microspheres and compared to free added celecoxib (10^{-6} M). We demonstrated no cytotoxicity, gradual celecoxib release of the PEA microspheres, and a PGE_2 suppression by the 10^{-4} M loaded microspheres for 28 days.

An innovative view on osteoarthritis is provided by looking into the differences between CD and NCD dogs on a cartilage level and studying the *in vitro* the applicability of celecoxib-loaded (PEA) microspheres as a treatment option for OA.

General introduction

During life, muscles and joints make it possible to perform physical exercise, ranging from taking a stroll to running a marathon. This comes with the inevitable wear and tear over time. In modern day society, however, it is not exercise that is the biggest treat to joints, but, in fact, the opposite, inactivity. This modern day bad habit is suspected to cause weaker muscles and cartilage, resulting in a more rapid break down of joints[1]. Furthermore, joints can be afflicted by diseases and trauma. It is estimated that 20% of the dogs over one year of age exhibits clinical signs of osteoarthritis (OA) and suffers from lameness, pain, and disability[2]. However, joint diseases do not only affect canines, but are also of great importance for humans. In Europe, one in four adults is affected by a musculoskeletal condition, which is also the most frequent cause of chronic pain[3]. In 2015 1,199,100 people in the Netherlands were estimated to be affected by osteoarthritis[4]. This not only results in chronic pain and disability among adults but also comes with economic, social, and psychological costs. In 2011 the healthcare costs in the Netherlands for osteoarthritis were 1.1 billion euros, 1.2% of the total health care costs[4]. OA shows comparable degenerative changes in the joint in both canines and humans and the canine is an accepted animal model for osteoarthritis[5,6]. Nonetheless, the etiopathogenesis between the two species show differences[7]. In humans, the cause of OA is predominantly primary and it is mainly the result of ageing, with no particular reason for the onset of the disease. While in dogs, the initial changes are mainly secondary, due to traumatic injury of the articular cartilage, infection and/or inflammation or abnormalities in conformation. Osteochondrosis seems to be an important predisposing factor in species that have a high prevalence of the disease[8]. Despite the widespread awareness of OA and the impact of the disease, the early pathogenesis of OA has yet to be elucidated. Before going into further detail about the characteristics of OA, the healthy canine articular joint is first discussed. Additionally, a subdivision within the canine species is made, namely in chondrodystrophic (CD) and non-chondrodystrophic (NCD), as this is believed to be of influence on articular cartilage and the development of OA.

The healthy synovial joint

Joints join skeletal structures to make movement possible. Synovial joints, also known as diarthrodial joints, also have a shock-absorbing function. Numerous synovial joints are present in the dog. Though all adapted for their own function, the basic building plan of a synovial joint consists of a joint cavity. This cavity is filled with synovial fluid, which separates the articulating bones. The boundaries of the joint cavity are completed by the synovial membrane, a delicate connective tissue. This membrane attaches around the periphery of the articular surfaces. In most synovial joints additional strength is given to the synovial membrane by a fibrous capsule and additional fibrous ligaments[9] (**Figure 1**).

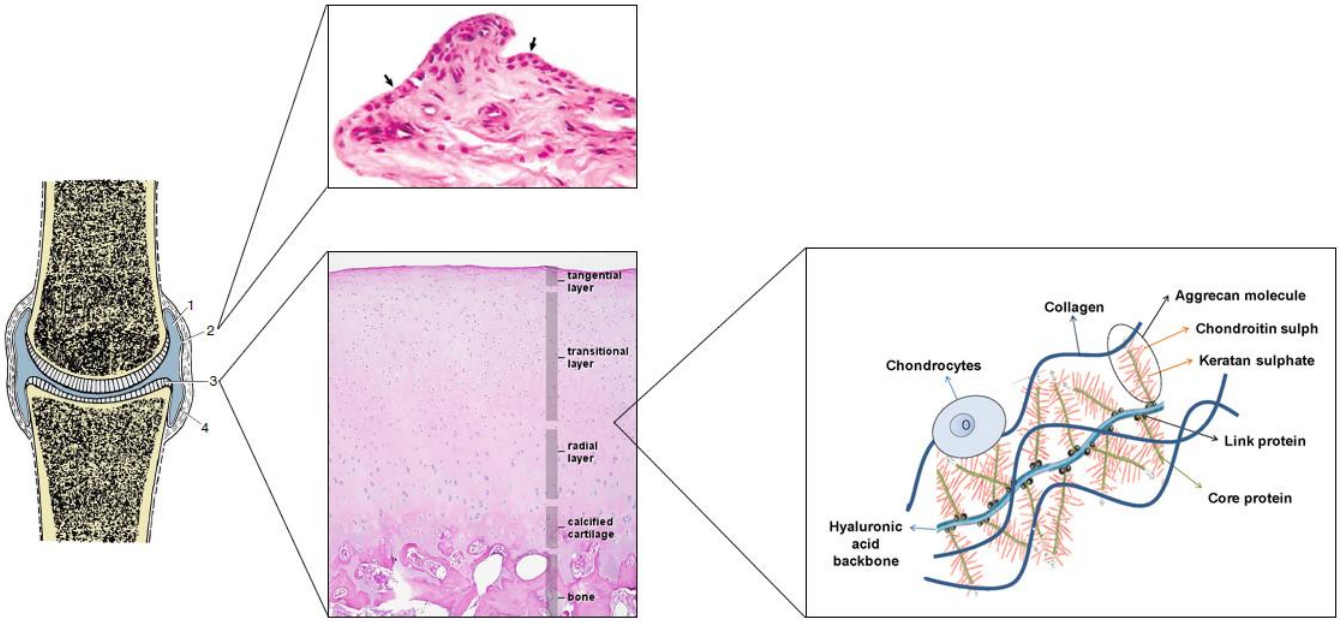

Figure 1. Schematic image of the anatomy of the synovial joint (**left**) showing (**1**) the joint cavity with synovial fluid, (**2**) synovial membrane, (**3**) articular cartilage and their matrix of glycosaminoglycans and collagens, and (**4**) fibrous layer of joint capsule. Redrawn with modification from Dyce *et al.* (2010)[9] and Buckwalter *et al.* (1994)[10].

Articular cartilage

Subchondral bone of synovial joints is covered by articular cartilage. The articular cartilage serves as the bearing substance. Articular cartilage forms only a thin (0.5-5.0 mm) layer[11]. It minimizes the friction created by movements to transmit mechanical forces to the underlying bone and to maximize the contact surface of the joint under load. Joints receive and absorb energy of impact and articular cartilage will deform under pressure[8].

Healthy cartilage consists of a solid matrix primarily composed of collagen (mostly type II) and proteoglycans, saturated with water (70-80% of dry weight). The largest proteoglycan is aggrecan, which is composed of long, non-polysulphated glycosaminoglycans (GAGs), hyaluronan, to which core proteins are attached in a branched arrangement. Similarly, smaller negatively charged polysulphated GAGs (chondroitin sulphate and keratin sulphate) are attached to the core proteins. The high content of negatively charged polysaccharide chains in the aggrecan aggregate is responsible for the water intake, resulting in an extremely high osmotic swelling pressure. Collagen type II counteracts the swelling pressure provided by aggrecan, giving cartilage its characteristic properties of having a high tensile strength and being able to resist compressive forces.

Functionally, articular cartilage can be divided in three zones: the superficial zone, the middle zone, and the mineralized deep zone. The superficial zone resists shearing forces, the middle zone functions in shock absorption, and the deep zone serves to attach the cartilage to the subchondral bone by its interlocking interfaces[8]. This matrix is heterogeneous. To function normally, interaction of physical and biochemical structures of the cartilage is necessary. By this the cartilage can provide frictionless motion, wear resistance, joint congruence, and transmission of load to subchondral bone. This material is synthesized and maintained by chondrocytes[12]. Chondrocytes are the only cell population present in articular cartilage. The membrane of these cells is in direct contact with the matrix and contains surface receptors for matrix components[8] (**Figure 1**).

Normal matrix turnover is enzymatic and enzyme inhibitors keep this process balanced. Aggrecanases are proteases capable of degrading aggrecans and are part of the 'a

disintegrin and metalloprotease' (ADAM) protein family. Collagenases are responsible for the breakdown of the peptide bonds in collagen. Articular cartilage has extremely poor regenerative capabilities compared to bone that possesses the ability to remodel itself[8].

Articular capsule

The articular capsule consists of out fibrous and inner synovial tissue layers. The outer layer is a heavy sheath that provides joint stability and attaches to the bone at its insertion at the margins of the joint. A segment of bone, variable in length, is thereby enclosed within the joint cavity. The inner tissue layer is the synovial membrane[8].

Synovial membrane

The synovial membrane, or synovium, completes the lining of the joint. This membrane can either be left entirely unsupported, rest directly on a tough outer fibrous capsule, or be separated from this by the interposition of fat pads. All three arrangements may occur in different regions of the same joint. The inner surface of the membrane carries many protrusions ranging in size and degree of permanency, which increase its surface area greatly. In contrast to mucous membranes, the synovial membrane lacks a continuous covering of cells[9].

The discontinuous surface layer is approximately one cell line thick and includes two types of cells: macrophages (A-cells) and fibroblast-like cells (B-cells). The B-cells are responsible for the production of synovial fluid[8]. The membrane is both vascular and sensitive[9]. The fibrous layer of the articular capsule contains beside blood vessels also lymph vessels. In this layer adipose tissue may accumulate, forming fat pads that function as soft cushions[8].

Synovial fluid

Synovial fluid fills the joint cavity. This viscous fluid is usually present in small amounts in a healthy joint. Synovial fluid has both lubricative and nutritive properties[9]. Furthermore, it provides oxygen, and removes carbon dioxide (CO_2) and metabolic wastes from the chondrocytes[8]. The way in which synovial fluid acts as a lubricant is disputed[9]. Nonetheless, it is very efficient as virtually no wear occurs in healthy joints despite the friction. The synovial fluid helps in providing nutrients for the articular cartilage, any intra-articular structures, and, possibly, the surface layer of the synovial membrane itself. This clear fluid has a colour that may range from pale straw to medium brown[9]. Healthy synovial fluid contains hyaluronic acid, lubricin (water-soluble glycoprotein), proteinases, and collagenase. Hyaluronic acid contributes to the viscous properties of synovial fluid. In the joint, synovial fluid mixed with water is released from the underlying cartilage whenever pressure from weight bearing is present and by so bears most of the load. As soon as the load is no longer present, the water returns to the cartilage due to the hydrophilic properties of the proteoglycans in the cartilage. This flushing of water in and out of the cartilage aids the exchange of nutrients and wastes[8].

Subchondral bone

The subchondral bone supports the overlying cartilage and dissipates concussive forces to the peripheral cortical bone. The degree of weight bearing positively correlates to the thickness of the subchondral bone plate. Larger, non-rodent, species often have subchondral bone that is composed of compact bone rather than trabecular bone[8].

The degenerated synovial joint

Not many diseases are as old as osteoarthritis (OA), which is also known as degenerative joint disease (**Figure 2**). OA has been described in fossils of large carnivorous dinosaurs that roamed the earth long ago[13]. Despite OA being an ancient problem, the fact that there is

widespread awareness of OA, and the impact of the disease, the early pathogenesis of OA is still unclear. OA was long thought to solely be a "wear and tear" disease leading to loss of cartilage. OA has, however, been found to rather be a process of abnormal remodelling of joint tissues driven by a host of inflammatory mediators within the affected joint[14].

The Osteoarthritis research society international (OARSI) has formulated the following definition for OA[15]: *"OA is usually a progressive disease of synovial joints that represents failed repair of joint damage that results from stresses that may be initiated by an abnormality in any of the synovial joint tissues including articular cartilage, subchondral bone, ligaments, menisci (when present), periarticular muscles, peripheral nerves or synovium. This ultimately results in the breakdown of cartilage and bone, leading to symptoms of pain, stiffness and functional disability."* The presence or absence of inflammation has been a point of discussion regarding appropriate terminology[12]. To summarize, OA is a disease that affects the joint as a whole.

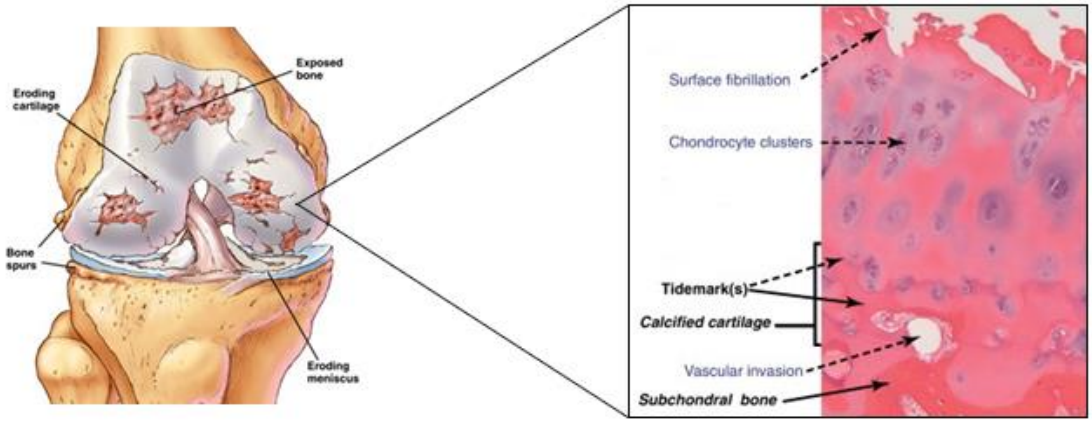

Figure 2. Visualisation of the degenerated joint (**left**) with a histological close-up of the degenerated cartilage showing the characteristics of osteoarthritic cartilage (**right**). Adaptation of Goldring *et al*. (2012)[16].

Clinical symptoms

As the cartilage is not innervated, OA only affecting the cartilage will not give rise to clinical symptoms. Bone, however, is well innervated and as soon as this is affected, due the generated friction for instance, clinical symptoms are seen. OA will usually present itself with a sudden onset of lameness, often painful, and deterioration of the muscles. This can especially be seen after more stressful exercise or after misplacement of the extremities. The resulting lameness will eventually fade gradually, but this takes some time. Sometimes lameness is only seen after rest and will disappear after more exercise is performed. Animals with OA will often reduce their activity due to pain, which will lead to a vicious cycle of decreased flexibility, loss of strength, joint stiffness, and decreased cardiovascular fitness[17]. The process of OA is irreversible, which often results in an end-stage clinical syndrome of the joint.

Pathobiology

OA is highly prevalent in dogs and certain risk factors have been identified for developing OA. Risk factors for OA associated with hip dysplasia are weight and hip joint laxity. The probability of having OA also increases with age[6]. Furthermore, joint luxations, (articular) fractures, ligament injuries[18], and excessive joint loading[19] are important universal risk factors. These injuries all have in common that they lead to alteration of the original morphology and construction of the joint.

Though OA is a disease which can affect every joint, certain joints are more frequently affected in canines. The affected joint and the pathological process responsible for the onset of OA are mentioned below: hip (hip dysplasia); elbow (elbow dysplasia); knee (anterior cruciate ligament degeneration); scapula and tarsus (osteochondrosis); scapula, elbow, hip, and toe articulations (primary OA), and spinal cord (OA in the facet joints, often accompanied by spondylosis due to intervertebral disk degeneration).

When the homeostasis of the normal cartilage is disrupted, OA can occur. OA leads to biochemical and biomechanical factors that occur concurrently and cause degenerative changes to this material. These progressive changes have not only been characterized for articular cartilage, but may also be seen in periarticular tissues. There are probably multiple etiologies sharing common pathways of physical and chemical disruption[12] (**Figure 3**[20] and **Figure 4**[21]).

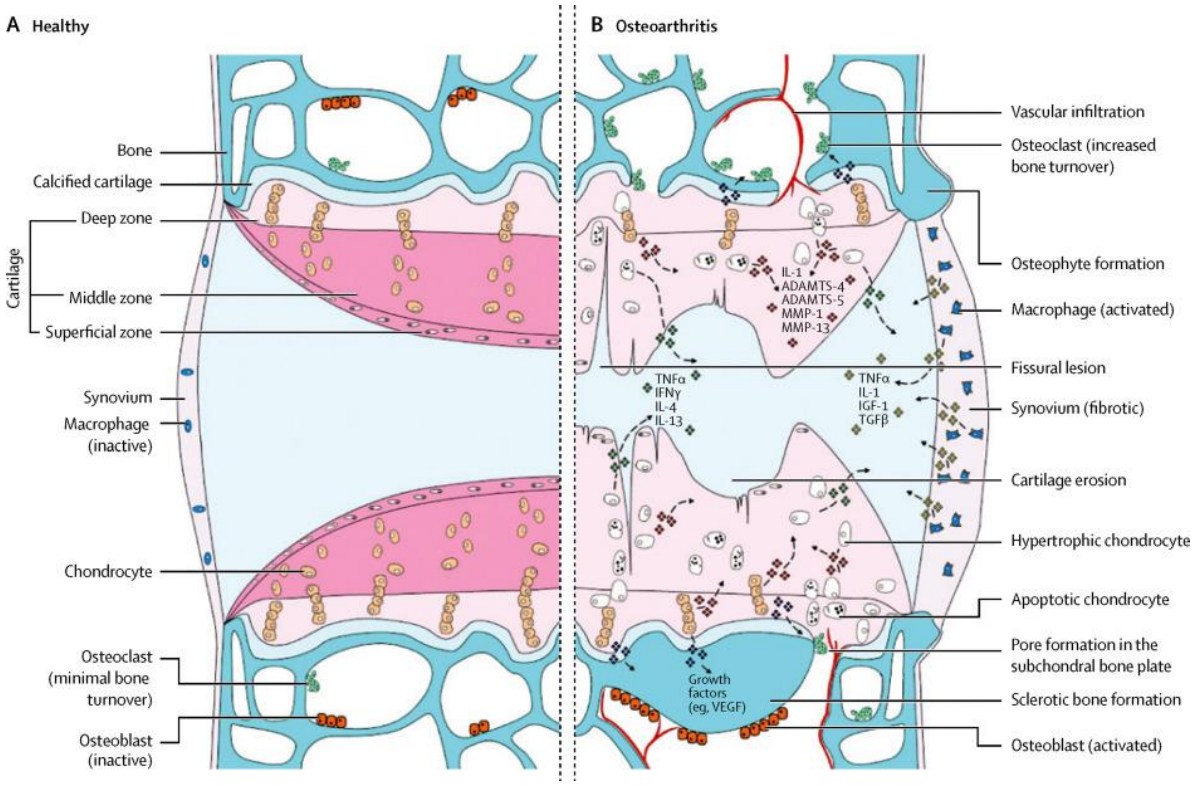

Figure 3. Signalling pathway and structural changes in the development of osteoarthritis. **A.** Healthy joint. **B.** Osteoarthritic joint. *ADAMTS* = a disintegrin and metalloproteinase with thrombospondin-like motifs. *IL* = interleukin. *IGF* = insulin-like growth factor. *MMP* = matrix metalloproteinase. *TGF* = transforming growth factor. *TNF* = tumour necrosis factor. *VEGF* = vascular endothelial growth factor. Reprinted from Glyn-Jones *et al.* (2015)[20].

After most cartilage injuries, regeneration of the lost original tissue cannot be achieved[21]. There is limited healing of the tissue damage. Even though stimuli are present, regeneration cannot take place properly. Production of healing tissue with the same properties as the original – morphological, biochemical and biomechanical – is unlikely. Consequently, the regenerated fibrous tissue is of inferior quality. Successful healing is mainly limited by failure of the osteochondral defects to heal with normal hyaline cartilage. The degree of healing is heavily influenced by the size of the defect, depth of the injury, location and relation to weight-bearing areas, and age of the patient. Superficial defects may often not progress and will not necessarily compromise joint function. Full-thickness defects are, however, repaired by ingrowth of subchondral fibrous tissue, which may also undergo metaplasia to

fibrocartilage. Furthermore, adherence of repair tissue to surrounding non-affected cartilage is often incomplete[19].

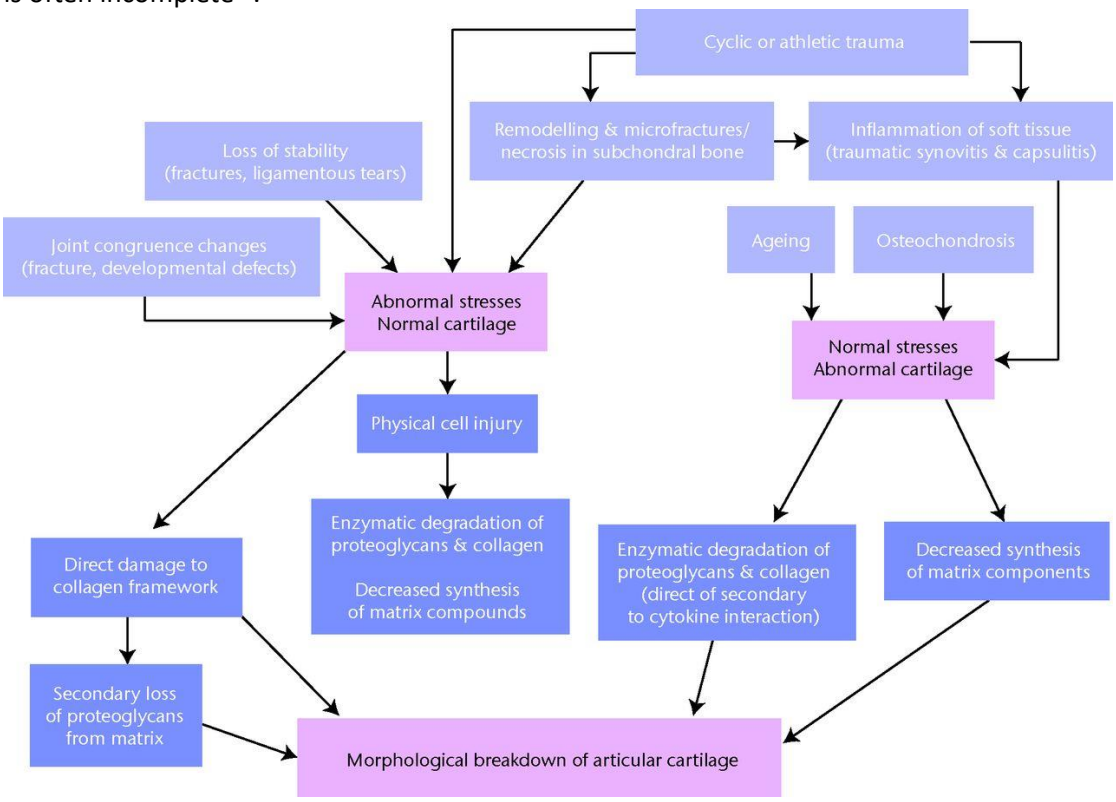

Figure 4. Factors involved in articular cartilage degeneration in osteoarthritis. Reprinted from McIlwraith *et al.* (2012)[22].

Changes in articular cartilage

The development of OA is gradual. Traumatic events may lead to OA in certain ways. First, there is direct trauma to the cartilage, which will lead to changes in the cartilage morphology. Some injuries, however, may cause cartilage damage more indirectly. For instance, ligamentous injury may result in abnormal stress on normal cartilage, leading to joint instability and by this cartilage damage[19]. Repetitive mechanical injury is considered as the critical signal for the initiation and progression of OA[23].

Characteristic for OA is the gradual loss of articular cartilage in which chondrocyte-matrix associations are disturbed and ECM metabolism is shifted towards a more catabolic state[23]. In the early stages of OA, chondrocytes try to counteract the enhanced catabolic processes by increasing the collagen and proteoglycan content of the ECM. This increased proteoglycan content is followed by an increase in water content. As OA progresses, the more catabolic state of the articular cartilage can no longer be compensated. This results in a disrupted matrix homeostasis driven by cytokine cascades and inflammatory mediators[23]. Matrix metalloproteinases (MMPs), such as aggrecanases (a desintegrin and metalloproteinase with trombospondin-like motifs (ADAMTS)) produced by the chondrocytes and synovial membrane, break down the matrix. The loss of proteoglycans impairs the hydraulic and lubricating functions of the cartilage. This leads to further mechanical injury of the cartilage. These abnormal loads can generate shear stresses that break the collagen crosslinks. The collagen fibres on the surface of the articular cartilage get disrupted and fibrillated. This fibrillation is accompanied by erosion and thinning of the articular cartilage. Necrosis of individual chondrocytes eventually leads to hypocellularity of the cartilage and the remaining chondrocytes cluster or clone[8]. These clustered hypertrophic chondrocytes will produce a different type of collagen, collagen X, a proteoglycan subtype[24].

Collagen X is a short non-fibril forming collagen predominantly found in the hypertrophic chondrocyte region of the growth plate during normal endochondral ossification[25] and produced by OA chondrocytes as soon as they differentiate towards the hypertrophic phenotype[26].

Several inflammatory factors, such as nitric oxide (NO), cyclooxygenase 2 (COX-2), and phospholipase A_2, modulate chondrocyte function. When stimulated by IL-1 or in combination with TNF-α chondrocytes are able to express these enzymes[27-29]. It has been reported that OA cartilage expresses COX-2 and NO synthesis, resulting in the spontaneous release of NO and prostaglandin E_2[30,31].

Changes in synovial tissue

The contribution of the synovium in OA pathogenesis is widely accepted. During OA, the synovium displays clear signs of inflammation: hypertrophy and hyperplasia of the synovial lining with an increased number of synovial lining cells, fibrosis, inflammatory synovial infiltrates of macrophages and lymphocytes, and neoangiogenesis[32]. Angiogenesis and inflammation may either accompany each other or facilitate the potential of one another[33,34]. Not only the cartilage itself produces destructive factors, the synovium also plays a major role in the production of inflammatory mediators and metalloproteinases[35].

Inflammatory factors, such as TNF-α, have a multifaceted role in the degeneration process. They are potent inducers of cartilage degeneration in several ways: upregulation of MMP activity, decrease of collagen synthesis, induction of chondrocyte apoptosis, and the increase in other inflammatory mediators (IL-8, IL-6, prostaglandin E_2 (PGE_2), and NO)[36-38]. Inflammatory mediators may affect prostaglandin formation by upregulation of COX-2. The increased expression of COX-2 and its main product PGE_2 have been found in cartilage tissue from OA patients[29,39,40]. The COX-2 mediated prostaglandin synthesis results in an increased proteoglycan release by the articular cartilage, leading to a decreased proteoglycan content[40].

As soon as the synovium or subchondral bone is involved pain will be evident. While the articular cartilage does not participate directly in the inflammatory response, it is rather affected by the inflammation in the synovium and/or subchondral bone[8].

Changes in synovial fluid

The osteoarthritic joint tends to be an unfavourable setting for local cellular health and viability. Osteoarthritic synovial fluid comprises of pro-inflammatory mediators that destroy cartilage and/or induce apoptosis. Such pro-inflammatory mediators are cytokines [41,42] (tumour necrosis factor alpha (TNF-α), interleukins (IL)[43]), chemokines (NO[44]), MMPs[45] and growth factors[41], which play a major role in the etiopathology of the disease. Even in the absence of classic inflammation, characterized by infiltrations of neutrophils and macrophages in the joint tissues, elevated levels of inflammatory cytokines have been measured in OA synovial fluid[23]. There is a clear indication of an inflammatory mediator/proteinase inhibitor imbalance in OA compared to healthy individuals[46]. Inflammatory cell infiltrates in the synovial membrane may impair fluid drainage from the joint. This can cause synovial fluid to lose some of its lubricating properties due to the degradation of hyaluronic acid by the superoxide generating systems of neutrophils[8]. Furthermore, there is evidence that the composition of synovial fluid may influence the structure of the repair tissue formation[47,48].

Current treatment options osteoarthritis

Currently, the conservative treatment for OA remains symptomatically. As the development of OA is gradual, OA can exist for quite some time before diagnosis. In this time significant damage may already have occurred. Therefore, the main aim of treatment is to put a hold

on the inflammation and break through the vicious circle of the degenerative disease. With this further damage may be avoided. Self-evidently, if OA is secondary, the main cause should be eliminated when possible. Conservative treatment is composed of painkillers[49-52], weight control [53], controlled exercise[54,55], and a correct diet either with or without supplementation[56-60]. Furthermore, surgery is also one of the treatment options[61-63]

The decision on which treatment to use differs from patient to patient and depends on the intensity of the pain, degree of joint degeneration, functional limitation, etiology, systemic condition, joint location, limb alignment, bone quality, ligament stability, and age. The key issue is precise patient selection.

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used for the treatment of OA. Besides the pain relief, NSAIDs have an anti-inflammatory affect, which is also beneficial for OA. Unfortunately, the application is accompanied by several drawbacks both cyclooxygenase (COX) inhibition dependent and independent[64,65]. Non-selective inhibitors inhibit both COX1 and COX2, COX1 being the constitutive form. Inhibition of COX1 is the main cause of negative side effects, as COX1 is expressed in various tissues for homeostatic functions. COX2 on the other hand is the inducible enzyme during inflammation[66] (**Figure 5**).

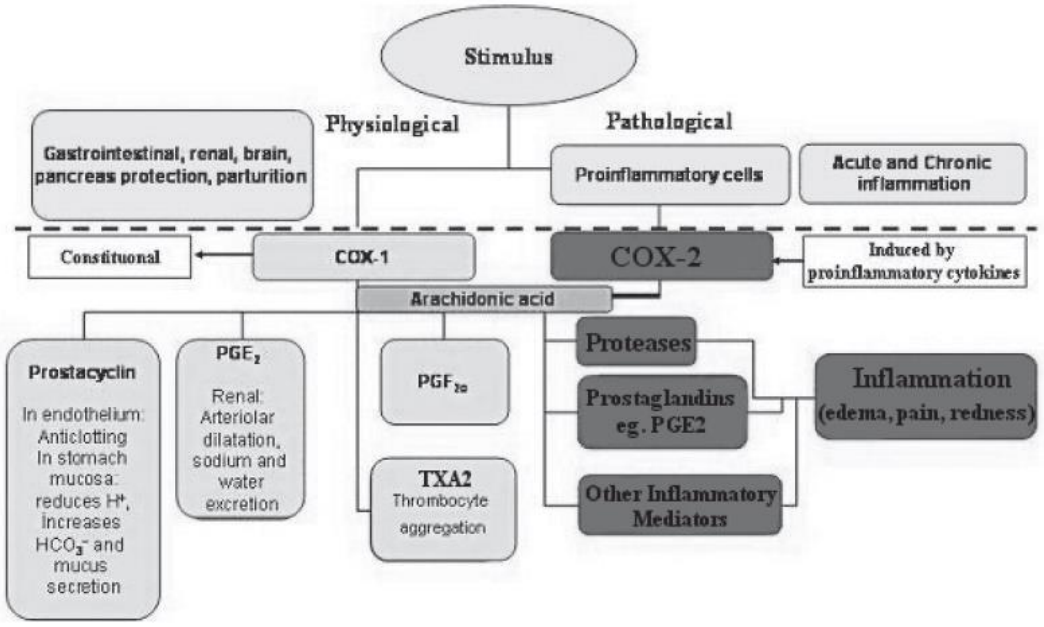

Figure 5: Roles of cyclooxygenase (COX) 1 and COX2. *PGE_2* = prostaglandin E_2. *$PGF_{2\alpha}$* = prostaglandin $F_{2\alpha}$. *TXA2* = thromboxane A_2. Reprinted from Süleyman *et al. (*2007)[64].

Biomaterial-based regenerative approach as a novel treatment option

The undesirable effects of COX-1 inhibition include gastro-intestinal side effects, gastrointestinal (gastric ulcers) side effects being the most common but also renal and hepatic side effects may be observed[64]. Highly selective COX-2 inhibitors, such as celecoxib and rofecoxib, show less adverse side effects than (unselective) inhibitors of both COX-1 and COX-2 and yet remain as potent[64](**Figure 5**). These selective inhibitors have, however, been shown to induce systemic toxicity, mainly regarding (thrombotic) cardiovascular problems[64] and nephrotoxicity[67,68]. These adverse side effects are even more relevant to the ageing patient that suffers from OA, making the use of celecoxib controversial these days. Since articular cartilage is initially an avascular structure, the question remains whether the therapeutic dose will be reached after oral administration. Local administration of anti-

inflammatory agents, therefore, seems like a sensible approach as the plasma concentration of celecoxib remains below the toxic level and celecoxib can reach the avascular cartilage. Single intra-articular administration only has a temporary effect, possibly because of the short half-life of pharmaceutical drugs[69] or rapid clearance from the joint. A solution to this problem is provided by the use of biomaterial-based controlled release systems for the continuous delivery of anti-inflammatory therapeutics over prolonged periods of time[70-72].

Current treatments are still unsatisfying, underlining the importance of developing novel long-acting treatments for degenerative joint diseases. A biomaterial based controlled release system may meet the expectations for such a novel treatment. The biomaterial must be completely degradable in a sufficient prolonged way for the sustained drug delivery to occur efficiently. Moreover, the biomaterials should not provoke any negative effects by themselves. Polyester amide (PEA) based injectable microspheres are proposed as an intra-articular drug delivery system with auto regulatory behaviour (**Figure 6**). Certain advantages are obtained by the amino acid-based building blocks in PEA microspheres. These provide 1) imparting chemical functionality; 2) possible improvement of biological properties of materials; 3) enhanced thermal and mechanical properties; and 4) metabolizable building blocks[73]. Moreover, the local pH is not affected by the degradation of the polymer backbone of PEA microspheres, as this degradation is via a surface erosion mechanism.

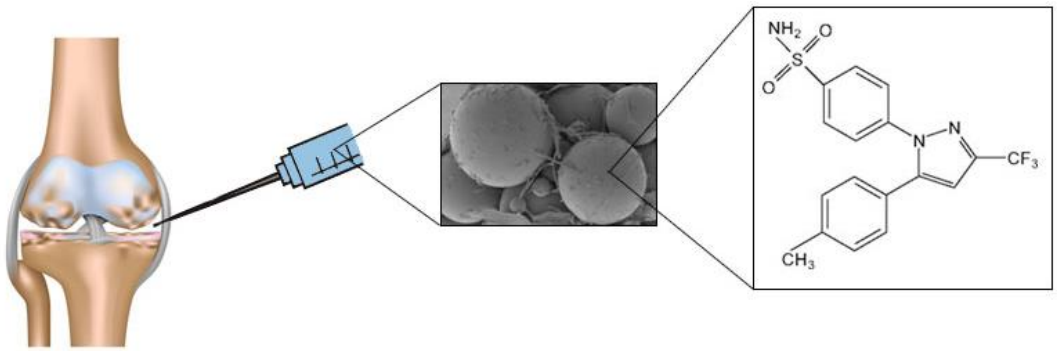

Figure 6. Celecoxib-loaded polyester amide (PEA) microspheres are being injected in an osteoarthritic joint cavity (**left**), scanning electron microscope image of PEA microspheres (**middle**), and the structure of celecoxib (**right**). Adaptation of Janssen *et al.* (2016)[70].

Dogs develop OA, but is all canine joint cartilage the same?

The domestic dog (*Canis familiaris*) is undoubtedly the most morphological diverse mammal species. The majority of phenotypic variation in domestic dogs is found among, rather than within, the over 350 recognized dog breeds. One aspect of variation is leg length, also known as chondrodystrophy. This is defined by dysplastic, shortened long bones and is a characteristic for breeds such as the Dachshund (**Figure 7**). Long bone length in dogs is a unique example of multiple disease-causing retrocopies of the same parental gene in a mammalian species[74].

Figure 7. Left an example of a non-chondrodystrophic (NCD) breed (Labrador Retriever) and right an example of a chondrodystrophic (CD) breed (Shorthaired Dachshund). Beeldbank UKG.

The phenotype primarily affects the length of the long bones, with growth plates calcifying early in development. Endochondral ossification is responsible for the formation of most of the vertebrate appendicular and axial skeleton, in this process cartilage is replaced with bone in de developing limb. The long bone growth plates show disorganisation of the proliferative zone and reduction in depth of the maturation zone[74] (**Figure 8**).

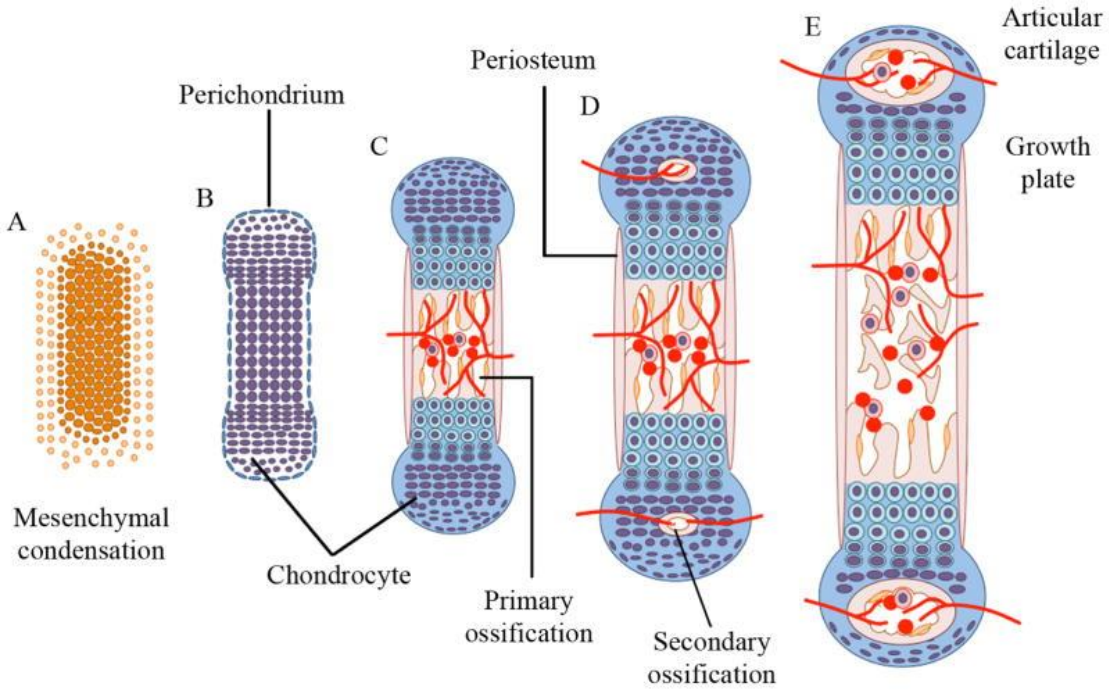

Figure 8. Cartilage development and endochondral ossification. (**A**) The mesenchymal condensation of bipotential osteochondroprogenitors at prospective skeleton sites marks the beginning of the process of endochondral ossification. (**B**) The primordial cartilage continues to grow and forms an avascular cartilaginous template surrounded by perichondrium. (**C**) Hypertrophic chondrocytes are calcified and invaded by micro blood vessels resulting in primary ossification. (**D**) Blood vessels invade the epiphysis and a secondary ossification centre is formed with osteoblasts and bone marrow. (**E**) Ossification of the epiphysis. When completed, hyaline cartilage remains only in the epiphyseal plates and articular cartilage. The epiphyseal plates promote longitudinal growth until young adolescence. Reprinted from Usami *et al.* (2016)[75].

The retrogene fibroblast growth factor 4 (*FGF4*) has been appointed as the genetic basis for the disproportional short limbs in CD dogs[76]. This is a retrogene insertion on CFA18. This insertion, however, failed to explain breeds such as the American Cocker Spaniel, Beagle, and French Bulldog. Even though these breeds were originally classified as CD based on histopathologic and morphologic analysis[77,78]. The CFA18 insertion leads to the atypical expression of the *FGF4* transcript, resulting in excessive synthesis of *FGF4*. Moreover, it induces the activation of fibroblast growth factor receptors 3 (*FGFR3*) in chondrocytes inappropriately, resulting in chondrodysplasia[76]. In humans, inappropriate expression of *FGFR3* causes dwarfism that resembles chondrodysplasia as seen in dogs[79]. In humans, it is associated with the early onset of OA[80]. *FGF* and *FGFR3* regulate the maintenance of articular cartilage and ECM proteins[81]. Recently, a region on CFA12 has been identified due to association with a segregating form of skeletal dysplasia in the Nova Scotia Duck Tolling Retriever. This novel retrogene leads to an increase in *FGF4* expression in neonatal intervertebral discs. It is probable that these expression changes also impact endochondral ossification. While the *FGF4* retrogene on CFA18 impacts limb length, the *FGF4* retrogene on CFA12 explains the chondrodystrophic phenotype, including limb length and intervertebral disc disease[74]. The chondrodystrophy of the America, Cocker Spaniel, Beagle, and French Bulldog can now be explained by the CFA12 insertion. Breeds that have both the CFA18 and CFA12 insertion have the most dramatic decrease in height: Basset Hound, Cardigan Welsh Corgi, and Dachshund.

Osteoarthritis can occur in all dog breeds; however, little is known concerning the occurrence of OA in certain dog breeds. Mele documented in 2007[82] the incidence of hip arthritis in 206 dogs of various breeds admitted to the Dover Veterinary Clinic, to the authors knowledge no other incidence reports are available. As NCD and CD dog breeds have been shown to display different characteristics for other degenerative joint diseases, such as intervertebral disc degeneration[78], a distinction in the incidence of OA between these two subpopulations would be of interest. Moreover, it has been demonstrated in mice that FGF3 delays OA progression[83]. CD dogs have already been proposed as a model for human degenerative disc disease[77,84]. Underlining the importance of distinguishing between the two subpopulations when choosing the most appropriate animal model. We have reason to believe that these subpopulations also differ on a cartilage level.

Validating the possible molecular and biochemical differences between CD and NCD dogs is of great importance when choosing the most appropriate breed in animal models. Especially since the beagle dog, a CD breed, is most often chosen in canine models.

Outline and aims of the Honours Programme project

The main aim of this Honours Programme project

The main aims of this Honours Programme project are to study the differences between chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs on a biomolecular and biochemical level and to evaluate a new local treatment strategy with microspheres for the inhibition of inflammation, pain, and degeneration in osteoarthritis (OA).

Aim 1 – Develop a synovial fluid co-culture based *in vitro* culture model mimicking osteoarthritis.

Rationale: The perfect *in vitro* model for OA has not been established yet. Developing an *in vitro* model with a close resemblance to the physiological joint is of interest to better evaluate new treatment options. As OA is a disease that comprises the whole joint, synovial fluid also participates in the pathobiology.
Approach: To establish a synovial fluid co-culture based *in vitro* culturing model, synovial fluid from healthy and osteoarthritic donors was collected. Healthy articular cartilage micro-aggregates from CD and NCD will be cultured in the presence of healthy synovial fluid or osteoarthritic synovial fluid, or in a chondrogenic medium (control).

Aim 2 – Determine the differences between chondrodystrophic and non-chondrodystrophic dogs on a cartilage level in an *ex vivo* cartilage model.

Rationale: Chondrodystrophy has already been associated with a degenerative joint disease, namely intervertebral disc degeneration. This results in a display of different characteristics in NCD and CD dogs. However, a distinction between these subpopulations is currently not made in articular cartilage. We have reason to believe that not only articular cartilage differs between NCD and CD dogs; this also influences their susceptibility for osteoarthritis and their response to treatment strategies.
Approach: Healthy cartilage derived from canine knees from both CD and NCD donors will be cultured alone or in co-culture with healthy canine knee synovial tissue. Moreover, healthy canine synovial tissue is cultured alone to serve as control. These three tissue combinations are studied in an *ex vivo* cytokine based culturing model. In order to assess the differences at the cartilage level in a healthy environment and in the presence of a pro-inflammatory stimulus, biochemical and biomolecular parameters related to cartilage will be assessed.

Aim 3 – Determine the *in vitro* effects of the controlled release of celecoxib by loaded PEA microspheres on articular cartilage cells in a pro-inflammatory environment.

Rationale: Controlled and sustained release of the selective COX-2 inhibitor celecoxib should be able to suppress inflammation and pro-inflammatory cytokines and mediators over a prolonged period of time. This proof of concept creates a justifiable basis for the intra-articular application of celecoxib-loaded polyester amide (PEA) microspheres for NCD patients suffering from OA.
Approach: The effects of controlled release of a COX-2 inhibitor from a biomaterial will be assessed in a tumour necrosis factor alpha (TNF-α) stimulated articular cartilage monolayer *in vitro* cell culture model. Three main read out parameters will be assessed: 1) the release of celecoxib-loaded PEA microspheres in a pro-inflammatory environment over prolonged period of culture in the presence of cartilage cells, 2) the anti-inflammatory effect of celecoxib-loaded PEA microspheres over time, and 3) the possible dose-dependent effect from the celecoxib-loaded PEA microspheres.

Embedding in faculty research programme

This project is part of the faculty's Regenerative Medicine and stem cells research programme. This is a collaborative project in which the group of Orthopaedics participates in a consortium funded by the TOP sector (LSH Impulse) with UMC Orthopaedics, DSM Biomedical, and the Dutch Arthritis Foundation as partners.

An innovative *in vitro* model for osteoarthritis: cartilage micro-aggregates cultured in the presence of osteoarthritic synovial fluid

Abstract

Background. Osteoarthritis affects the joint as a whole. All tissues of the synovial joint participate in the pathogenesis of this degenerative disease. Logically, not only chondrocytes but also the other tissues and components should be taken into account when mimicking the joint *in vitro*. Therefore, we propose a possible *in vitro* culturing model with the presence of osteoarthritic synovial fluid.

Materials and methods. Articular cartilage micro-aggregates of four canine donors, two non-chondrodystrophic (NCD) and two chondrodystrophic (CD), were cultured in the presence of 20% healthy synovial fluid for 7 days or in the presence of 20% osteoarthritic synovial fluid for 14 days. Read-out parameters were histology, qRT-PCR, and biochemical assays (glycosaminoglycan- (GAG) and DNA content, and prostaglandin E_2 (PGE_2) release).

Results. Against expectations, micro-aggregates cultured with healthy synovial fluid seemed to have a higher PGE_2 content in the medium after 7 days of culturing than micro-aggregates cultured with osteoarthritic synovial fluid. Interestingly, micro-aggregates derived from NCD dogs disintegrated at an earlier time point of culture compared to micro-aggregates from CD dogs.

Conclusions. This study highlighted the difficulties of designing a suitable *in vitro* model for osteoarthritis and more specifically the difficulties of culturing with synovial fluid. In the current study, osteoarthritic synovial fluid did not promote a pro-inflammatory environment. There were indications that NCD cartilage micro-aggregates were influenced in a greater extent by inflammatory conditions compared to CD cartilage micro-aggregates.

Keywords: chondrocytes, synovial fluid, co-culture, micro-aggregates, chondrodystrophic, non-chondrodystrophic.

Introduction

Osteoarthritis (OA) is a degenerative joint disease that affects the joint as a whole. It is usually a progressive disease of synovial joints representing failed repair of joint damage that may be initiated by an abnormality by any of the synovial joint tissues[15]. OA is highly prevalent in canines and is often seen secondary to joint malformations, luxations, (articular) fractures, and ligament injuries[19]. It seems that certain (genetic) traits predispose for the development of OA in dogs. The canine species can be divided into two subpopulations; chondrodystrophic (CD) and non-chondrodystrophic (NCD). Chondrodystrophy causes abnormally short limbs and is a characteristic for several dog breeds, such as the dachshund and basset hound. Differences between CD and NCD dogs have already been found on a gene expression level in intervertebral discs[85]. These two dog subpopulations exhibit differences in the process of endochondral ossification, resulting in the difference in appearance[86]. Whether CD and NCD dogs also differ on a cartilage level is thus far unexplored. Currently, the symptomatic treatment of OA for canines consists of (mostly oral) non-steroidal anti-inflammatory drugs (NSAIDs)[50], steroidal injections[51], weight control[53], controlled exercise[54], customised diets[56] and supplements[57-60], or surgery[61-63]. A curative therapy for osteoarthritis has yet to be found.

Articular cartilage is avascular and chondrocytes meet their requirements by diffusion from three sources: vessels in the tissues at the periphery of the cartilage, vessels in the subjacent marrow spaces, and synovial fluid within the joint cavity. The porosity of the cartilage matrix aids in this diffusion, as it absorbs and releases fluid while the cartilage is alternately unloaded and compressed during movements of the joint[87]. Synovial fluid provides the largest part of nutritive components[88]. Evidence exists that poor cellular nutrition in cartilaginous tissues is a primary source of tissue degeneration, which may result in OA[89-91]. If cells no longer receive proper nutrition to maintain viability, matrix remodelling and synthesis becomes dysregulated. Thus, the ability of cartilage to act in its primary role of load support is affected by changes in tissue composition and structure, which occur as a result of degeneration[11].

The composition of healthy synovial fluid is similar to that of dialyzed blood plasma with the addition of components provided by synovial cells and catabolic products from the surrounding tissues. This viscous fluid is clear and its colour ranges from pale straw to medium brown[87]. Furthermore, white blood cell count and differentiation with microscopic synovial fluid evaluation has long been recognized to be useful in the assessment and diagnosis of arthritis[92].

The osteoarthritic joint tends to be an unfavourable setting for local cellular health and viability, as osteoarthritic synovial fluid consists of mediators that promote inflammation, destroy cartilage, and/or induce apoptosis. It contains inflammatory mediators such as cytokines[41,42] (tumour necrosis factor alpha (TNF-α), interleukins (IL)[43]), chemokines (nitric oxide[44]), matrix metalloproteinases (MMPs)[45], and growth factors[41] which play a major role in the etiopathology of the disease. There is a clear indication of an inflammatory mediator/proteinase inhibitor imbalance in OA, compared to healthy individuals[46].

The composition of synovial fluid may influence the structure of the repair tissue formation. It has been shown that bovine chondrocytes stimulated with synovial fluid from donors suffering from OA, triggered the proteoglycan synthesis to a higher extent than the synovial fluid of rheumatoid arthritis patients[47]. Moreover, the composition of synovial fluid from acutely injured joints seems to differ from that of chronically injured joints, as synovial fluid from acutely injured knees stimulated chondrogenesis, whereas synovial fluid from chronically injured joints inhibits this[48]. Culturing with synovial fluid has been done in the past with human chondrocytes, in monolayers[93] or seeded in an agarose suspension[94]. With

this current culture system, chondrocytes are cultured in an environment enriched with synovial fluid as would be the case in an *in vivo* situation.

At the preclinical stage, innovative therapies are being tested *in vitro.* For this purpose, the most representative culture model, and close to the *in vivo* situation, thus far, are cartilage explants. These are being cultured either in the presence of foetal bovine serum (FBS) or with chemically defined media containing growth factors, commonly transforming growth factor beta (TGF-β) at a supra-physiological concentration of 10 ng/ml[95,96]. To mimic the joint more accurately a culturing system with synovial fluid may prove valuable, since the synovial fluid itself may, for instance, affect drug degradation. Therefore, the aims of this study were to: 1) create a synovial fluid based *in vitro* culture model for osteoarthritis by culturing healthy cartilage cells in the presence of osteoarthritic synovial fluid; 2) to identify possible differences in cartilage metabolism in micro-aggregates from chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs; and 3) to assess whether addition of 2 ng/mL TGF-β would be beneficial for culturing. Given the extent of the culture conditions and technical limitation with respect to the available quantity of healthy synovial fluid, initial experiments were conducted with two CD and two NCD donors.

Materials and methods

Sources of canine tissue

Osteoarthritic synovial fluid was collected during standard of care surgery for stifle pathology at the Companion Animals Clinic of the University of Utrecht. Healthy synovial fluid was collected from experimental dogs that had been euthanised in unrelated studies. For the collection of healthy synovial fluid samples, the joint was left intact to guarantee a sterile work space. Synovial fluid was collected from the knee and/or shoulder joints. Viable cells were counted using a TC20™ Automated Cell Counter (1450102, Bio-Rad, Veenendaal, the Netherlands) after staining with 0.40% Trypan Blue Dye (1450021, Bio-Rad, Veenendaal, the Netherlands). Healthy synovial fluid samples typically contained less than two million cells per ml, while osteoarthritic synovial fluid contained more than two million cells. After aspiration, all synovial fluid samples were centrifuged at 1500 rpm for 5 minutes to remove cells, aliquoted, and stored at -20 °C until further use. For culturing in order to have a representative healthy and OA environment, two pools were eventually composed, one with healthy synovial fluid and one with osteoarthritic synovial fluid (**Table 1.1**).

Donor	Breed	NCD/CD	Gender (M/F)	Age (months)	Weight (kg)	Joint	Healthy/ OA joint
1409326	Rottweiler	NCD	Fc	35	33.4	Knee	OA
1609029	Pit bull	NCD	M	54	38.3	Knee	OA
1609865	Labrador Mix	NCD	Mc	127	24.2	Knee	OA
992178	Mixed breed	NCD	M	18	26	Shoulder, knee	Healthy
993506	Mixed breed	NCD	M	5	24	Shoulder, knee	Healthy
100642	Mixed breed	NCD	F	15	26	Shoulder, knee	Healthy

Table 1.1. Canine synovial fluid pool donor characteristics. A pool of osteoarthritic (OA) synovial fluid (**top three donors**) was made, as well as a pool of healthy synovial fluid (**bottom three donors**). *NCD* = non-chondrodystrophic. *CD* = chondrodystrophic.

***In vitro* micro-aggregate culture of articular cartilage cells**

Passaging

Cryopreserved articular cartilage cells (AC, Passage 0) of four donors (**Table 1.2**), two chondrodystrophic (CD) and two non-chondrodystrophic (NCD) donors, were separately passaged for a total of 14 days. Passage 1 (P1) and 2 (P2) were established on day 7 and 10, respectively. For passaging four T175 culture flasks (660175 Cellstar® Greiner Bio-one, Alphen aan de Rijn, the Netherlands), one for each donor, containing expansion medium, were cultured under normoxic conditions (37°C, 5% carbon dioxide (CO_2)). The expansion medium consisted of hgDMEM (high glucose, GlutaMAX, pyruvate (Invitrogen, 31966)), 100 µL/mL FBS (Gibco, 16000-044), 10 µL/mL penicillin/streptomycin (p/s, PAA laboratories, P11-010), 5 µL/mL ascorbic acid 2-phosphaten (asap, Sigma, A8960), 1 µL/mL dexamethasone (Sigma, D1756), 1 µL/mL basic fibroblast growth factor (AbD Serotec, PHP105), and fungizone 5 µL/mL (15290-018, Invitrogen). After 14 days, the flasks were aliquoted with 2 million cells (TC20™ Automated Cell Counter) per cryotube and stored in at -150 °C.

Donor	Breed	NCD/CD	Gender (M/F)	Age (months)
938	Mixed breed	NCD	F	18
911	Mixed breed	NCD	F	18
1305	Beagle	CD	F	18
1310	Beagle	CD	F	17

Table 1.2. Canine articular cartilage (AC) cells donor characteristics. *NCD* = non-chondrodystrophic. *CD* = chondrodystrophic.

Culturing

AC cells (P2) were expanded for three days in four T175 culture flasks in expansion medium under normoxic conditions (37°C, 5% CO_2). After the expansion time, AC cells were counted and microaggregates of approximately 35,000 cells were formed (n=16 per condition per donor) on day 0. The ACs were plated in a 96-wells plate (655180, Cellstar®, Greiner Bio-One, Alphen aan de Rijn, the Netherlands) in 50 µl chondrogenic medium. The chondrogenic medium consisted of hgDMEM (high glucose, GlutaMAX, pyruvate (Invitrogen, 31966)), 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 asap, and 10 µL/mL bovine serum albumin (BSA, Sigma, A9418). The following day, the d0 samples and medium were collected, medium was changed and the conditions were established. The following six groups were formed: chondrogenic medium (control), transforming growth factor beta (TGF-β) medium (chondrogenic medium with the addition of 2 ng/mL TGF-β), chondrogenic medium with 20 healthy synovial fluid (HSF), chondrogenic medium with osteoarthritic synovial fluid (OASF), TGF-β medium with healthy synovial fluid (THSF), and TGF-β medium with osteoarthritic synovial fluid (TOASF). The synovial fluid, whether healthy or OA was supplemented in a concentration of 20% (200 µL/mL). The TGF-β medium consisted of chondrogenic medium with the addition of 2 ng/mL TGF- β (**Figure 1.1**).

The medium was changed and simultaneously collected on day 0, 4, 7, 10, and 14. Additionally, on day 0, RNA (n=3 per donor) and glycosaminoglycan (GAG) and DNA samples (n=3 per donor) were collected. On day 4 RNA samples were also collected (n=6 per condition per donor), while on day 7 and 14 GAG and DNA samples (n=6 per condition per donor) were collected. After 7 days, the micro-aggregates from HSF and THSF were terminated, due to insufficient resources. After 14 days, the other conditions of the experiment were ended.

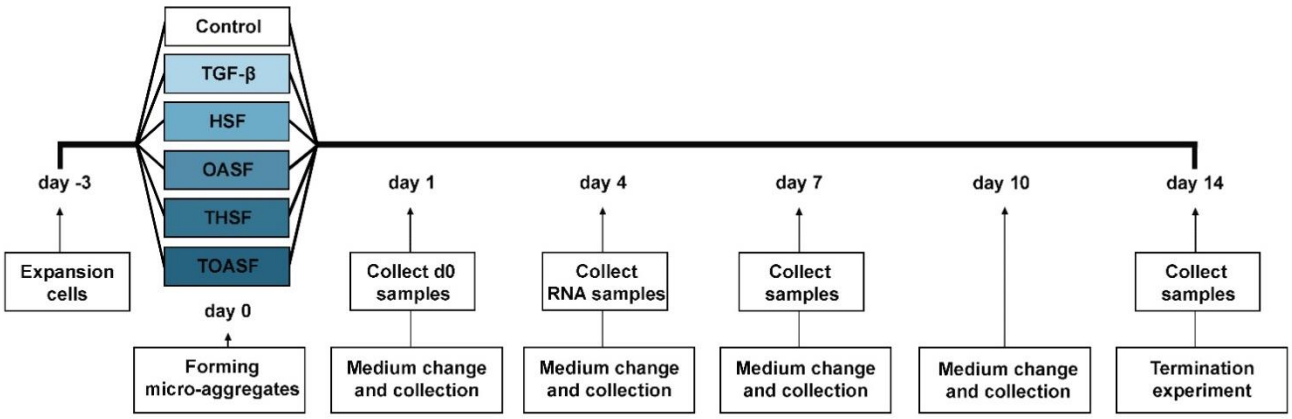

Figure 1.1. Diagram showing the time schedule of the experiment, whereby six conditions were created. On day 0 and 4 samples for RNA isolation and on day 0, 7 and 14 samples for glycosaminoglycan (GAG) and DNA samples and histology were collected. The experiment was terminated after 14 days of culturing. The healthy synovial fluid (HSF) and healthy synovial fluid with the addition of transforming growth factor beta (THSF) conditions were terminated after 7 days of culturing. *Control* = chondrogenic medium. *TGF-β* = 2 ng/mL transforming growth factor beta. *HSF* = healthy synovial fluid. *OASF* = osteoarthritic synovial fluid. *THSF* = healthy synovial fluid with the addition of TGF-β. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β.

Cell proliferation and extracellular matrix production

GAG and DNA content

On day 0, 7, and 14 of the experiment, samples were collected to measure DNA and GAG content of the cartilage micro-aggregates (n=6 per donor and condition and n=3 for day 0 per donor). The medium, too, was collected on day 0, 4, 7, 10, and 14, and was stored at -20°C until further measurements. Before assessing DNA and GAG content, papain digestion was performed.

Samples were lyophilized for 1 hour in the Savant SpeedVac® System (AES2010 Concentrator). Each micro-aggregate received 75 µL of papain digestion solution, consisting of a papain buffer (200 mM $H_2NaPO_4 \cdot 2\ H_2O$ (21254, Boom B.V., Meppel, the Netherlands), pH 6) and 10 mM EDTA (100944, Merck Millipore, Amsterdam, the Netherlands, pH 6.0), 10 mM Cysteine HCL (C7880, Sigma-Aldrich, Saint Louis, USA), and 10 mM Papain (P3125, Sigma-Aldrich, Saint Louis, USA). The micro-aggregates were incubated over night at 60 °C. The following day all samples were vortexed and incubated for an additional hour.

A dimethylmethylene blue (DMMB) assay was performed with a DMMB staining solution: 16 mg/L DMMB (Sigma-Aldrich 341088), 0.5% ethanol (Klinipath 4099.9005), 2.37 g/L NaCl (Merck 106404), 3.04 g/L Glycine (Sigma-Aldrich G8898); to measure the GAG content of the micro-aggregates and their medium. A stepwise dilution of chondroitin sulphate (C4384, Sigma-Aldrich, Saint Louis, USA) was created to generate a standard curve. Absorption was measured at an extinction of 540 and 595 nm with a microplate reader (TECAN plate reader) directly after the addition of DMMB. The amount of GAG per sample was calculated according to the polynomic formula obtained by the standard curve.

The DNA content of the micro-aggregates was measured using the Qubit™ dsDNA High Sensitivity Assay Kit (Q32851, Invitrogen, Eugene, USA), according to the guidelines of the manufacturer.

Histology

Cartilage micro-aggregates (n=2 per condition, per donor) were collected on day 0 (n=2 per donor), 7, and 14 and fixed in neutral buffered formalin (NBF, 4%, 4286, Klinipath B.V., Duiven, the Netherlands) and stained with 10% Eosin (K8886235, Boom B.V., Meppel, the Netherlands) for at least 24 hours. Then, the samples were embedded in 2.4% alginate (A2033, Sigma-Aldrich, Saint Louis, USA) and subsequently in formalin with addition of 102 mM $CaCl_2$. The samples were dehydrated according to the protocol of the pathology department of the University of Utrecht, and embedded in paraffin. 5 µm sections were fixed on Microscope KP plus slides (KP-3056, Klinipath B.V., Duiven, the Netherlands). Next, slides were left on a heating plate for one hour and left in the incubator overnight at 37 °C.

Safranin-O/Fast Green staining

Sections were first deparaffinised and hydrated by the use of xylene (5 minutes, twice) and graded ethanol (96%, 80%, 70%, and 60%, 5 minutes each). This was followed by washing by Milli-Q for 5 minutes, and incubation with citrate (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 10 minutes, while being gently shaken. Then, the sections were subjected to Weigert's Haematoxylin (solution 1: alcoholic Haematoxylin, Klinipath 640495; solution 2: acidified ferric chloride, Klinipath 640505) for 5 minutes. After this, the sections were gently washed by running tap water for 10 minutes and rinsed with demineralized water. Thereafter, sections were stained with 0.4% aqueous Fast Green (F7252, Sigma-Aldrich, Saint Louis, USA) for 4 minutes, rinsed with three changes (for 2, 2, and 1 minute) of 1% Acetic Acid (76051830, Boom), followed by staining with 0,125% Aqueous Safranin-O (S8883, Sigma-Aldrich, Saint Louis, USA) for 5 minutes. Lastly, sections were dehydrated by briefly dipping them in 96% ethanol and placed in 100% ethanol (5 minutes). Finally, the sections were placed in Xylene (5 minutes, twice) and mounted (Vectamount, VWR 631-0146).

Collagen type I and II immunohistochemistry

Sections were deparaffinised for collagen type I and II immunohistochemistry according to the protocol previously described. Subsequently, sections were washed with phosphate buffered saline (PBS) for 5 minutes. Next, slides were incubated with 0.3% H_2O_2 (51008600.9025, Boom B.V., Meppel, the Netherlands) for 10 minutes at room temperature and washed twice in PBS-Tween (PBS-T) 0.1% for 5 minutes each. Antigen retrieval was achieved by 1 mg/mL pronase (11459643001, Roche Diagnostics, Almere, the Netherlands) followed by 10 mg/mL hyaluronidase (H3506, Sigma-Aldrich, Saint Louis, USA) for 30 minutes each at 37 °C. After each antigen retrieval step, sections were washed twice with PBS-T 0.1% for 5 minutes each. Then, sections were blocked with PBS/BSA 5% for 30 minutes at room temperature. Thereafter, the sections were incubated overnight at 4 °C with a collagen type I mouse monoclonal antibody (100 mg/mL, ab6308, Abcam, Cambridge, UK) diluted 1:1500 in PBS/BSA 5%, or a collagen type II mouse monoclonal antibody (42 µg/ml, II-II6B3, DSHB, Iowa City, IA) diluted 1:2000 in PBS/BSA 5%. A normal mouse IgG_1 antibody (50µg/ml, 3877, Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:750 in PBS/BSA 5%, was used as a negative control.

The next day, sections were washed twice with PBS-T 0.1%, 5 minutes each. Thereafter, the sections were incubated for 60 minutes at room temperature with a secondary antibody (EnVision+ System-HRP Goat Anti-Mouse, K4001, Dako, Glostrup, Denmark) conjugated with horseradish peroxidase (HRP). The sections were washed twice for 5 minutes each in PBS. Thereupon, sections were incubated in 3,3'-Diaminobenzidine (DAB) peroxidase substrate solution (K3468, DAKO, Glostrup, Denmark) for 1 minute, and, subsequently, rinsed in Milli-Q and demiwater for 5 minutes each. Haematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) was added for 1 minute for counterstaining. Then, the sections were rinsed in running tap water for 10 minutes and dehydrated with graded alcohol (70%, 80%, 96%, 96%, 100%, 5 minutes each) followed by xylene (5 minutes, twice) and, lastly, mounted with Vectamount.

Gene expression

After 4 days of culturing, micro-aggregates (n=6 per donor and condition and n=3 for d0 samples, for each donor) were collected for RNA isolation. The micro-aggregates were snap frozen with liquid nitrogen and crushed with pellet pestles (P9951-901, Argos Technologies, Elign, USA). Then, a RNAeasy® Micro Kit (74004, Qiagen, Hilden, Germany) was used according to the guidelines of the manufacturer, in order to isolate the RNA. DNA removal was safeguarded in this process by performing a DNase step (RNAse free DNase Set, 79254, Qiagen, Valencia, USA). Quality of the isolated RNA was not tested, since micro-aggregates were used and inputs for the RNA Nanochip analyser were too low. The iScript™ cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands) was used according to the guidelines of the manufacturer. Six reference genes were chosen to normalize for the target gene expression of ECM anabolism, ECM catabolism, cytokines, PGE_2 pathway enzymes, and apoptotic markers (**Table 1.3**). IQT™ SYBR Green Supermix Kit (Bio-Rad, Veenendaal, the Netherlands) and the CFX384 Connect™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands) were used to perform qRT-PCR. Relative quantitative gene expression was determined by the Normfirst method. By which the mean Ct-values of the reference genes normalized the mean Ct-values of the genes of interest (GOI): $\Delta Ct = Ct_{mean\ ref} - Ct_{GOI}$. Thereafter, $E^{\Delta Ct}$ were calculated whereby E indicates the efficiency of amplification from the GOI/reference gene.

Inflammatory response

PGE_2 release

Medium samples of day 0, 4, 7, 10, and 14 were used to measure prostaglandin E_2 (PGE_2) concentrations by ELISA (n=1). The PGE_2 released in the medium was measured with a competitive colorimetric ELISA (Prostaglandin E2 monoclonal ELISA kit, 514010, Cayman Chemical, Ann Arbor, USA) according to the manufactures instructions. The colorimetric intensity was determined using microplate reader (TECAN plate reader).

Statistical analysis

All data was statistically analysed using IBM SPSS statistics 24. A normality check was performed to determine normal distribution of the GAG and DNA content and gene expression using a Shapiro Wilks test. Not normally distributed data was subjected to the Kruskal Wallis and Mann Whitney U test, while ANOVA was used for the normally distributed data. Post-hoc tests (Benjamini & Hochberg) were performed for multiple comparisons. Significance was achieved at *p*-value <0.05. Biological significance of differences in gene expression was set at the threshold of a two-fold change.

Since multiple factors (donor, dog subpopulation, treatment and day) could influence the outcome of the present results, a multivariate regression model, the COX proportional hazard model, was used for the gene expression. For each main comparison the most suitable statistical model was designed. All the comparisons in which the two dog subpopulations, CD and NCD dogs, were compared, the donor was incorporated as a random effect.

Results

To mimic the *in vivo* situation of a synovial joint the cartilage micro-aggregates of CD and NCD donors were cultured in the presence of 20% healthy or osteoarthritic synovial fluid. Due to quantity limitations of healthy synovial fluid, healthy synovial fluid culture conditions where studied until 7 days of culture, while OASF was continued for an additional 7 days (14 days of culturing in total).

Viability and cell counting

OA synovial fluid had a viable cell count ranging from $1.16 \cdot 10^6$ – $5.62 \cdot 10^6$ cells (40-73% alive). Healthy joints contain limited amounts of synovial fluid and as such healthy synovial fluid could be collected only in limited amounts. Therefore, from only one healthy donor cell count and viability was directly assessed before centrifugation and aliquotation. This synovial fluid had a viable cell count of $4.21 \cdot 10^5$ cells (60% alive).

Cell proliferation and extracellular matrix production

GAG and DNA content

Given that synovial fluid itself contains GAGs, the measurements of the medium were corrected for this. After this correction, however, it became clear that the total GAG release of micro-aggregates cultured in medium with addition of synovial fluid could not be reliably measured due to the high amount of background interference of the synovial fluid itself. As such, the GAG release in the medium over the culture period represents primarily the GAGs already present in the collected synovial fluid rather the GAGs secreted by the cultured ACs. While GAG content of the micro-aggregates represents the GAGs deposited within the matrix.

The GAG content, without taking the culture conditions into account (healthy versus OASF), did not significantly differ between NCD and CD. Therefore, further analysis of the GAG content was conducted excluding donor type (NCD or CD) as a fixed effect. Culturing in synovial fluid, HSF or OASF, resulted in a significant higher GAG deposition, compared to the control after 7 days of culturing. Addition of TGF-β, whether to the control, HSF or OASF, resulted in a similar significant increase. 14 days of culturing in the presence of osteoarthritic synovial fluid resulted in a significant increase in GAG content compared to the control but did not further increase GAG production. The same was the case for TOASF when compared to TGF-β (**Figure 1.2**).

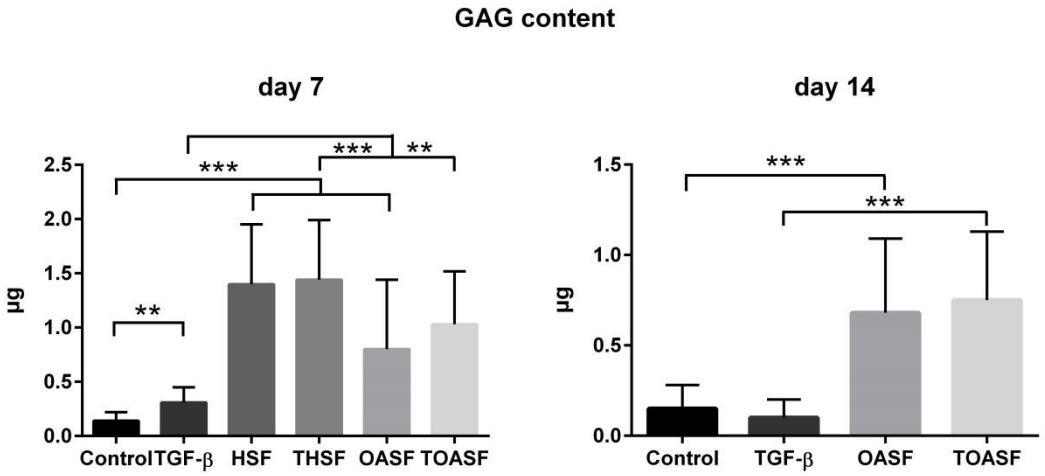

Figure 1.2. Glycosaminoglycan (GAG) content in cartilage micro-aggregates (mean±SD) after 7 days of culturing (**left**) and 14 days of culturing (**right**). The GAG content did not significantly differ between non-chondrodystrophic (NCD) and chondrodystrophic (CD) derived micro-aggregates and, therefore, analysis of the GAG content was conducted excluding donor type as a fixed factor. Healthy synovial fluid conditions were terminated after 7 days of culturing. Culturing in synovial fluid resulted in significantly higher GAG deposition, with or without the addition of 2 ng/mL transforming growth factor beta (TGF-β). *Control* = chondrogenic medium. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with addition of TGF-β. *OASF* = osteoarthritic synovial fluid. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β. ****** = *p*-value < 0.01. ******* = *p*-value < 0.005.

Since the DNA content of the micro-aggregates was influenced by the subpopulation of the donor (NCD or CD), donor was regarded a fixed factor and, therefore, conditions are compared within the subpopulation. Both micro-aggregates derived from NCD as derived from CD donors, contained significantly more DNA when cultured in the presence of synovial fluid, HSF or OASF. Only in the NCD donors did synovial fluid (HSF or OASF) with supplementation of TGF-β significantly increase the DNA content when compared to solely TGF-β. After 14 days of culturing the DNA content in CD was significantly increased by culturing in TGF-β compared to the control, as was seen in TOASF when compared to TGF-β (**Figure 1.3**).

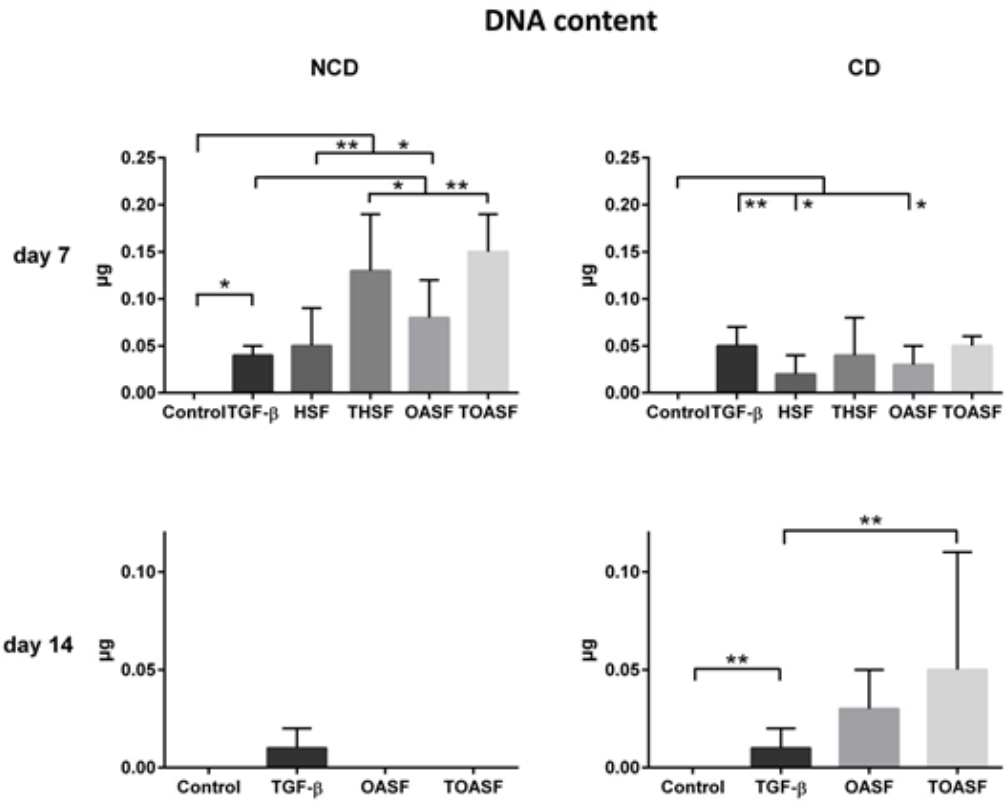

Figure 1.3. DNA content (mean±SD) of the micro-aggregates after 7 (**top**) and 14 (**bottom**) days of culturing in the non-chondrodystrophic (NCD) (**left**) and chondrodystrophic (CD) (**right**) donors. *Control* = chondrogenic medium *TGF-β* = chondrogenic medium with the addition of 2 ng/mL transforming growth factor beta. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with addition of TGF-β. *OASF* = osteoarthritic synovial fluid. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β. ***** = *p*-value <0.05. ****** = *p*-value < 0.01

Furthermore, GAG content was corrected for DNA content. After 7 days of culturing, GAG/DNA was significantly upregulated in THSF compared to TGF-β in the NCD donors. An upregulation in THSF compared to TGF-β was also observed in the CD micro-aggregates, but this was not significant. In the CD donors GAG/DNA after 7 days of culturing was only significantly downregulated in TGF-β when compared to the control. After 14 days of culturing, GAG/DNA of TOASF was significantly upregulated compared to TGF-β in the NCD donors. In CD donors similar changes were observed, GAG/DNA in TOASF was significantly upregulated compared to TGF-β, though, GAG/DNA of TOASF in CD donors seemed to remain lower than that of the NCD donors. In the CD micro-aggregates, after 14 days of culturing, the GAG/DNA of TGF-β was still significantly lower than the control (**Figure 1.4**).

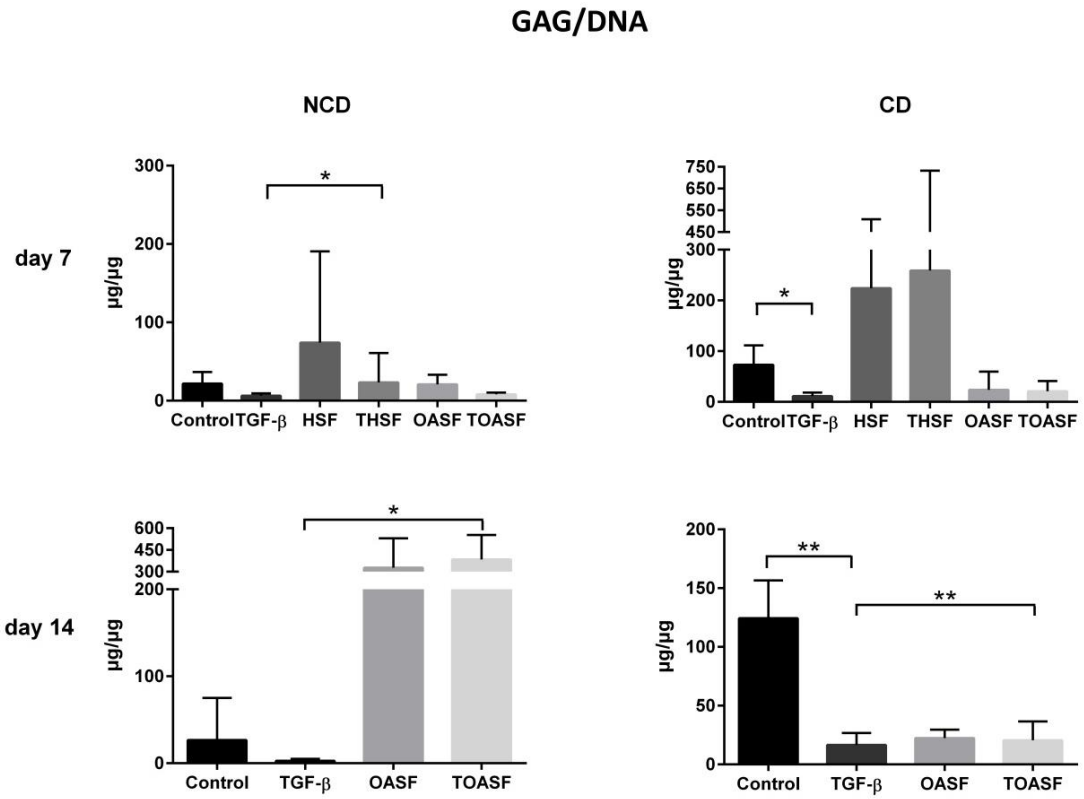

Figure 1.4. Glycosaminoglycan (GAG) content (mean±SD) corrected for DNA of the cartilage micro-aggregates after 7 (**top**) and 14 (**bottom**) days of culturing within the non-chondrodystrophic (NCD) (**left**) and chondrodystrophic (CD) (**right**) donors. GAG/DNA is significantly lower in TGF-β (chondrogenic medium with 2 ng/mL transforming growth factor beta) compared to control (chondrogenic medium) after 7 days of culturing and this remains the case after 14 days of culturing. In both NCD and CD donors the GAG/DNA after 14 days of culturing in TOASF is significantly upregulated compared to TGF-β. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with addition of TGF-β. *OASF* = osteoarthritic synovial fluid. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β. ***** = *p*-value < 0.05. ****** = *p*-value < 0.01.

Safranin-O/Fast Green staining

The Safranin-O/Fast Green staining indicated that interdonor differences, not only between CD and NCD micro-aggregates, were present. During culturing, it was observed that the micro-aggregates cultured with synovial fluid, whether HSF or OASF, lost their intercellular adhesion. This caused the micro-aggregates to fall apart. Histology showed that particularly NCD dog cartilage micro-aggregates were majorly disintegrated after 14 days of culturing in OASF. The addition of TGF-β seemed to result in slightly more coherent micro-aggregates (**Figure 1.5**). Micro-aggregates were not stained positively for Safranin-O as hardly any red staining was visualised. Only on day 0 in the CD donors and after culturing with OASF and TOASF conditions some slight purple staining was visualised.

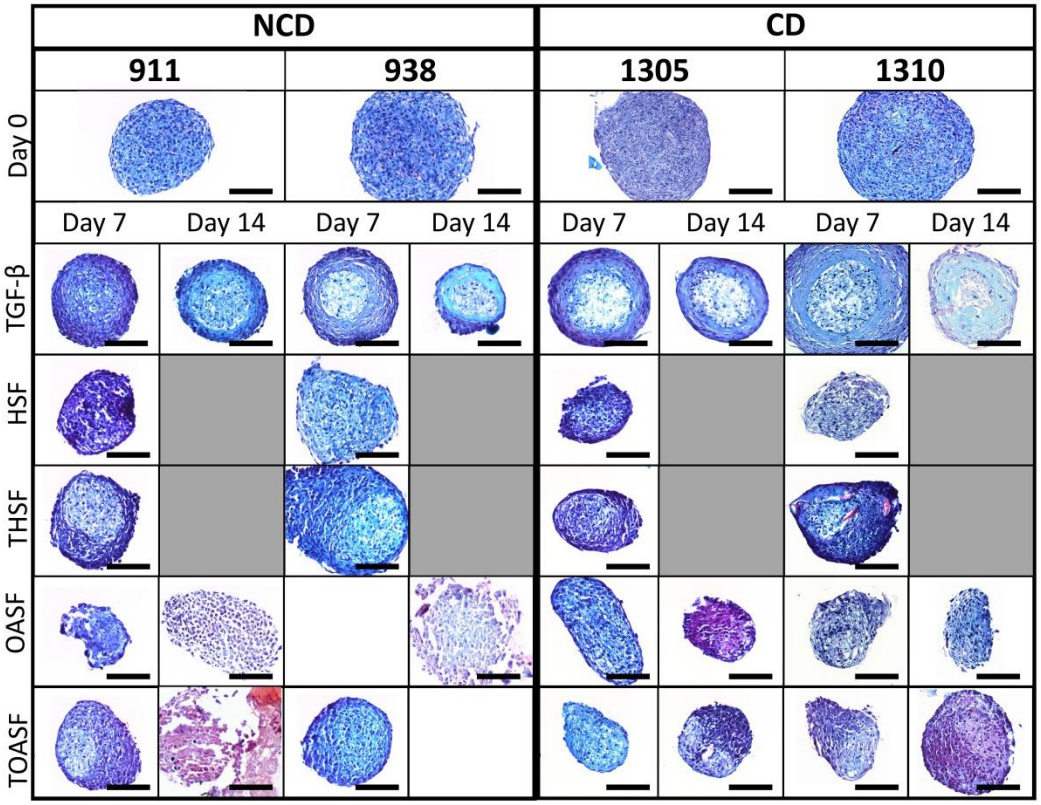

Figure 1.5. Safranin-O/Fast Green staining of articular cartilage micro-aggregates derived from non-chondrodystrophic donors (NCD) (**left**) and chondrodystrophic (CD) (**right**) donors on day 0 and after 7 and 14 days of culturing. The healthy synovial fluid (HSF) and 2 ng/mL transforming growth factor beta (TGF-β) added to healthy synovial fluid (THSF) conditions were terminated after 7 days of culturing. Culturing with synovial fluid resulted in loss of intercellular adhesion. Particularly NCD derived micro-aggregates majorly disintegrated after 14 days of culturing in osteoarthritic synovial fluid (OASF). The micro-aggregates cultured in chondrogenic medium (control) were lost for all donors. Empty boxes indicate that the subsequent micro-aggregates were lost for histology. A glycosaminoglycan (GAG) rich matrix would is visualized by red staining. Scale bar indicates 100 µm. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β condition.

Collagen type I and type II immunohistochemistry

Culturing with the addition of TGF-β showed an increase in collagen type I staining (**Figure 1.6**) when compared to the control, resulting in a ring of fibrosis. After 14 days of culturing the collagen type I has, however, shifted from the outer rim to the centre of the micro-aggregate. Notably, synovial fluid, whether osteoarthritic or healthy, seemed to counter this effect. No collagen type II was visualised in any of the conditions of the micro-aggregates (data not shown).

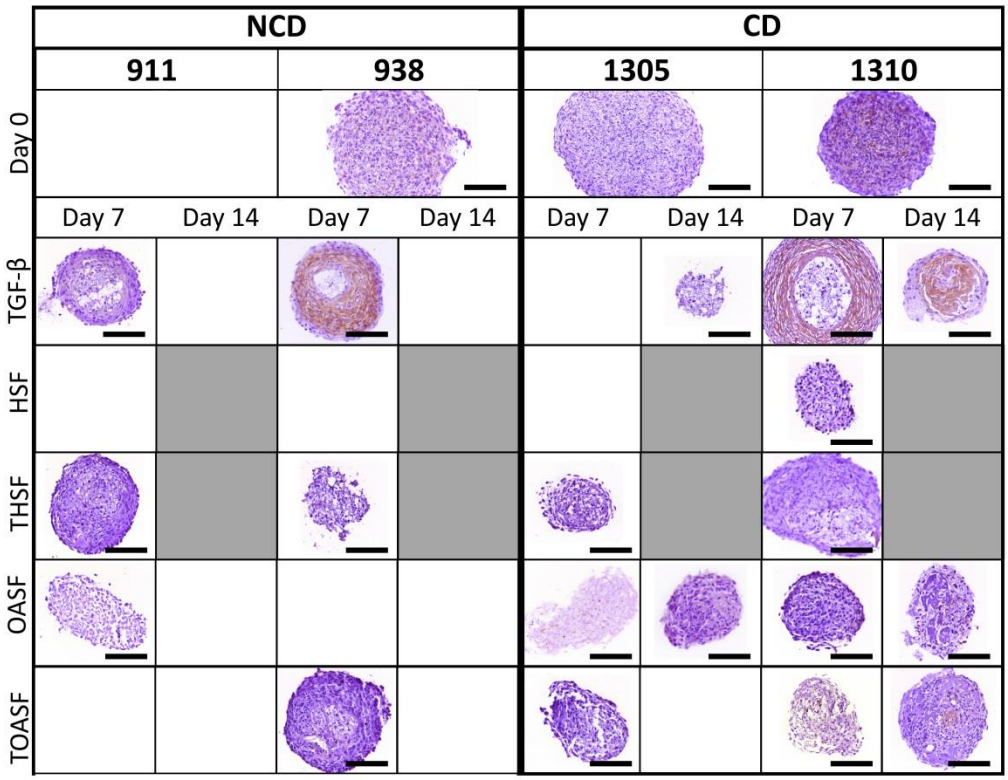

Figure 1.6. Collagen type I immunohistochemistry of articular cartilage micro-aggregates derived from non-chondrodystrophic donors (NCD) (**left**) and chondrodystrophic (CD) (**right**) donors on day 0 and after 7 and 14 days of culturing. After 7 days of culturing in chondrogenic medium with the addition of 2 ng/mL transforming growth factor beta (TGF-β) induced a fibrotic rime, while after 14 days the collagen had shifted more central in the micro-aggregate. The micro-aggregates cultured in chondrogenic medium (control) were lost for all donors. Empty boxes indicate that the subsequent micro-aggregates were lost for histology. Collagen type I positive staining is visualized by brown staining. Scale bar indicates 100 µm. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with the addition of TGF-β condition. *OASF* = osteoarthritic synovial fluid with the addition of TGF-β condition. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β condition.

Gene expression

Day 0 and 4 samples were employed to evaluate gene expression. The gene expression levels of *BCL2*, *BAX*, *IL1β*, *FASL*, *ADAMTS5*, *TNFα*, *COL10*, and *PTGES2* were inconsistently expressed or undetectable in the majority of the samples, and are, therefore, not shown.

Micro-aggregates derived from CD dogs generally showed a higher gene expression when compared to micro-aggregates derived from NCD dogs, which is in line with the higher DNA levels detected in the day 0 samples (**Figure 1.3**).

COL1A1 was significantly upregulated in CD derived micro-aggregates on day 0 compared to the NCD derived micro-aggregates. On day 0 a trend ($0.10 > p\text{-value} > 0.05$) was observed in *CASP3* in which the expression was significantly upregulated in NCD derived micro-aggregates compared to the CD ones. Moreover, on day 0 *PTGES1* expression was significantly higher in NCD cartilage compared to CD cartilage (**Figure 1.7**).

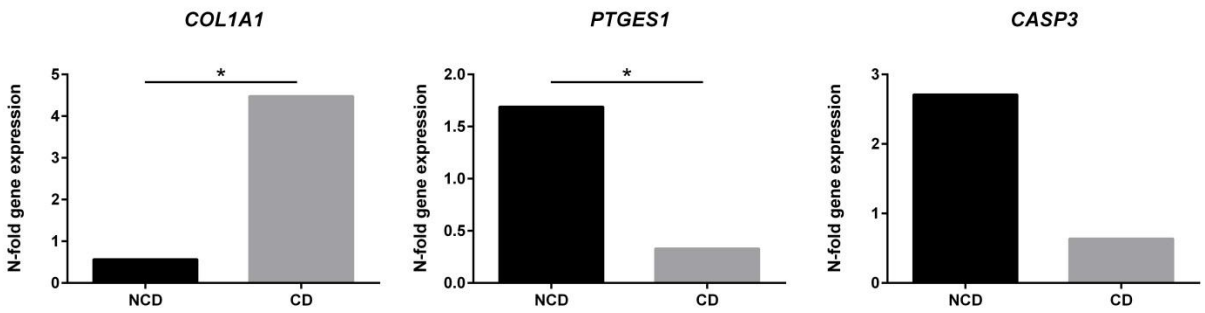

Figure 1.7. N-fold gene expression (relative to normal±SE) on day 0 for *COL1A1, CASP3,* and *PTGES1*, comparing non-chondrodystrophic (NCD) articular cartilage micro-aggregates with chondrodystrophic (CD) micro-aggregates. ***** = *p*-value <0.05.

Furthermore, to evaluate the differences between NCD and CD micro-aggregate metabolism the conditions of both subpopulations were compared. Significantly more *COL2A1* expression in the OASF condition was found in CD derived micro-aggregates. *SOX9*, also an anabolic marker, was significantly upregulated in the CD donors compared to the NCD donors in OASF. *CASP3* was significantly downregulated in the control in the CD donors compared to the NCD donors, but was significantly upregulated in TGF-β and OASF in CD micro-aggregates compared to the NCD ones. *PTGES1* and *COX2*, both part of the PGE_2 pathway, also showed some significant differences between NCD and CD donors. *PTGES1* was significantly upregulated in CD micro-aggregates compared to the NCD ones in the control. Significantly more expression in CD micro-aggregates compared to NCD micro-aggregates was also observed in THSF and OASF. While in TOASF *PTGES1* was significantly downregulated in CD derived micro-aggregates compared to NCD derived micro-aggregates in TOASF. *COX2* expression was significantly more upregulated in the CD donors compared to the NCD donors in TGF-β and THSF (**Figure 1.8**).

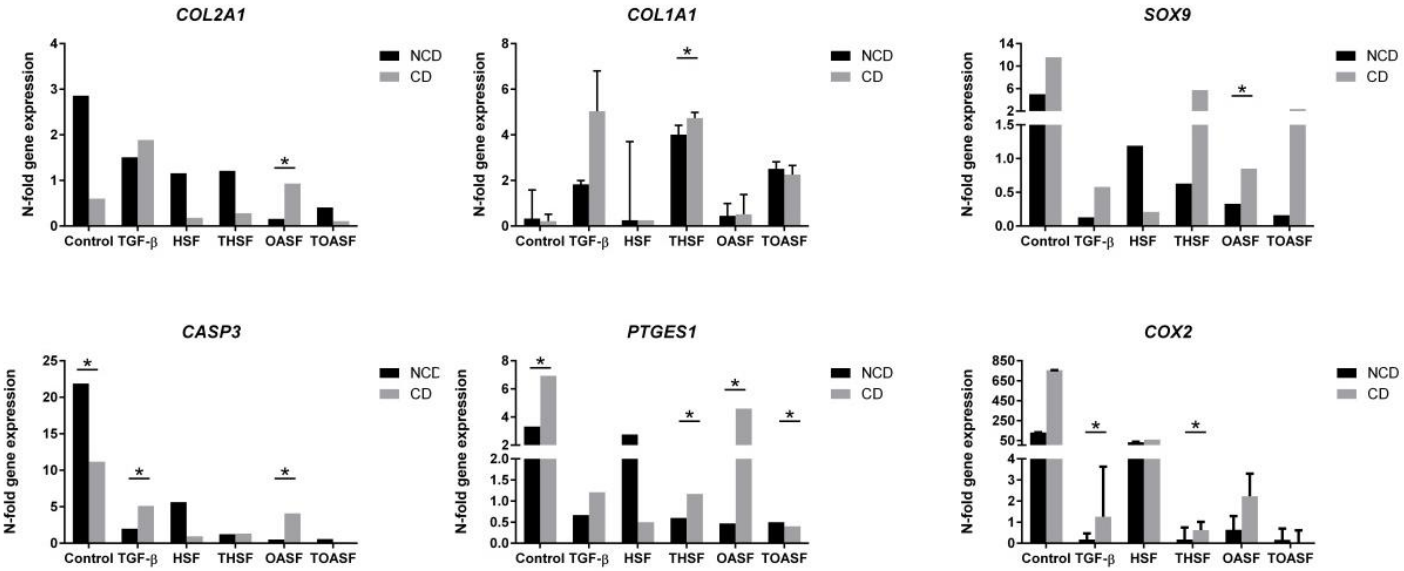

Figure 1.8. N-fold gene expression (relative to normal±SE) of *CASP3, COL1A1, COL2A1, COX2, PTGES1, and SOX9* after 4 days of culturing comparing non-chondrodystrophic (NCD) and chondrodystrophic (CD) articular cartilage micro-aggregates within the conditions. *Control* = chondrogenic medium. *TGF-β* = chondrogenic medium with the addition of 2 ng/mL transforming growth factor beta. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with the addition of TGF-β. *OASF* = osteoarthritic synovial fluid. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β. ***** = *p*-value < 0.05.

Moreover, the gene expression levels after 4 days of culturing in the established conditions were compared. *COL1A1, COL2A1,* and *SOX9* are import to evaluate the matrix quality. *Col1A1* gene expression was significantly more expressed in TGF-β when compared to the control. Furthermore, *COL1A1* was also significantly more upregulated in THSF compared to HSF, as in TOASF compared to OASF. Notably, *CASP3*, an apoptotic marker, was significantly downregulated in OASF compared to HSF. The control micro-aggregates showed a significantly higher expression of *COX2* compared to TGF-β (**Figure 1.9**).

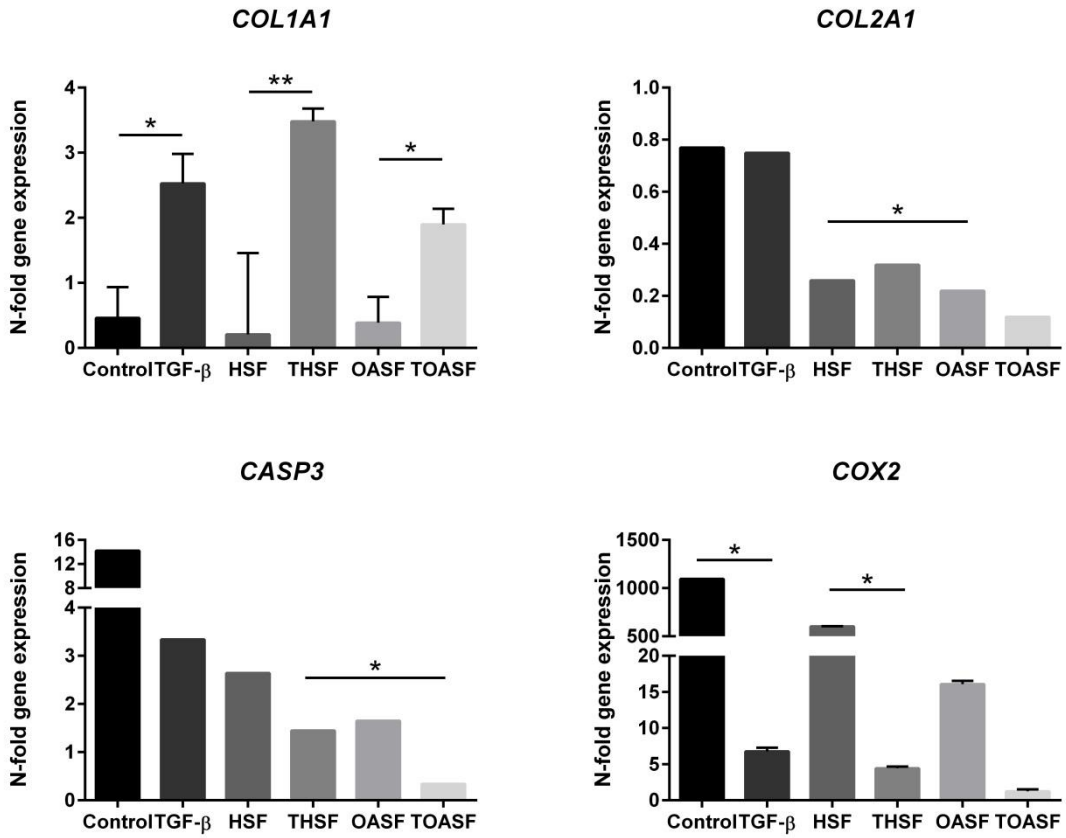

Figure 1.9. N-fold gene expression (relative to normal±SE) of *CASP3, COL1A1, COL2A1,* and *COX2* of articular cartilage micro-aggregates after 4 days of culturing. *Control* = chondrogenic medium. *TGF- β* = chondrogenic medium with the addition of 2 ng/mL transforming growth factor beta. *HSF* = healthy synovial fluid. *THSF* = healthy synovial fluid with the addition of TGF-β. *OASF* = osteoarthritic synovial fluid. *TOASF* = osteoarthritic synovial fluid with the addition of TGF-β. ***** = *p*-value < 0.05. ****** = *p*-value < 0.01.

Inflammatory response

Presumably, synovial fluid itself contained PGE_2, making it hard to descern what was actually produced by the micro-aggregates. However, the percentage of PGE_2 in the culture medium remained the same, while the release in the medium differed between the time points, indicating that the micro-aggregates did produce PGE_2 on their own.

In the control the production of PGE_2 in total was higher in the NCD micro-aggregates. In TGF-β the opposite was the case (**Figure 1.10, top**). The PGE_2 content in the medium appeared highest in the first week in the micro-aggregates cultured in HSF. This was similar in the NCD and CD micro-aggregates. In both subspecies, the amount of PGE_2 in the medium did not really increase due to culturing OASF until 7 days of culturing (**Figure 1.10, bottom**). Culturing in OASF gave more PGE_2 in the medium of CD micro-aggregates than in NCD micro-aggregates. The PGE_2 content in the synovial fluid conditions (HSF and OASF) was higher compared to the control.

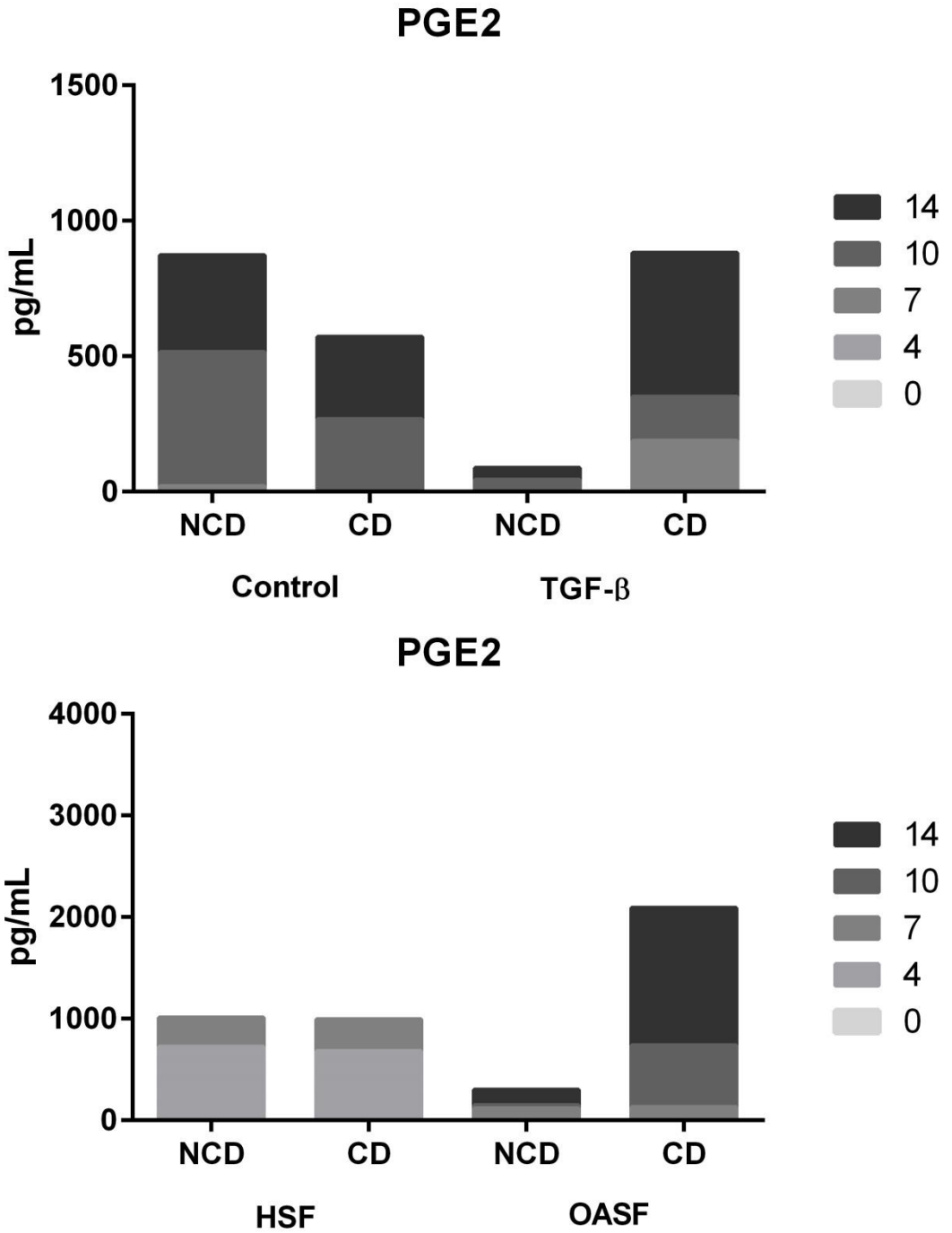

Figure 1.10. Total prostaglandin E_2 (PGE_2) content (mean) in the medium in total after 14 of culturing in chondrogenic medium (control) and chondrogenic medium with the addition of 2 ng/mL transforming growth factor (TGF-β) (**top**), and healthy synovial fluid (HSF) and osteoarthritic synovial fluid (OASF) (**bottom**) in non-chondrodystrophic (NCD) (n=2) and chondrodystrophic (CD) (n=2) micro-aggregates. Presumably, the synovial fluid itself contained PGE_2, making it hard to descern what was actually produced by the micro-aggregates. However, the percentage of PGE_2 in the culture medium remained the same, while the release in the medium differed over time.

Discussion

Mimicking the osteoarthritic joint by culturing with osteoarthritic synovial fluid

Mimicking osteoarthritis proves to be challenging as it is a degenerative disease of the whole joint in which multiple tissues take part in the pathogenesis[15]. The most suitable model to study new treatments for OA in dogs to truly mimic the diseased joint, therefore, should employ material from canine OA patients (i.e. OA cartilage and synovium). Unfortunately, within the limitations of veterinary practice, little material is available of OA patients. This is due to the fact that total joint replacements, a suitable source for all joint tissues for *in vitro* studies, by prostheses are rarely performed. A culturing system with more easily accessible OA patient derived tissues, namely synovial fluid, would, therefore, be of value. With this culture system, chondrocytes are cultured in an environment enriched with synovial fluid as would be the case in an *in vivo* situation. A co-culture set-up of synovial fluid with canine chondrocyte micro-aggregates, a 3D-environment mimicking cartilage, has, to the author's knowledge, not been performed yet.

Cultures where synovial fluid is being supplemented to determine the effect on a cellular level have several technical challenges and limitations. Synovial fluid requires special handling due to its high viscosity. Synovial fluid contains vast amounts of GAGs and other factors, and by combining it with the micro-aggregate culture systems, makes it unreliable to determine the GAGs released by the cells in the medium. During normal metabolism, joint tissue macromolecules or derived fragments are released into the synovial fluid. Increased release of GAGs from the articular cartilage in the environment indicates cartilage degeneration and would be expected to be more prominent in case of OA. Our findings are in line with previous reports where no difference between normal and diseased joints with regard to GAG levels in synovial fluid have been reported[97]. However, GAG content in synovial fluid has even been opted as an early diagnostic biomarker of OA in horses[97]. Indeed, Little *et al*[98]. found that the sulphated GAG concentration was markedly high in several of their synovial fluid samples from arthritic horses, but this did not correlate with the degree of articular cartilage erosion. Given the multifactorial background of OA and donor variation, more canine synovial fluid samples (healthy and OA) need to be collected to have a more representative population of synovial fluid samples. Furthermore, contamination of the medium in the micro-aggregate preparation may interfere with the GAG content measurements of the micro-aggregates themselves. Machado *et al*.[99] deemed dye-binding assays with DMMB as unreliable for measuring the GAG content of horse synovial fluid, as ions and other anionic macromolecules interfere. As such, higher dilutions of synovial fluid-supplemented medium need to be studied lowering the detection limits. For future culturing, washing of the micro-aggregates may be beneficial to minimalize the interference of the synovial fluid with GAG measurements in the micro-aggregates.

Moreover, the micro-aggregates cultured in the presence of synovial fluid (healthy or OA) lost their cell adhesion and fell apart, which was histologically already visible after 7 days. This made the collection of (complete) samples challenging. This loss of cell adhesion may be due to aggrecanases in the synovial fluid. Especially in the early and late stages of OA aggrecanases are elevated in synovial fluid[100]. An increased cellularity has been observed before when culturing healthy human articular cartilage with synovial fluid from injured knee joints for 14 days. In this culture set-up, the proteoglycan content did also decrease[93]. In our study, changes in DNA could not be measured reliably, due to the disintegration of the micro-aggregates.

Synovial fluid, whether HSF or OASF, seemed to counter the effects on collagen type I formation. Possibly also due to the aggrecanases in the synovial fluid. Less collagen type I staining was seen in micro-aggregates cultured with synovial fluid together with TGF-β.

While in gene expression only TOASF seemed to have a lower expression of *COL1A1* than TGF-β, though this change was not significant.

The aim of this experiment was to determine whether osteoarthritic synovial fluid could mimic the osteoarthritic joint environment in the articular cartilage cells. If this desired effect was accomplished, a new model would be possible for testing in an osteoarthritic environment *in vitro*. However, OASF did not always promote a pro-inflammatory environment, while this was the case for HSF. Presumably, PGE_2 release by the NCD micro-aggregates stayed low because the cells were no longer viable. DNA content supports this, as this could not be reliably detected in the NCD micro-aggregates after 14 days of culturing. The PGE_2 content in the medium of the synovial fluid conditions does seem to be higher than in the control. Against our expectations, *COX2* gene expression did not increase in the presence of OASF. PGE_2 content in the medium did not increase until day 7 of the culturing. Though the PGE_2 measurements should be interpreted cautiously as no correction was made for the PGE_2 content of the synovial fluid itself. Moreover, *CASP3* was significantly downregulated in OASF compared to HSF, possibly indicating less apoptosis in OASF. However, *BAX, BCL2,* and *FASL*, all also apoptotic markers, were not expressed. OASF did result in the disintegration of the micro-aggregates indicating a catabolic environment.

To optimise the current co-culture set-up different synovial fluid concentrations should be tested, as concentrations of 5%[46], 10%-50%[101] have also been applied in co-cultures. Moreover, micro-aggregates are, due to their small size, more susceptible for the aggrecanases in the synovial fluid and pellets (200,000 cells) or chondrocytes in agarose gels[94] may be a more suitable fit for this culturing set-up. Moreover, interference of synovial fluid in the data analysis should be taken account and uncultured synovial fluid should be used for corrections. An alternative approach to mimic the (osteoarthritic) joint more closely would be to culture with synovium conditioned medium instead of synovial fluid. Unfortunately, little material is available of OA patients. Synovium conditioned medium would contain the mediators produced by the synovium, which are likely present in the synovial fluid too, without the drawbacks of culturing with synovial fluid.

Differences in cartilage homeostasis between CD and NCD dogs

It has already been shown that degenerative joint diseases, such as intervertebral disc degeneration, have different characteristics between CD and NCD dogs[85]. These two dog subpopulations exhibit differences in the process of endochondral ossification, resulting in their difference in appearance (i.e. long bone length). Therefore, possible differences in cartilage homeostasis between CD and NCD dogs, within the presently used culture system, were analysed.

The presence of *CASP3* closely correlates with the OA grade and chondrocyte apoptosis may be determined by the expression of this gene[102]. In the control condition *CASP3* was significantly downregulated in the CD micro-aggregates compared to the control NCD micro-aggregates. A similar trend was visible at the initiation of the experiment (day 0 samples). Altogether, this may indicate that NCD cartilage cells are in a further degenerative state compared to CD cartilage cells. This relates with the observation that micro-aggregates derived from NCD dogs disintegrated at an earlier time point of culture compared to micro-aggregates from CD dogs. This was most obvious in NCD cartilage micro-aggregates after 14 days of culturing in OASF. It is plausible that NCD cells either produced less matrix, as indicated by the prominent staining of collagen type I present primarily in CD micro-aggregates, or produced matrix that could be easily degraded by active components of the synovial fluid. These observations are in line with our hypothesis that NCD dogs are more prone to develop OA when compared to chondrodystrophic dogs. While from a clinical perspective osteoarthritis seems to be more frequently diagnosed in larger dog (NCD)

breeds compared to smaller (CD) dog breeds, there are no comparative studies in this respect.

What the underlying reason is of the susceptibility of NCD dogs in cartilage degeneration remains elusive. The inflammatory components seem to be at play. *COX2,* a key player in the prostaglandin biosynthesis, gene expression did not differ significantly on day 0 or after culturing in chondrogenic medium (control) between CD and NCD micro-aggregates. However, *COX2* was significantly upregulated in CD dogs compared to NCD when cultured in TGF-β or THSF. Moreover, we observed that basal (on day 0) *PTGES1*, important for COX2 regulation, expression was significantly upregulated in NCD derived micro-aggregates. Interestingly, in the control after 4 days of culturing the contrary is the case; CD micro-aggregates then express *PTGES1* more significantly than NCD micro-aggregates. Still there are indications that NCD cartilage micro-aggregates are more susceptible for inflammation, though this may not immediately show in a control culture set-up. A COX2 immunohistochemistry staining could possibly confirm these gene expression results on a protein level.

Culturing with the addition of transforming growth factor β (TGF-β)

TGF-β addition to chondrogenic medium stimulated fibrosis, evident in the higher gene expression of *COL1A1* and visible in the deposition of collagen type I as the pronounced fibrous rim. *COL1A1* gene expression was significantly more expressed in TGF-β compared to the control. Such a significantly higher gene expression of *COL1A1* was also observed when comparing THSF to HSF and when comparing TOASF to OASF. Though in THSF and TOASF a fibrous rim did not show in immunohistochemistry staining. Similar results due to TGF-β have been shown in the past[103]. This observation, relates to the metalloproteinase activity of synovial fluid that possibly degrades this collagen deposition driven by TGF-β.

Conclusions

Not much is known about the differences in cartilage metabolism between CD and NCD dogs when cultured with synovial fluid. Culturing with synovial fluid comes with challenges. This study was a pilot to evaluate whether co-culturing cartilage micro-aggregates with synovial fluid would be suitable for an osteoarthritis model *in vitro*. Due to resource limitations, no follow-up experiments were conducted. For now, we have observed that synovial fluid give more PGE_2 in the medium after more than 7 days of culturing, but this seemed most prominent in healthy synovial fluid. Future work would be needed to optimise this co-culture system. Though it should be bore in mind that this experiment was only conducted with 4 donors (n=2 CD and n=2 NCD).

Gene category	Gene	Primer sequence (5'-'3)	Product size (bp)	qRT-PCR temperature condition (°C)
Anabolic	*ACAN*	Fw GGACACTCCTTGCAATTTGAG Rv GTCATTCCACTCTCCCTTCTC	110	61-62
	COL1A1	Fw GTGTGTACAGAACGGCCTCA Rv TCGCAAATCACGTCATCG	109	61
	COL2A1	Fw GCAGCAAGAGCAAGGAC Rv TTCTGAGAGCCCTCGGT	150	60.5-65
	SOX9	Fw CGCTCGCAGTACGACTACAC Rv GGGGTTCATGTAGGTGAAGG	105	62-63
Catabolic	*ADAMTS5*	Fw CTACTGCACAGGGAAGAG Rv GAACCCATTCCACAAATGTC	148	61
	MMP13	Fw CTGAGGAAGACTTCCAGCTT Rv -TTGGACCACTTGAGAGTTCG	250	65
Inflammatory mediators	*IL1β*	Fw TGCTGCCAAGACCTGAACCAC Rv TCCAAAGCTACAATGACTGACACG	115	68
	TNFα	Fw CCCCGGGCTCCAGAAGGTG Rv GCAGCAGGCAGAAGAGTGTGGTG	61	65
PGE_2 pathway	*COX2*	Fw TTCCAGACGAGCAGGCTAAT Rv GCAGCTCTGGGTCAAACTTC	112	60
	PTGES1	Fw CCAGTATTGCCGGAGTGACCAG Rv AAACGAAGCCCAGGAACAGGA	97	68
	PTGES2	Fw GCTCTCAAGACCTACCTGG Rv AGTCACTTCCTTTCCCTGG	98	60-62
Apoptotic markers	*BAX*	Fw CCTTTTGCTTCAGGGTTTCA Rv CTCAGCTTCTTGGTGGATGC	108	58-59
	BCL2	Fw GGATGACTGAGTACCTGAACC Rv CGTACAGTTCCACAAAGGC	80	61.5-63
	CASP3	Fw CGGACTTCTTGTATGCTTACTC Rv CACAAAGTGACTGGATGAACC	89	61
	FASL	Fw ATGTTTCAGCTCTTCCATCTACAG Rv CAGAAGGTGGATTGGGTTGAC	114	60
Osteoarthritis	*COL10*	Fw CCAACACCAAGACACAG Rv CAGGAATACCTTGCTCTC	80	61
Reference gene	*GAPDH*	Fw TGTCCCCACCCCCAATGTATC Rv CTCCGATGCCTGCTTCACTACCTT	100	58
	HNRPH	Fw CTCACTATGATCCACCACG Rv TAGCCTCCATAACCTCCAC	151	61
	RPL13	Fw GCCGGAAGGTTGTAGTCGT GGAGGAAGGCCAGGTAATTC	87	61
	RPS19	Fw CCTTCCTCAAAAAGTCTGGG Rv GTTCTCATCGTAGGGAGCAAG	95	61-63
	SRPR	Fw GCTTCAGGATCTGGACTGC Rv GTTCCCTTGGTAGCACTGG	81	61.5
	YWHAZ	Fw CGAAGTTGCTGCTGGTGA Rv TTGCATTTCCTTTTTGCTGA	94	58

Table 1.3. Gene-specific primer sequences with associated amplification temperatures.

An explant culture model to evaluate the difference between chondrodystrophic and non-chondrodystrophic dogs on a cartilage level

Abstract

Background. Chondrodystrophy causes abnormally short limbs and is a characteristic for several dog breeds. It has been shown that chondrodystrophic (CD) dogs differ from non-chondrodystrophic (NCD) dogs on an intervertebral disc level, as CD dogs suffer from accelerated disc degeneration. Whether CD and NCD dogs differ on a cartilage level has remained unexplored. Possibly the Wnt signalling pathway may play a role in this. Animal studies have already shown that the Wnt signalling pathway has multiple roles in the regulation of cartilage development, growth, and maintenance. The aim of the present study was to explore at the biochemical and biomolecular level the (dis)similarities between cartilage derived from CD and NCD dogs overall and look specifically in the Wnt signalling pathway.

Materials and methods. Healthy cartilage from NCD and CD, were cultured in an *ex vivo* explant setup for 21 days in the presence of chondrogenic (control) medium with or without a pro-inflammatory stimulus (tumour necrosis factor alpha (TNF-α)) and/or celecoxib (CXB), a specific cyclo-oxygenase 2 (COX2) inhibitor. Addition of TNF-α simulated *in vitro* the osteoarthritis environment, while the addition of the COX2 inhibitor simulates oral anti-inflammatory medication given to osteoarthritic patients. Read-out parameters were histology, immunohistochemistry (including COX2 staining), qRT-PCR focussing on representative genes from the canonical Wnt signalling pathway, and biochemical assays (glycosaminoglycan- (GAG) and DNA content, and prostaglandin E_2 (PGE_2)).

Results. CD derived cartilage appeared to be better at retaining GAGs in a pro-inflammatory environment. NCD cartilage responded more to treatment with CXB in an inflammatory environment and PGE_2 was suppressed by CXB. More COX2 seemed to be present in NCD cartilage. Wnt signalling pathway associated genes were significantly different expressed between NCD and CD cartilage and culture conditions were also of influence. Based on the *AXIN2* expression the Wnt signalling pathway appears to be more expressed in CD cartilage.

Conclusions. Cartilage differs between NCD and CD dogs at the biochemical and biomolecular level. NCD cartilage seems to be more susceptible for inflammation and to respond better to the treatment with anti-inflammatory drugs. Moreover, the Wnt signalling pathway appears to be differently expressed between CD and NCD derived cartilage and may play a role in the cartilage matrix differences.

Keywords: chondrodystrophic, non-chondrodystrophic, cartilage explants, osteoarthritis, matrix metabolism, canine, Wnt pathway, inflammation.

Introduction

Osteoarthritis (OA) is one of the most important musculoskeletal diseases accompanied by chronic pain and disability. The disease comes with economic, social, and psychological costs. The disease is facilitated by ageing, trauma, mechanical forces, conformation, and hormonal and genetic factors[104]. The pathophysiology of OA has not been fully elucidated and, the treatment remains symptomatically. Several methods, *in vitro* and *in vivo*, have been developed to study the pathophysiology of and treatment options for OA. Where *in vivo* studies allow for a closer resemblance to the (naturally occurring) whole-joint disease, *in vitro* systems contribute to the reduction of laboratory animal use and can be easier manipulated[105]. Lastly, *in vitro* allow the usage of tissue from target species, which is not always possible *in vivo.* Therefore, both come with their own advantages, as well as disadvantages. An intermediary model between these *in vivo* and *in vitro* models would incorporate the advantages from both and would be provided by an *ex vivo* explant culture model.

A great advantage of the use of explant cultures is that cells can be examined in their natural extracellular matrix (ECM). In cell cultures, cells are removed from their ECM, altering their cell phenotype. Chondrocyte-matrix associations are of major importance in cartilage homeostasis and this homeostasis becomes disrupted in OA. Examination in an explant model more accurately reflects naturally occurring OA[105]. A way to study OA in an explant based model is by cytokine stimulation. It has been demonstrated that cartilage is sensitive to tumour necrosis factor alpha (TNF-α) stimulation. A release of GAGs from the cartilage tissue has been observed in OA human cartilage explant model after TNF-α stimulation[106]. This is in line with earlier findings that articular chondrocytes can enter a catabolic state by TNF-α (and interleukin 1 beta (IL1β)) stimulation as is seen in OA[35,107].

This *ex vivo* model makes it possible to test certain factors, such as the effects of drugs. Furthermore, differences between donors may also come to light, for instance between subpopulations from the canine species. It has been observed as early as 1952 that chondrodystrophy results in a different expression of intervertebral disc (IVD) degeneration, as the age of onset, frequency, and spinal location are different in chondrodystrophic (CD) dogs[78]. Currently no distinction is made between these two subpopulations (CD and non-chondrodystrophic (NCD)) when it comes to articular cartilage, particularly when developing treatment strategies for OA. However, the difference on IVD level together with the fact that CD and NCD dogs are distinctly different when it comes to the process of endochondral ossification[86] sprouted the hypothesis that these two subpopulations also vary on a cartilage level. Currently, two different insertions in *FGF4* have been appointed as the cause of chondrodystrophy in dogs. Interestingly, it has been observed in adult mice that FGF3 delays OA progression in knee joints[83]. Indicating even more that chondrodystrophy leads to a different cartilage metabolism and that this even may influence the susceptibility for the development of OA.

More recently it has been shown that the canonical Wnt signalling pathway, or β-catenin-dependent signalling, is higher in IVDs of CD dogs when compared to NCD dogs[85], causing early disc degeneration. Animal studies have shown that the Wnt signalling pathway has multiple roles in the regulation of cartilage development, growth, and maintenance[75]. For instance, the canonical Wnt signalling is required for progression of endochondral ossification[108] and growth of the axial and appendicular skeleton[108,109]. Several studies indicate that higher expression of the canonical Wnt signalling in cartilage may be the cause of cartilage damage as seen in OA[108]. Human genetic studies even suggest the canonical Wnt signalling as a risk factor for OA[75].

Taken together, there are thus far no studies addressing differences at cartilage between CD and NCD dogs. Therefore, the aim of this study was to identify possible

variations in cartilage metabolism and more specifically in the canonical Wnt signalling pathway between CD and NCD dogs in an explant culture model. This was performed under basal conditions and in the presence of a pro-inflammatory stimulus to simulate the OA environment and/or in the presence of a selective cyclo-oxygenase 2 (COX2) inhibitor, namely celecoxib, to simulate the standard of care anti-inflammatory medication that OA canine patients receive on the course of clinical OA.

Materials and methods

Sources of canine tissue

Cartilage from the knee joint of healthy CD (n=7) and NCD (n=8) donors was collected. Donor information is listed in **Table 2.2**, all dogs had been euthanised in unrelated studies. The joint was opened under sterile conditions in order to precisely separate cartilage from bone and other structures during collection. From donor 401 onwards, only tissue from the femur epicondyle, optionally with the addition of tissue from the tibia plateau, were harvested separately. This distinction was made since adult articular cartilage has noticeable biochemical and biomechanical characteristics based on the differences in topographical loading within the joint[110]. Therefore, the experimental set up employed tissues from the same location in a matched manner. The obtained tissue was collected in 50 mL tubes with 25 mL hgDMEM (high glucose, GlutaMAX, pyruvate (Invitrogen, 31966)) + 20 µL/mL Penicillin/Streptomycin (p/s, PAA laboratories, P11-010). After washing with hgDMEM + 10 µL/mL p/s, an overnight rest at 37 °C in a Petri dish (353803, Corning, Durham USA) with 25 mL hgDMEM + 10 µL/mL p/s was included.

***Ex vivo* explant culture of canine cartilage and joint capsule tissue**

After an overnight acclimation period, cartilage was cut into pieces with mean ± SD wet weights of 10.4 ± 4.9 mg per cartilage explant. From the tibia one cartilage explant from each donor was cultured per well in a non-adherent 96-wells plate (Corning Costar, 7007). From the medial femur epicondyle two cartilage explants from each donor were cultured in each well of a 24-wells plate (662160, CELLSTAR® Greiner Bio-one, Alphen aan de Rijn, the Netherlands). Day 0 samples from the cartilage were also collected. Both wells-plates were cultured for 21 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). To prevent adhesion to the 24-wells plates, they were coated with an agarose coating (V3121, Promega Corporation, Madison, USA). Explants were subjected to a pro-inflammatory stimulus by adding 10 ng/mL TNF-α and/or treated with different dosages of celecoxib (CXB) (10^{-7} M, 10^{-6} M, and 10^{-5} M). Initial experiments where conducted with CXB in a 10^{-7} M concentration, however, the effect was minimal. Therefore, in follow up experiments the CXB concentration was increased to a 10^{-6} and 10^{-5} M concentration. Furthermore, an ethanol control was included in the 96-wells plate cartilage explant culture since higher dosages of celecoxib had to be diluted in ethanol. The ethanol concentration corresponded to the concentration used to dilute celecoxib 10^{-6} M and 10^{-5} M concentrations (**Table 2.1**).

96 wells-plate (tibia cartilage)	24-wells plate (femur cartilage)
Control	Control
TNF	TNF
TNF +CXB-7	TNF+CXB-7
CXB-7	CXB-7
TNF +CXB-6	TNF+CXB-6
CXB-6	CXB-6
TNF+CXB-5	
CXB-5	
EtOH	

Table 2.1. Tibia derived cartilage was cultured in a 96-wells plate; while corresponding femur derived cartilage was cultured in a 24-wells plate in the following conditions. *Control* = chondrogenic medium. *TNF* = tumour necrosis factor alpha. *CXB* = celecoxib.

Donor	Breed	NCD/ CD	Gender (M/F)	Age (months)	Weight (kg)	Details
120	German Shepherd	NCD	M	122	26	Cartilage from whole joint. Clinical signs of hip OA, on section no signs of OA in hip
257	Foxhound	NCD	F	29	25	Cartilage from whole joint. Received antibiotics systemically for infection
401	Foxhound	NCD	F	20	23	Only cartilage from medial femur epicondyle or tibia plateau
447	Foxhound	NCD	F	22	22	Cartilage from whole joint. Received antibiotics systemically for infection
459	Mixed Breed	NCD	F		24	Cartilage from whole joint
482	Mixed Breed	NCD	F		24	Cartilage from whole joint
692	Foxhound	NCD	F	19	28	Only cartilage from medial femur epicondyle or tibia plateau
804		CD				
808	Beagle/ Bedlington cross	CD	F	49	14.9	
809	Beagle/ Bedlington cross	CD	F	49	15.9	
840	Mixed Breed	NCD	F		24	
936	Beagle/ Bedlington cross	CD		26		Only cartilage from the femur epicondyle
5222	Beagle	CD	F	21	10.5	Only cartilage from medial femur epicondyle or tibia plateau
5550	Beagle	CD	F	22	12.4	Only cartilage from medial femur epicondyle or tibia plateau. Appeared to have thicker cartilage macroscopically
5053	Beagle	CD	F	22	12.4	Only cartilage from medial femur epicondyle or tibia plateau
5466	Beagle	CD	M	23	9.3	Cartilage healthy hip joint
862	Beagle	CD	M	48	10	Only cartilage from medial femur epicondyle or tibia plateau
801	Beagle	CD	F	45	10	Only cartilage from medial femur epicondyle or tibia plateau
362	Beagle	CD	F	48	10	Only cartilage from medial femur epicondyle or tibia plateau
17316	Mixed Breed	NCD	F	19	23	Cartilage healthy hip joint
3516	Mixed Breed	NCD	F	19	22	Cartilage healthy hip joint
721	Mixed Breed	NCD	F	21	21	Cartilage healthy hip joint

Table 2.2. Donor characteristics for articular cartilage explant culture. Donors 862, 362, 181, 17316, 3516, and 721 were only used for the cyclo-oxygenase (COX) 2 immunohistochemistry staining. *OA* = osteoarthritis. *NCD* = non-chondrodystrophic. *CD* = chondrodystrophic.

The medium was changed and simultaneously collected on day 2, 7, 10, 14, and 17; medium from day 21 was also collected. Additionally, on day 7, 3 cartilage explants per donor per condition from the 96-wells plate were collected for RNA isolation. On day 21 GAG/DNA samples (n=3 per donor for the 24-wells plate and n=3 per condition per donor for the 96-wells plate) were also collected. After 21 days the experiment was terminated and histology samples were also collected (n=2 per donor for the 24-wells plate, n=2 per condition per donor for the 96-wells plate) (**Figure 2.1**).

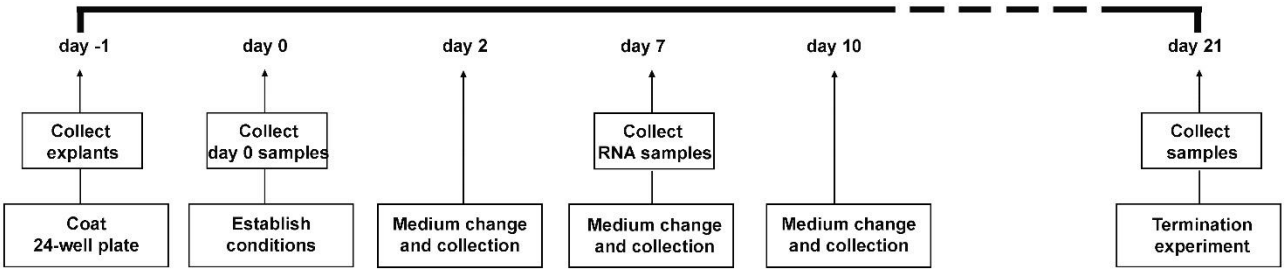

Figure 2.1. Diagram showing the time schedule of the experiment, whereby the different culture conditions were created. On day 7, samples for RNA isolation were collected. On day 0 and 21, samples for glycosaminoglycan (GAG)/DNA and histology were collected. The experiment was terminated after 21 days of culturing.

Cell proliferation and extracellular matrix production of the canine cartilage explants

GAG and DNA content

Cartilage explants from day 0 were collected to measure DNA, GAG, and GAG/DNA from both the tibia and femur of all donors. With the termination of the experiment, on day 21, explants were also collected to measure DNA and GAG content. Three cartilage explants derived from the tibia per condition per donor were pooled and two explants derived from the femur per condition per donor were pooled for analysis. The collected medium from days 3, 7, 10, 14, 17, and 21 was stored at -20 °C until GAG release was measured. The pooled explants per condition per donor were lyophilized for 2 hours in the Savant SpeedVac® System (AES2010 Concentrator) to measure dry weights of the cartilage explants. Thereafter, papain digestion was conducted on the cartilage explants with 600 µL of papain digestion solution (papain buffer (200 mM $H_2NaPO_4 \cdot 2\ H_2O$ (21254, Boom B.V., Meppel, the Netherlands), pH 6) and 10 mM EDTA (100944, Merck Millipore, Amsterdam, the Netherlands, pH 6.0), 10 mM Cysteine HCL (C7880, Sigma-Aldrich, Saint Louis, USA), and 10 mM Papain (P3125, Sigma-Aldrich, Saint Louis, USA)). DNA analysis and dimethylmethylene blue (DMMB) assays were performed accordingly to the protocol as termed in Chapter 1.

Histology

Cartilage explants (n=2 per condition, per donor) were collected on day 0 and 21 and fixed in neutral buffered formalin (NBF 4%, 4286, Klinipath B.V., Duiven, the Netherlands) for at least 24 hours. The samples were thereafter embedded in paraffin. Sections of 5 µm sections were fixed on Microscope Ultra plus slides (KP-3056, Klinipath B.V., Duiven, the Netherlands). Next, slides were left on a heating plate for at least three hours and dried in the incubator overnight at 60 °C.

Safranin-O/Fast Green staining

The Safranin-O/Fast Green staining was performed on the explants as described previously (Chapter 1) with slight modifications: the 10-minute citrate step was skipped as cartilage

explant were large enough and were not pre-embedded in alginate. Furthermore, sections were rinsed twice for 5 minutes with 1% Acetic Acid (76051830, Boom), instead of in three changes (for 2, 2, and 1 minute) of 1% Acetic Acid.

Collagen I and II immunohistochemistry staining
The collagen type I and type II immunohistochemistry staining was performed on the explants as described in Chapter 1. The primary antibody dilutions was accordingly modified as follows: Collagen type I mouse monoclonal antibody was diluted 1:500 in PBS/BSA 5% and collagen type II mouse monoclonal antibody was diluted 1:2000 in PBS/BSA 5%.

Cyclooxygenase (COX) 2 immunohistochemistry
Deparaffinisation and dehydration of the explants was accomplished by two times xylene and graded alcohol (96%, 80%, 70%, and 60%) for 5 minutes each. Sections were washed in Milli-Q for 5 minutes. After addition of an endogenous enzyme block (S2003, Dako, Heverlee, Belgium) the sections were incubated for 10 minutes at room temperature. Then, sections were washed twice with Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) (5 minutes each) and a second block was achieved by adding TBS-BSA 5% for 60 minutes at room temperature. Next samples were incubated with 1:50 diluted COX2 monoclonal antibody (160112, Cayman, Michigan, USA) or a normal mouse IgG_1 negative control (sc-3877, Santa Cruz Biotechnology) overnight at 4 °C. The following day, sections were washed twice with TBS-T (5 minutes each). Thereafter, sections were incubated with secondary anti-mouse antibody (K4001, Dako, Heverlee, Belgium) for 30 minutes at room temperature. The sections were then rinsed twice in TBS for 5 minutes and incubated with DAB for 5 minutes at room temperature. Sections were rinsed twice in Milli-Q (5 minutes) and counterstained with Haematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) for 1 minute. The samples were rinsed a final time by running tap water for 10 minutes. Lastly, sections were dehydrated by graded alcohol (70%, 80%, 96%, 96%, and 100%) and two times xylene (5 minutes each) and mounted.

After staining, images of the slides were made with the Colorview IIIU digital camera (Olympus, Zoeterwoude, the Netherlands) mounted on a BX-40 microscope (Olympus). Images were taken in magnification 10X. The number of positively stained cells in the cartilage explants was manually counted in Photoshop CC as a percentage of the total amount of cells present. COX2 is a cytoplasmic staining.

Gene expression
Explants were collected and stored at -70 °C after seven days of culturing for RNA isolation (n=3 per condition for each donor). The three samples were pooled (n=3) per condition per donor. The samples were snap frozen in liquid nitrogen and crushed by use of a hammer and anvil for at least three times. After this, the sample was collected and 600 µL of Lysis solution (17209, Exiqon, Woburn, USA) was added and samples were stored at -70 °C. For RNA isolation a miRCURY™ RNA Isolation Kit (300110, Exiqon, Woburn, USA) was used according to the guidelines of the manufacturer. DNA removal was safeguarded in this process by performing a DNase on-column step (RNAse free DNase Set, 79254, Qiagen, Valencia, USA). Quality of the isolated RNA was tested with the RNA nanodrop analyser.

Primer sequences were selected based on their role in anabolic and catabolic cartilage metabolism, apoptosis, bone morphogenetic protein (BMP) pathway, transforming growth factor beta (TGF-β) pathway, inflammation, osteoarthritis, and Wnt signalling pathway (**Table 2.6**). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by using the maximum amount of ng cDNA possible and IQT™ SYBR Green Supermix Kit (Bio-Rad, Veenendaal, the Netherlands). Six reference genes were chosen: GAPDH, HNRPH, RPL13, RPS19, SRPR, and YWHAZ. qRT-PCR was performed on the CFX384

Connect™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands) for 40 cycles at the desired temperatures for the different plates. Relative quantitative gene expression was determined by the Normfirst method. The Mean Ct-values of the genes of interest (GOI) were normalized by the mean Ct-values of the reference genes as an internal control: $\Delta Ct = Ct_{mean\ ref} - Ct_{GOI}$. Thereafter, $E^{\Delta Ct}$ were calculated whereby E indicates the efficiency of amplification from the GOI/reference gene.

Inflammatory response

PGE_2 analysis

The replicates of the medium collected on day 4, 7, 14, and 21 were pooled for that day subsequently to measure the total PGE_2 released per mL medium (pg/mL) by ELISA (Prostaglandin E2 monoclonal ELISA kit, 514010, Cayman Chemical, Ann Arbor, USA) as previously described in Chapter 1 (n=6 per condition for CD cartilage and n=4 per condition for NCD dogs).

Statistics

All data were statistically analysed using IBM SPSS statistics 24 and R Studio (http://www.rstudio.com/, Foundation for Open Access Statistics). A normality check was performed to determine normal distribution of the gene expression, using a Shapiro Wilks. Data that was not normally distributed was subjected to the Kruskal Wallis and Mann Whitney test. Normally distributed data was subjected to the ANOVA. Post-hoc tests (Benjamini & Hochberg) were performed for multiple comparisons. Since multiple factors (donor, NCD-CD, treatment and day) could influence the outcome of the present results, a multivariate regression model, the COX proportional hazard model, was used, when corresponded. Concretely, the donor and experiments as a random effect, and culture condition and breed as fixed effects were tested for the *goodness of fit*. Then, the model that retrieved the lowest *Akaike Information Criterion* (AIC) values was chosen for the analysis of the corresponding data set. The effect size (ES) and ES's confident intervals (CI set at 95%) were also taken into consideration to evaluate the significances. The definitions of the effect sizes, provided by Sawilowsky (2009)[111], are illustrated in **Table 2.3**. When data was normally distributed, ES were calculated and subsequently provided as Hedge's g (Cohen's delta correction for a low sample size[112], **Table 2.3**); and when not normally distributed, it was provided as a Cliff's delta[113] in the present study. Every *p*-value with a lower ES than the described in **Table 2.4** was considered not relevant

Given that only gene expression data from the lower CXB concentration (CXB 10^{-7} M) was available for NCD dogs, CXB 10^{-6} could not be compared between CD and NCD cartilage.

Effect size (ES)	Hedge's g	Cliff's delta
None	ES<0.01	
Very Small	0.01≤ES<0.2	ES<0.11
Small	0.2≤ES<0.5	0.11≤ES<0.28
Medium	0.5≤ES<0.8	0.28≤ES<0.43
Large	0.8≤ES<1.2	0.43≤ES<0.7
Very Large	1.2≤ES<2	ES≥0.7
Huge	ES≥2	

Table 2.3. A definition of the effect sizes (ES). Table derived from Vargha *et al.* (2000)[113], Sawilowsky (2009)[111], and Miranda-Bedate *et al.* (manuscript in preparation).

p-value	Effect size (ES)	Interpretation
Independent of *p*-value	None, very small, small	Uncertain
***p*≤0.05**	Medium and larger	Substantive significant
0.05<*p*≤0.1	Large and larger	Substantive significant
***p*≤0.15**	Very large and huge	Substantive significant

Table 2.4. Threshold and interpretation to determine whether or not data is relevant for this study. Table derived from Ellis (2010)[114], Greenland *et al*. (2016)[115], and Miranda-Bedate *et al.* (manuscript in preparation).

Results

GAG and DNA content

The total GAG produced by the cartilage explants includes the GAG content of the cartilage explant and the total amount of GAGs released per time point. Femur and tibia did not significantly differ in GAG and DNA content on day 0 and at the end of the culture period and no further distinction was made between these locations.

Although, no significant difference was found for GAG content, DNA content, and GAG corrected for DNA content between CD and NCD dogs on day 0 and of control samples of day 21, trends were observed in which CD derived explants contained a higher GAG and GAG/DNA content, and NCD derived cartilage contained a higher DNA content (**Figure 2.2**)

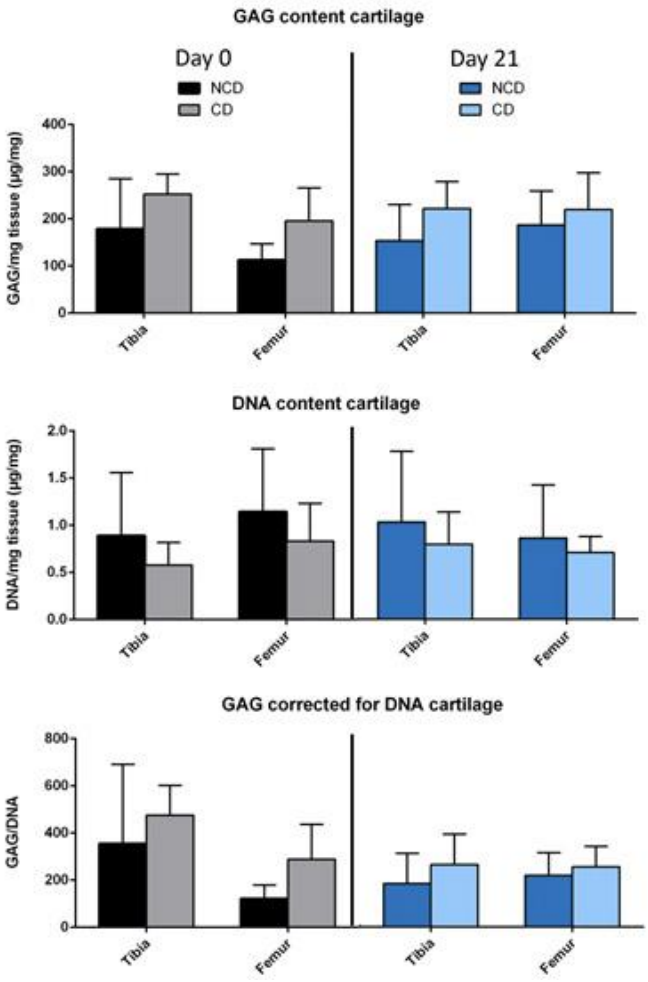

Figure 2.2. Articular cartilage of non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs do not differ from one another with regard to proteoglycan (GAG) and DNA content, nor do they differ between locations. Moreover, the GAG (**top**), DNA (**middle**), and GAG/DNA (**bottom**) content (mean±SD) of cartilage explants from day 0 (**left**) and control (chondrogenic medium) samples of day 21 (**right**) show no differences. Day 0: n=5 for NCD tibia, n=6 for CD tibia, n=3 for NCD femur, n=6 for CD femur. Day 21: n=9 for NCD tibia, n=9 for CD tibia, n=9 for NCD femur, n=9 for CD femur.

Differences between CD and NCD dogs regarding cartilage homeostasis were also analysed after 21 days of *ex vivo* culturing. Only cartilage explants treated with TNF+CXB-7 had a significantly higher GAG content in CD cartilage compared to NCD cartilage. The other conditions did, however, show clear trends in which CD dogs also appeared to have a higher GAG content than NCD dogs (**Figure 2.3, top**). DNA content did not significantly differ between CD and NCD cartilage regardless of the culture condition. However, it is intriguing that DNA content of NCD derived cartilage explants appeared higher in control, TNF, and TNF+CXB-7 conditions (**Figure 2.3, middle**). Moreover, GAG corrected for DNA content

appeared to be higher in cartilage explants from CD dogs, but this was only significant in CXB-7 and TNF+CXB-7. Altogether, this implies that cartilage explants from CD dogs contain relatively more GAGs per cell in their matrix when compared to NCD dogs (**Figure 2.3, bottom**). No differences could be obtained between culture conditions within NCD and CD derived cartilage of GAG content, DNA content, and GAG/DNA content (**Figure 2.3**).

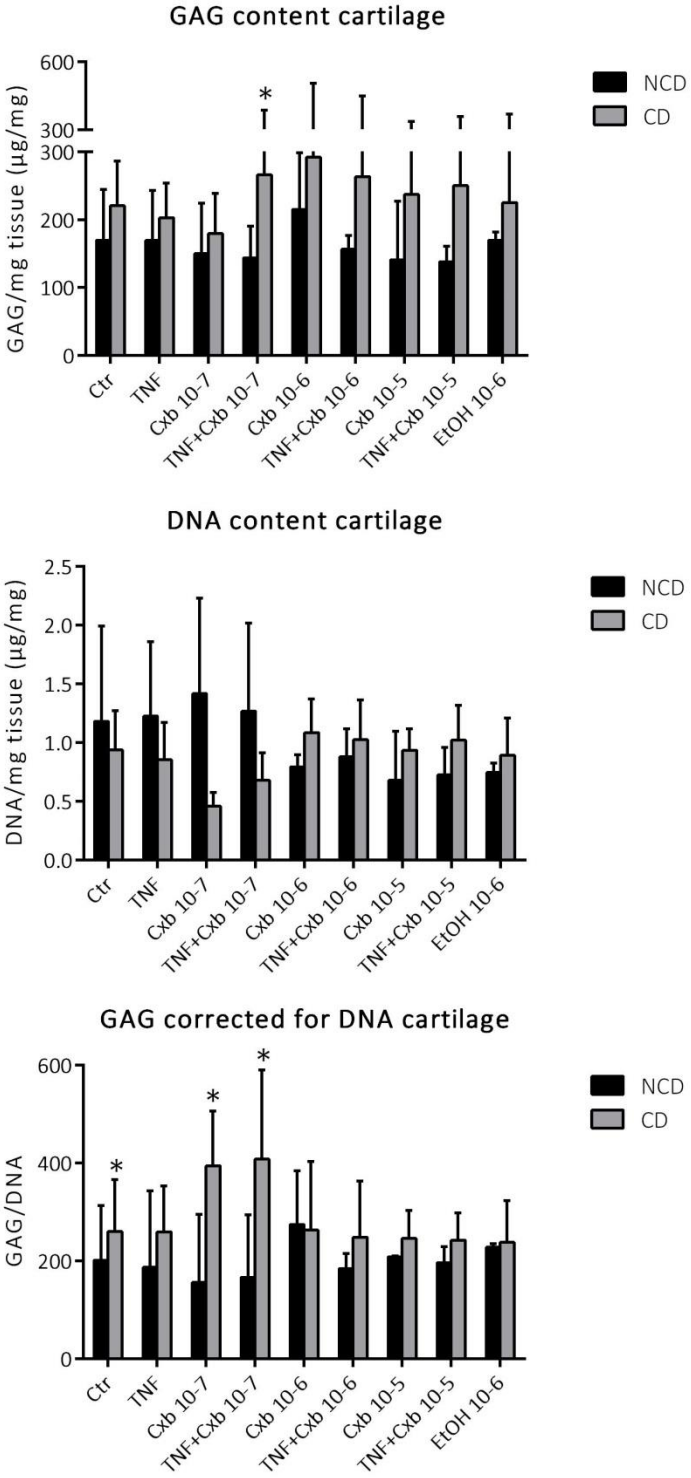

Figure 2.3. Glycosaminoglycan (GAG) (**top**), DNA (**middle**), and GAG/DNA (**bottom**) content (mean±SD). Chondrodystrophic (CD) explants show a trend of a higher GAG content. No difference was found between CD and non-chondrodystrophic (NCD) cartilage with regard to DNA content. GAG/DNA content was significantly higher in CD cartilage in the control, celecoxib (CXB) 10^{-7} M, and tumour necrosis factor alpha (TNF) + CXB 10^{-7} M. * = *p*-value < 0.05. *Ctr* = control (chondrogenic medium). *TNF* = tumour necrosis factor alpha. *Cxb* = celecoxib.

GAG release did not differ between CD and NCD on the overall, although at every measured time point NCD cartilage released more GAGs in the medium than CD cartilage. GAG release seemed to diminish over the 21-day culture period; as less GAGs were released at the end (day 21) for both NCD and CD derived explants. NCD and CD derived cartilage did not differ from one another in their GAG release profiles over the course of days in both the control and TNF condition. Control NCD cartilage explants released less GAGs on day 17 compared to day 3. The same was true for CD cartilage, where at multiple time points (i.e. day 10, 14, 17, and 21) GAG release was significantly lower compared to day 3. More differences in GAG release between days were obtained after TNF stimulation for both NCD and CD cartilage, especially NCD cartilage displayed less release on day 10, 14, 17, and 21 compared to day 3 (**Figure 2.4**). Taken together, NCD and CD cartilage did not show differential GAG release profiles over the course of 21 days. However, NCD derived explants appeared to display a higher GAG release and a more pronounced reaction to the inflammatory stimulus, than CD derived explants.

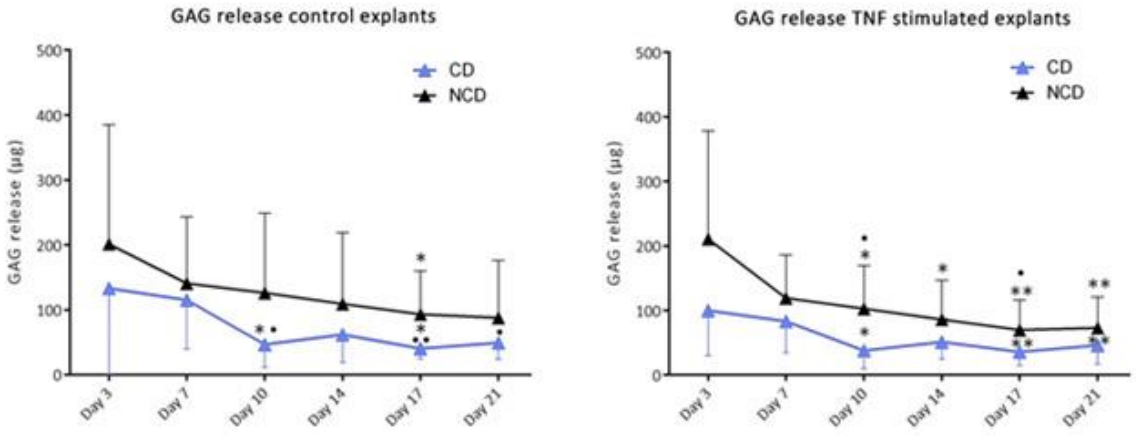

Figure 2.4. Both non-chondrodystrophic (NCD) and chondrodystrophic (CD) derived explants showed corresponding glycosaminoglycan (GAG) release profiles (mean±SD) over the course of 21 days in both the control (**left**) and tumour necrosis factor alpha (TNF) stimulated (**right**) conditions. The GAG release was lower at the end of the 21-day culturing period. ***** = *p*-value <0.05 and ****** = *p*-value <0.01, both compared to the GAG release of day 3; • = *p*-value <0.05 and •• = *p*-value <0.01 both compared to the GAG release of day 7.

Safranin-O/Fast Green staining of cartilage explants

Safranin-O/Fast Green staining of the cartilage explants showed no clear difference in the presence of GAGs (Safranin-O positive staining) was visualised between NCD and CD donors on day 0 (**Figure 2.5, top row**). After 21 days of culturing the qualitative staining of GAGs in the NCD cartilage seemed to indicate the presence of less GAGs compared to the CD cartilage. This was observed in all conditions with the exception of TNF+CXB-6. The original zonal structure of articular cartilage was present in the explants from both NCD and CD dogs. Furthermore, TNF-α or CXB supplementation did not appear to influence the GAG deposited in the cartilage explants **(Figure 2.5**).

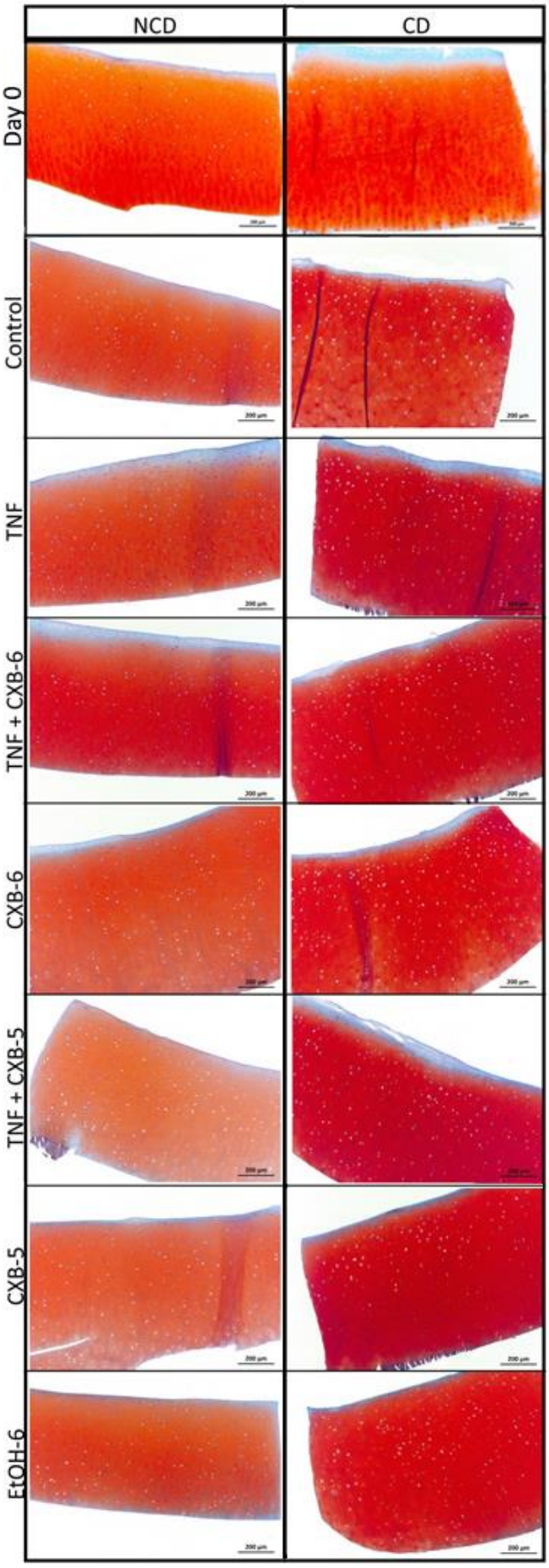

Figure 2.5. Safranin-O/Fast Green staining of the tibia cartilage explants. This qualitative staining indicates less glycosaminoglycans (GAGs) are present in the non-chondrodystrophic (NCD) (**left**) cartilage after 21 days of culturing compared to chondrodystrophic (CD) (**right**) cartilage, interestingly the tumour necrosis factor alpha (TNF) + celecoxib 10^{-6} M (CXB-6) seemed to improve the GAG content, based on the Safranin-O/Fast Green staining. Cartilage explants are all arranged with the superficial layer of the cartilage on the upper side. Red staining indicates a GAG rich matrix. A Representative donor from the whole donor set was chosen for the CD and NCD explant. Scale bar indicates 200 µm.

Collagen I and II immunohistochemistry staining

Collagen type I immunostaining was absent in day 0 cartilage explants of both NCD and CD donors (**Figure 2.6**), as was the case after 21 days of culturing in all culture conditions. Thus, no differences between the collagen type I protein were observed between the groups and subpopulations. No differences in content and distribution of collagen type II were visualised between the NCD and CD derived cartilage on day 0. Collagen type II was abundantly present in the matrix on day 0 and after 21 days of culturing (**Figure 2.7**).

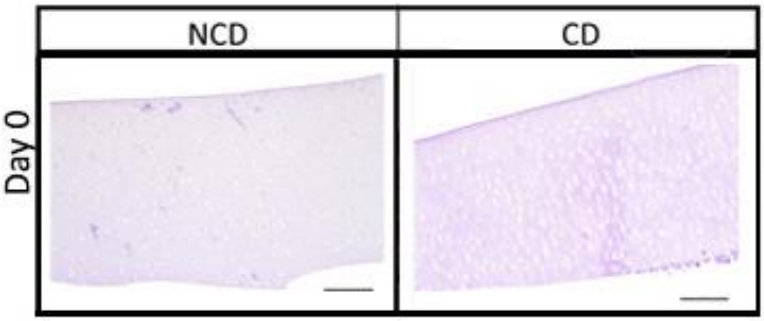

Figure 2.6. Representative images demonstrating that no clear differences in collagen type I content and distribution between non-chondrodystrophic (NCD) (**left**) and chondrodystrophic (CD) (**right**) dogs were observed on day 0 (**figure**) and after 21 days of culturing regardless of the culture condition (data not shown). Cartilage explants are all arranged with the superficial layer of the cartilage on the upper side. Brown staining indicates a collagen rich matrix. Scale bar indicates 200 µm.

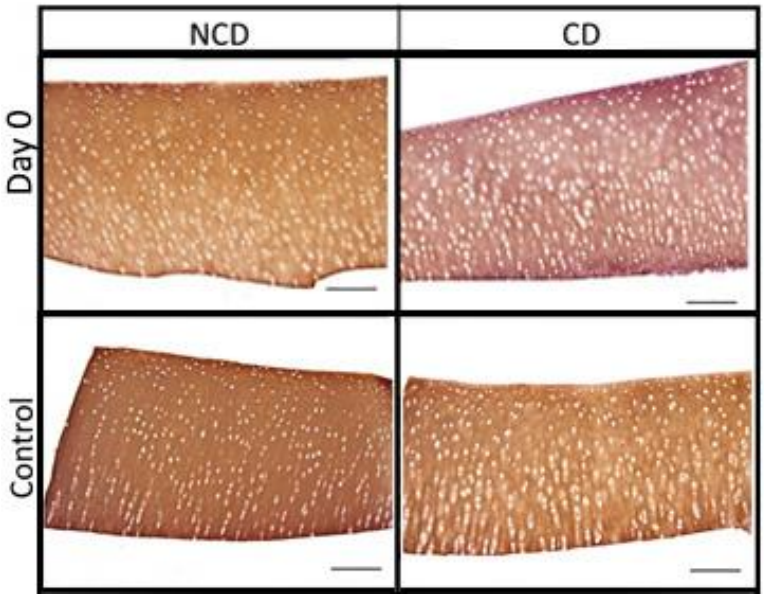

Figure 2.7. Representative images demonstrating that no clear differences in collagen type II content and distribution between non-chondrodystrophic (NCD) (**left**) and chondrodystrophic (CD) (**right**) dogs were observed on day 0 (**top**) and after 21 days of culturing regardless of the culture condition (only control is shown here, **bottom**). Furthermore, tumour necrosis factor alpha (TNF-α) and/or celecoxib appeared to have no distinct influence on the collagen content of cartilage explants. Cartilage explants are all arranged similarly with the superficial layer of the cartilage on the upper side. Brown staining indicates a collagen rich matrix. Scale bar indicates 200 µm.

COX2 immunohistochemistry staining

COX2 stained the cytoplasm of chondrocytes. Especially in the superficial zone cells were stained positively (**Figure 2.8**). On day 0, no significant difference was found between CD and NCD cartilage (**Figure 2.9, left**). As conditions did not give any significant influences in CD or NCD cartilage, it was taken out of the model. After 21 days of culturing the percentage of COX2 positively stained cells seemed to be significantly higher in NCD cartilage (*p*-value <0.01, small ES), this difference was, however, small. A great variety between donors was seen in both NCD and CD (**Figure 2.9**).

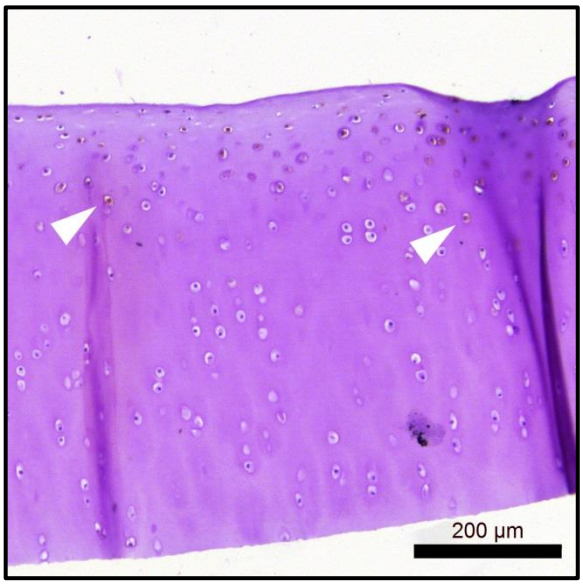

Figure 2.8. Cyclo-oxygenase 2 (COX2) positively stained cells in a cartilage explant (**arrowheads**). The cartilage explant is arranged with the superficial layer of the cartilage on the upper side. Scale bar indicates 200 µm.

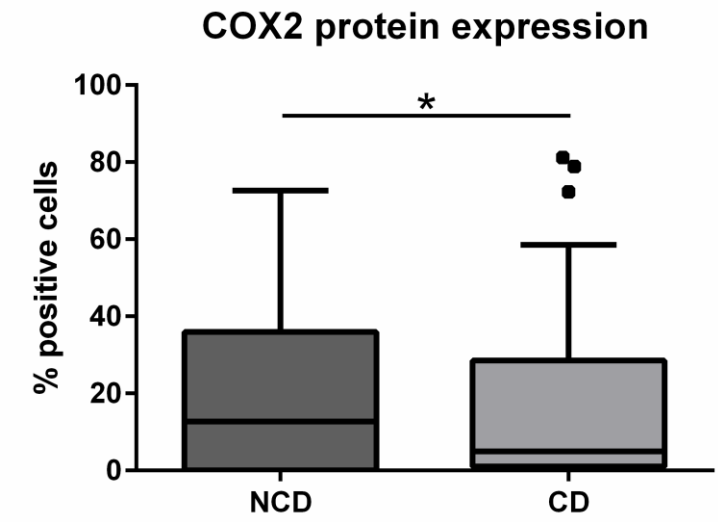

Figure 2.9. Percentage (mean±SD) of cells stained positively for cyclo-oxygenase 2 (COX2) after 21 days of culturing in cartilage explants of non-chondrodystrophic (NCD) dogs and chondrodystrophic (CD) dogs. As conditions did not give any significant influences in CD or NCD cartilage. There was great donor variability within the NCD and CD breeds. Day 0: n=5 for NCD and n=4 for CD. Day 21: n=6 for NCD and n=8 for CD. ***** = *p*-value <0.05.

Gene expression

Gene expression levels of *SOST*, *COL10*, *PAI1, ADAMTS5, IL1β, IL6,* and *WNT7B* could not be detected in the cartilage explants of all the conditions. All the Wnt signalling pathway cell markers, except for *SOST* and *WNT-7B*, were expressed. An overview of the genes, their change and their significances (*p*-value and ES) are given in **Table 2.5**. It was evaluated whether the culture conditions significantly differed within a subpopulation (NCD or CD). Where this was not the case, the differences between NCD and CD on the overall are given and discussed.

AXIN2, an important read-out parameter of Wnt signalling activity, was significantly (*p*-value <0.001, medium ES) upregulated in CD compared to NCD cartilage. Moreover, *DKK3* was also significantly (*p*-value < 0.001, very large ES) more expressed in CD compared to NCD cartilage. The same was the case for *CCND1,* as this was significantly (*p*-value <0.05, small ES) higher in CD compared to NCD cartilage. *CAV1*, a scaffolding protein and positive regulator of Wnt signalling, was also significantly (*p*-value <0.001, medium ES) more expressed in CD cartilage compared to NCD cartilage. *COL1A1* was significantly (*p*-value <0.001, medium ES) more expressed in the CD cartilage compared to NCD cartilage (**Figure 2.10**).

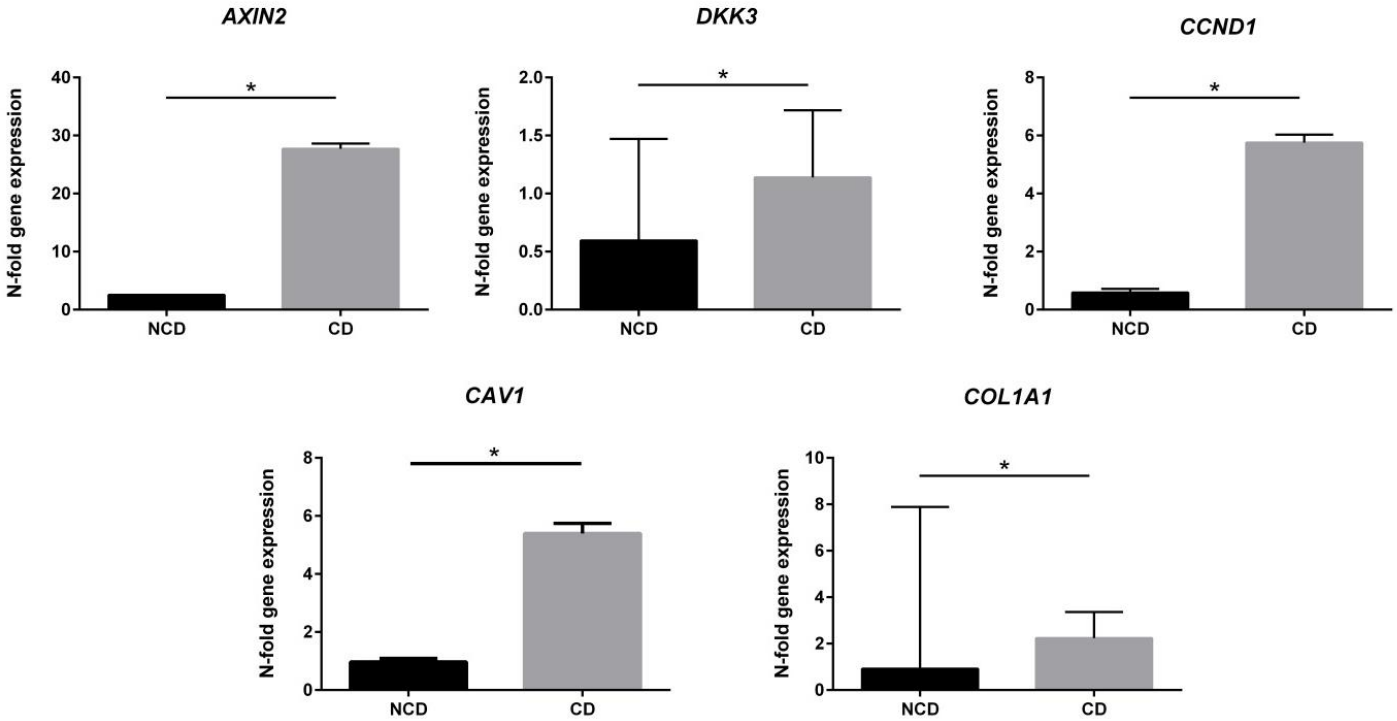

Figure 2.10. N-fold gene expression (relative to normal±SE) of *AXIN2, CAV1, CCND1, COL1A1, DKK3, ID1,* and in cartilage explants after 7 days of culturing. Non-chondrodystrophic (NCD) (n=32) compared to chondrodystrophic (CD) (n=28), with the exception of *CAV1* with n=12 for NCD and n=28 for CD. Culture conditions did not contribute to the statistical model as a fixed effect and therefore the data is presented for the CD and NCD cartilage samples as an overall. ***** = *p*-value <0.05.

There were genes in which the culture conditions did indeed significantly contribute to the statistical model and are, therefore, presented here within the CD and NCD cartilage groups.

Within the NCD subpopulation, *BAX*, a pro-apoptotic marker, was significantly downregulated in NCD cartilage in the presence of TNF (p-value <0.05, large ES), CXB-7 (*p*-value <0.06, very large ES), or TNF+CXB-7 (*p*-value <0.05, very large ES), compared to the control. The treatment conditions did not significantly differ from one another. *ACAN* did not significantly differ after treatment with TNF compared to the control. However, it was

significantly upregulated in the presence of TNF+CXB-7 compared to both TNF (*p*-value <0.06, medium ES) and control (*p*-value <0.05, medium ES). While CXB-7 alone showed a significant downregulation of *ACAN* compared to the TNF (*p*-value <0.07, medium ES) and TNF+CXB-7 (*p*-value <0.001, medium ES). *PTGES1* was significantly (*p*-value <0.05, medium ES) downregulated in TNF+CXB-7 compared to the control, though not when compared to TNF (**Figure 2.11**).

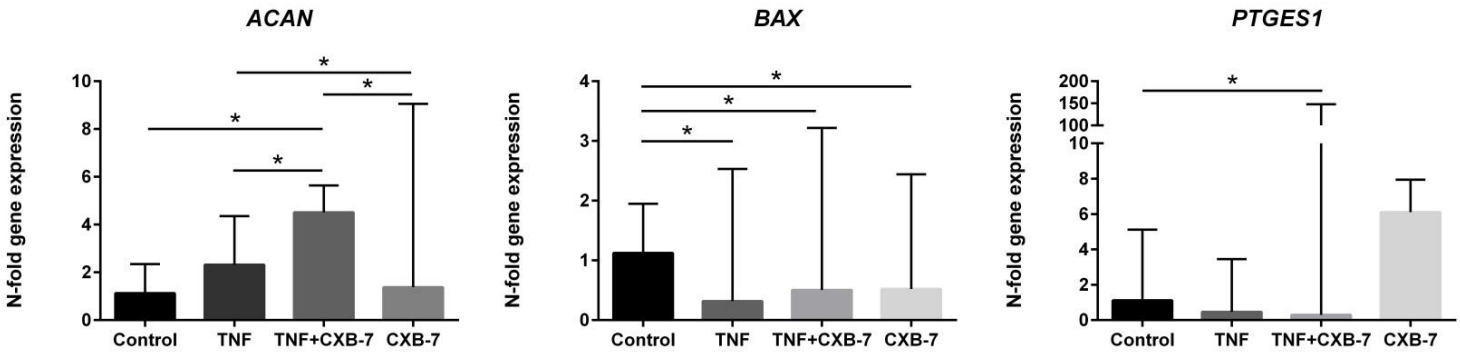

Figure 2.11. N-fold gene expression (relative to normal±SE) of *ACAN, BAX, and PTGES1* in non-chondrodystrophic (NCD) cartilage explants after 7 days of culturing. As conditions did significantly differ within this subpopulation, a direct comparison between NCD and chondrodystrophic (CD) cartilage in these genes was not possible. ***** = *p*-value <0.05.

Within the CD subpopulations *FZD1, WIF1, FGF2, BAX,* and *MMP13* differed significantly between the conditions. In *FZD1* the addition of CXB-7 gave a significant (*p*-value <0.01, very large ES) downregulation compared to the control, and TNF+CXB-7 was significantly (*p*-value <0.001, very large ES) downregulated compared to solely CXB-7. CXB-7 was significantly (*p*-value <0.05, very large ES) downregulated compared to TNF. CXB-6 was significantly (*p*-value <0.05, very large ES) upregulated compared to the lowest dose of CXB (CXB-7). This all in the *FZD1* gene. *WIF1* was significantly upregulated in CXB-7 compared to both control (*p*-value <0.1, very large ES) and TNF (*p*-value <0.1, very large ES); control and TNF did not differ. The combination of TNF+CXB-7 resulted in significant downregulation of *WIF1* compared to both control (*p*-value <0.1, large ES) and TNF (*p*-value <0.1, medium ES). *FGF2* was significantly (*p*-value <0.06, very large ES) downregulated by CXB-7 compared to the control. The pro-apoptotic marker *BAX* was significantly (*p*-value <0.2, medium ES) downregulated in the presence of CXB-6 compared to the control, but TNF+CXB-6 was unable to do this in a pro-inflammatory state (TNF). *MMP13* was significantly (*p*-value <0.08, large ES) upregulated in TNF+CXB-7 compared to CXB-7 (**Figure 2.12**).

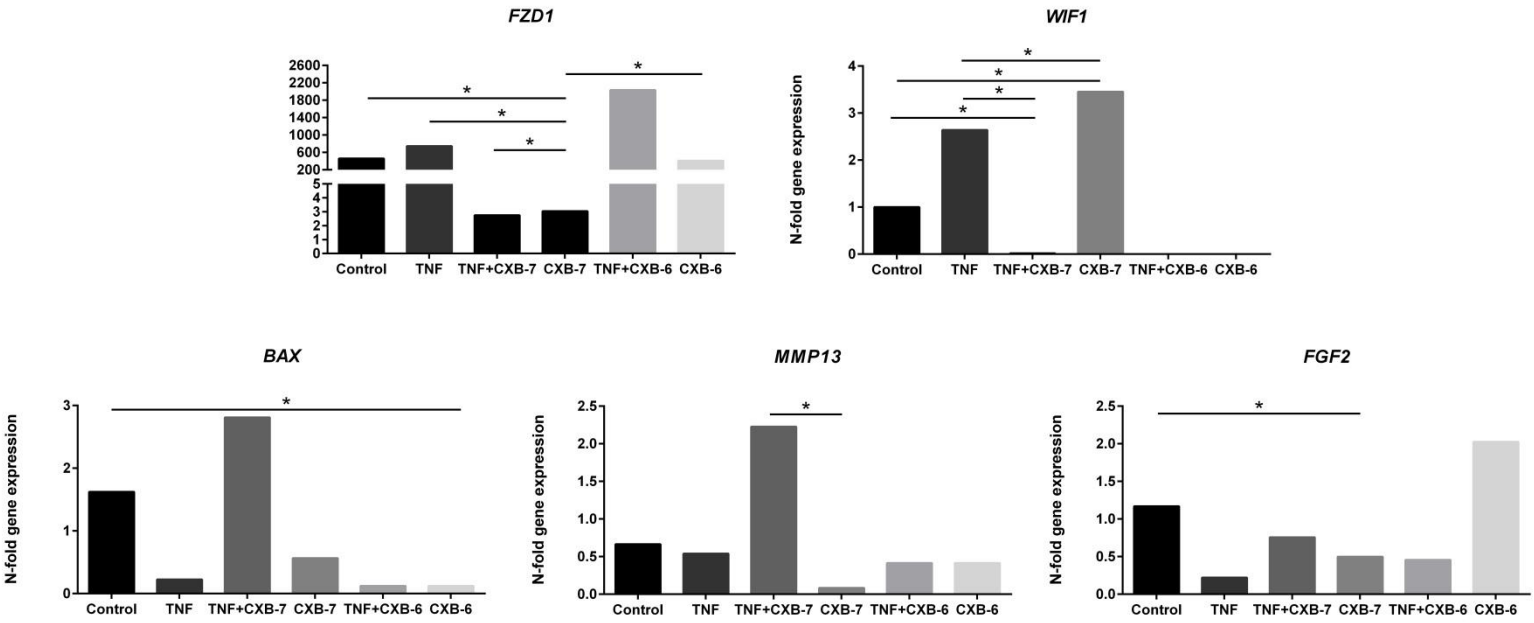

Figure 2.12. N-fold gene expression (relative to normal±SE) of *BAX, FGF2, FZD1, MMP13, and WIF1* in chondrodystrophic (CD) cartilage explants after 7 days of culturing. As conditions did significantly differ within the chondrodystrophic breed, a direct comparison between non-chondrodystrophic (NCD) and CD cartilage in these genes was not possible. ***** = *p*-value <0.05.

Gene	Within NCD or CD	Condition of interest	Change	Condition compared to	*p*-value	Effect Size (ES)	Interpretation of statistical difference
AXIN2		CD	↑	NCD	$6.32 \cdot 10^{-5}$	Medium	Significant
CAV1		CD	↑	NCD	$2.24 \cdot 10^{-6}$	Medium	Significant
COL1A1		CD	↑	NCD	$1.76 \cdot 10^{-5}$	Medium	Significant
CCND1		CD	↑	NCD	0.024	Small	Uncertain
DKK3		CD	↑	NCD	$8.89 \cdot 10^{-10}$	Very large	Significant
ACAN	NCD	TNF+CXB-7	↑	Control	0.016	Medium	Significant
		TNF+CXB-7	↑	TNF	0.058	Medium	Significant
		CXB-7	↓	TNF	0.069	Medium	Doubtful
		CXB-7	↓	TNF+CXB-7	0.001	Medium	Significant
BAX	NCD	TNF	↓	Control	0.003	Large	Significant
		CXB-7	↓	Control	0.051	Very large	Significant
		TNF+CXB-7	↓	Control	0.003	Very large	Significant
PTGES1	NCD	TNF+CXB-7	↓	Control	0.026	Medium	Significant
BAX	CD	CXB-6	↓	Control	0.109	Medium	Very doubtful
FGF2	CD	CXB-7	↓	Control	0.054	Very large	Significant
FZD1	CD	CXB-7	↓	Control	0.006	Very large	Significant
		CXB-7	↓	TNF	0.001	Very large	Significant
		TNF+CXB-7	↓	CXB-7	$1.12 \cdot 10^{-4}$	Very large	Significant
		CXB-6	↑	CXB-7	0.015	Very large	Significant
MMP13	CD	TNF+CXB-7	↑	CXB-7	0.070	Large	Significant
WIF1	CD	CXB-7	↑	Control	0.081	Very large	Significant
		CXB-7	↑	TNF	0.095	Very large	Significant
		TNF+CXB-7	↓	Control	0.096	Large	Significant
		TNF+CXB-7	↓	TNF	0.096	Medium	Doubtful

Table 2.5. Overview of the difference in gene expression level with *p*-value, effect size (ES) and interpretation as seen in chondrodystrophic (CD) derived cartilage explants compared to non-chondrodystrophic (NCD) explants (**top**). As well as the differences within the NCD (**middle**) and CD (**bottom**) breeds with their *p*-value, ES, and interpretation. *Control* = chondrogenic medium. *TNF* = tumour necrosis factor alpha. *CXB* = celecoxib.

PGE_2 release

TNF significantly upregulated PGE_2 release in both CD and in NCD cartilage explants. Moreover, only in NCD cartilage was CXB-6 able to suppress the PGE_2 release augmented by TNF-α treatment (**Figure 2.13**). No statistical differences were found regarding PGE_2 release between NCD and CD dogs. However, the ES analysis indicated possible trends. Very large and even huge ES were found when comparing the control, TNF, and TNF+CXB-6 between NCD and CD cartilage. The control (huge ES) and TNF (very large ES) were upregulated in NCD cartilage compared to CD cartilage, while in TNF+CXB-6 (very large ES) it was downregulated in NCD cartilage compared to CD cartilage.

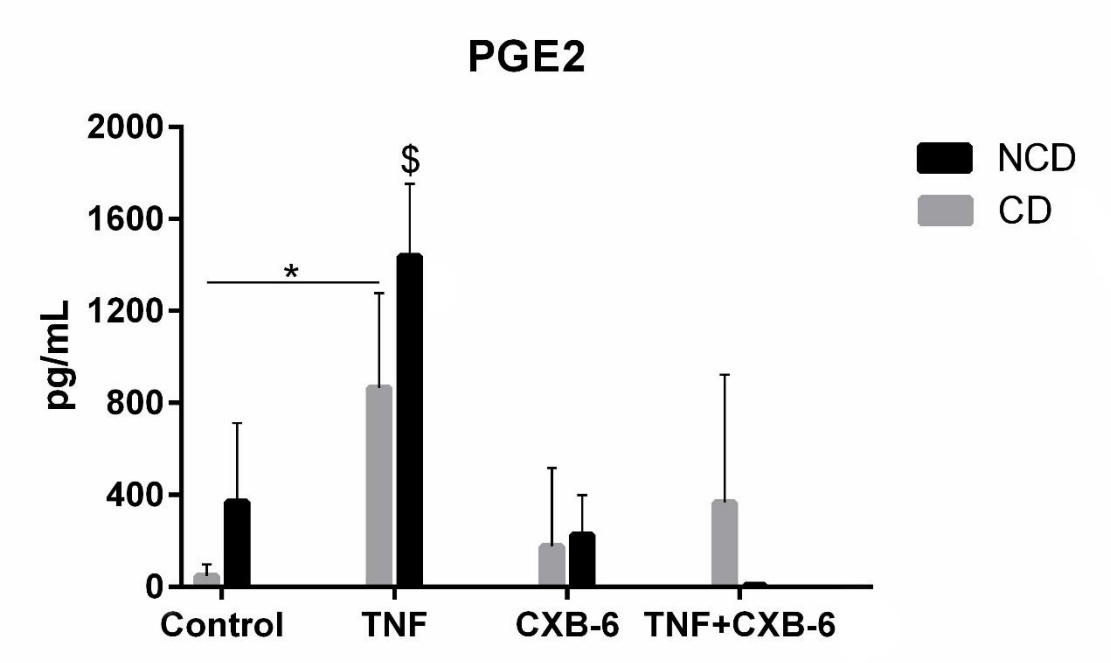

Figure 2.13. Total prostaglandin E_2 (PGE_2) release (pg/mL) (mean±SD) of the cartilage explants of day 4, 7, 14, and 21 together comparing the conditions within the subpopulations (chondrodystrophic (CD) and non-chondrodystrophic (NCD) dogs, respectively). Tumour necrosis factor alpha (TNF) significantly upregulates PGE_2 release in CD and in NCD cartilage. Moreover, only in NCD cartilage is CXB-6 able to suppress the elevated PGE_2 release elevated by TNF. ***** = *p*-value <0.05. **$** = *p*-value < 0.05 compared to all the other conditions within the NCD cartilage group.

Discussion

Biochemical analysis of an extensive set of cartilage samples derived from the knee joint from chondrodystrophic and non-chondrodystrophic dog breeds suggests that cartilage explants from CD dogs retains their GAGs in the matrix better than NCD dogs after 21 days in basal culture conditions and in the presence of a pro-inflammatory stimulus.

Although, at the gene expression level limited changes in the profile were observed, PGE_2 release was upregulated by the addition of TNF-α in both NCD and CD cartilage, indicating that the pro-inflammatory stimulus was able to generate an OA-like environment. In this environment, NCD cartilage seemed to favourably respond to the treatment with CXB, which displayed a chondroprotective effect on a gene expression level (*ACAN*) and was further confirmed by a more intense Safranin-O/Fast Green staining (TNF+CXB-6). Though this is a qualitative staining, NCD dogs displayed less intense staining for GAGs compared to CD dogs. Only in TNF+CXB-6 was the NCD matrix staining similar to that of CD, with good quality matrix. Chondroprotective properties have already been ascribed to CXB in past[116,117]. The possible mechanism of action is by counteracting the inflammatory process itself through COX2 inhibition. *PTGES1*, important for upregulation of COX2, was significantly downregulated in the presence of TNF+CXB-7 compared to the control in NCD cartilage. Even more so, PGE_2 release tended on the overall to be upregulated in the NCD cartilage compared to CD cartilage, which was in line with the higher COX2 immunopositivity of NCD cartilage cells. Interestingly, in this pro-inflammatory environment only in the NCD cartilage group PGE_2 levels were significantly downregulated by treatment with CXB-6.

CD and NCD cartilage responded differently to the same pro-inflammatory stimulus and anti-inflammatory medication at gene expression level. CD cartilage showed the most distinct changes. Only in CD cartilage, CXB-7 alone induced *WIF1* expression, an inhibitor of the Wnt pathway. Given that increased expression of *WIF1* has been correlated to reduced cartilage degeneration in TNF-transgenic mice[118], the present findings on *WIF1* expression would indicate that CXB treatment may aggravate degeneration. Specifically in CD cartilage, *BAX*, an apoptotic marker, was significantly downregulated in the presence of CXB-6 compared to TNF. The chondroprotective effects of CXB is further supported by the gene expression pattern of *FGF2,* that is released during cartilage injuries[119] and known to play a role in remodelling of damaged tissue[119]. *FGF2* expression was significantly downregulated in CXB-7 treated CD cartilage compared to control indicating that CXB-7 exerted protective effects. Follow up studies are needed to delineate the association between gene expression profiles and the working mechanism of the observed differential susceptibility between CD and NCD cartilage to inflammation.

Little to nothing is documented concerning a difference in the incidence of OA in NCD and CD dogs, but the present study suggests that CD dogs are better protected against OA. A possible explanation for the different cartilage homeostasis between NCD and CD cartilage could be attributed to the canonical Wnt signalling pathway. Yasuhara *et al.*[120] have shown that the canonical Wnt signalling has essential roles in the regulation of the organisation and function of the superficial zone *in vivo*. Activation of the Wnt/β-catenin signalling leads to a significant increase of proliferating cells in the superficial zone of the articular cartilage[120]. *FZD1*, a receptor of the Wnt ligands, remained unchanged throughout all culturing conditions in the NCD cartilage groups, while this was of influence in the CD cartilage. *FZD1* was downregulated in CXB-7 compared to the control, TNF and CXB-6. Addition of TNF (TNF+CXB-7) gave a downregulation compared to CXB-7. *WIF1* in CD cartilage was upregulated in CXB-7 and downregulated in TNF+CXB-7, both when compared to the control and TNF. This all indicates that Wnt may be differently regulated in CD cartilage. Interestingly, in our experiments we observed that *AXIN2*, an important read-out parameter of Wnt signalling activity, was significantly upregulated in CD cartilage compared

to NCD cartilage, regardless of the culture condition. *CAV1*, a scaffolding protein and positive regulator of Wnt signalling, was also higher expressed in CD compared to NCD cartilage. In the intervertebral disc, increased *CAV1* gene and protein expression has been associated with protective effects[85]. Moreover, *CCND1*, a downstream gene of the Wnt signalling pathway, was also significantly upregulated in CD cartilage compared to NCD cartilage. In this respect, protein levels of AXIN2 by Western Blot are required to give a more conclusive statement about Wnt activity.

Differences in the regulation of Wnt signalling between CD and NCD dogs may explain their differences in matrix composition. In the present study, *DKK3* was significantly more expressed in CD than NCD cartilage. Though Dickkopf (DKK) family members have been associated with inhibiting the Wnt signalling pathway, there are situations in which *DKK3* actually potentiates the Wnt signalling pathway[121]. Whether *DKK3* activates or inhibits the Wnt signalling pathway seems to be a tissue-dependent manner, thus, how *DKK3* impacts the Wnt signalling concretely in cartilage is unknown[121-123]. For example, *DKK3* has been associated with OA disease progression[124], because of its upregulation in OA, but may have a protective effect on cartilage integrity. Overall *DKK3* upregulation may be a defence mechanism to counteract disease-related dysregulation of cell signalling pathways, supporting our hypothesis of the Wnt pathway as an OA protective mechanism in CD dogs. In this way, pro-inflammatory cytokine effects, like those of TNF-α, on cartilage degradation would be reversed and TGF-β signalling enhanced, whilst activation of Wnt signalling is an attempt to counteract the changes in this pathway[125]. Snelling *et al.*[125] even suggest DKK3 supplementation at an early stage of disease or post-injury as a therapy.

Currently, no distinction is made between cartilage from different canine breeds. Bendele (2001)[5] has, however, described that very little overt cartilage degeneration has been observed in the Beagle dog over a 3-month period in an OA model based on anterior cruciate ligament transection. Furthermore, clinical OA is overrepresented in medium- and large breed dogs, rather than in chondrodystrophic dogs. Though only an observation, this is an indication that there may indeed be a difference in susceptibility to developing OA between these dog breeds on the basis of having chondrodystrophy or not. The present study provides some evidence that cartilage derived from CD dogs does indeed differ biomolecular and biochemically from NCD cartilage. The latter is in an *in vitro* setting less capable in retaining its GAG content and responds more pronounced to a pro-inflammatory stimulus. This may provide a possible explanation for the higher prevalence of OA in NCD dogs. More importantly, this would underline the importance of choosing the most appropriate dog breed for an osteoarthritic dog model, depending on the aim and desired effects. As NCD dogs seem to be more susceptible for developing OA and to respond to (an anti-inflammatory) treatment, it would be beneficial to choose a NCD dog breed for such models.

Conclusions and future work

Taken together, we have established that cartilage derived from CD and NCD dogs does indeed differ. More importantly, NCD cartilage seems to have a tendency for a pro-inflammatory state. Furthermore, NCD cartilage appears to release more PGE_2 in the presence of an inflammatory stimulus. We have reason to believe that the Wnt signalling pathway plays an important role in these found differences. Our current study reveals the first indications of a difference in the expression of the Wnt signalling pathway between CD and NCD dogs on a cartilage level. This was already established for intervertebral discs, but had not yet been described in articular cartilage. To elucidate this difference in expression more clearly, protein levels should be taken into account. Future work, therefore, will consist of Western Blot analysis of downstream molecules of the Wnt pathway of native cartilage from CD and NCD donors. Another way to confirm Wnt signalling pathway expression would be with immunohistochemistry staining of β-catenin. Moreover, cartilage explant co-cultures with synovium may even unravel more about these two subpopulations as such a co-culture would mimic the joint more closely. Therefore, based on the already found results we recommend that the distinction between CD and NCD should be taken into account when choosing the appropriate canine model.

Gene category	Gene	Primer sequence (5'-'3)	Product size (bp)	qRT-PCR temperature condition (°C)
Wnt signalling pathway	*AXIN2*	Fw GGACAAATGCGTGGATACCT Rv TGCTTGGAGACAATGCTGTT	128	60
	CAV1	Fw CGCACACCAAGGAAATCG Rv AAATCAATCTTGACCACGTCG	72	60
	CCND1	Fw ACTACCTGGACCGCT Rv CGGATGGAGTTGTCA	151	60
	DKK3	Fw CATCCAGTCCAGTGCTCTCA Rv GGGCCAGGATTGTAAGTGAA	140	58
	FZD1	Fw GGCGCAGGGCACCAAGAAG Rv GAGCGACAGAATCACCCACCAGA	97	61.5
	LRP5A	Fw GCTCCATCCACGCCTGTAA Rv ACCATTGTCCTCCGCACAC	137	61
	SOST	Fw TCTCTTGCTCTGTGTCTCG Rv TACTCGGATGCGTCTTTGG	223	59
	WIF1	Fw CCGAAATGGAGGCTTTTGTA Rv ATGCAGAACCCAGGAGTGAC	135	61.5
	WNT7B	Fw AACACGCACCAGTACACCAA Rv CACTTGCAGGTGAAGACCTC	110	60
Osteoarthritis	*Col10*	Fw CCAACACCAAGACACAG Rv CAGGAATACCTTGCTCTC	80	61
	FGF2	Fw TTCTTCCTGCGGATCCA Rv GTTGCAATTTGACGT GGG	76	61
Anabolic	*ACAN*	Fw GGACACTCCTTGCAATTTGAG Rv GTCATTCCACTCTCCCTTCTC	110	61-62
	COL1A1	Fw GTGTGTACAGAACGGCCTCA Rv TCGCAAATCACGTCATCG	109	61
	COL2A1	Fw GCAGCAAGAGCAAGGAC Rv TTCTGAGAGCCCTCGGT	150	60.5-65
	SOX9	Fw CGCTCGCAGTACGACTACAC Rv GGGGTTCATGTAGGTGAAGG	105	62-63
Catabolic	*ADAMTS5*	Fw CTACTGCACAGGGAAGAG Rv GAACCCATTCCACAAATGTC	148	61
	MMP13	Fw CTGAGGAAGACTTCCAGCTT Rv -TTGGACCACTTGAGAGTTCG	250	65
Inflammatory mediator	*IL1β*	Fw TGCTGCCAAGACCTGAACCAC Rv TCCAAAGCTACAATGACTGACACG	115	68
	IL6	Fw GAGCCCACCAGGAACGAAAGAGA Rv CCGGGGTAGGGAAAGCAGTAGC	123	65
PGE_2 pathway	*PTGES1*	Fw CCAGTATTGCCGGAGTGACCAG Rv AAACGAAGCCCAGGAACAGGA	97	68
	PTGES2	Fw GCTCTCAAGACCTACCTGG Rv AGTCACTTCCTTTCCCTGG	98	60-62

Apoptotic marker	*BAX*	Fw CCTTTTGCTTCAGGGTTTCA Rv CTCAGCTTCTTGGTGGATGC	108	58-59
	BCL2	Fw GGATGACTGAGTACCTGAACC Rv CGTACAGTTCCACAAAGGC	80	61.5-63
	CASP3	Fw CGGACTTCTTGTATGCTTACTC Rv CACAAAGTGACTGGATGAACC	89	61
	FASL	Fw ATGTTTCAGCTCTTCCATCTACAG Rv CAGAAGGTGGATTGGGTTGAC	114	60
TGF-β pathway	*PAI1*	Fw AAACCTGGCGGACTTCTC Rv ACTGTGCCACTCTCATTCAC	98	61.5
Reference gene	*GAPDH*	Fw TGTCCCCACCCCCAATGTATC Rv CTCCGATGCCTGCTTCACTACCTT	100	58
	HNRPH	Fw CTCACTATGATCCACCACG Rv TAGCCTCCATAACCTCCAC	151	61
	RPL13	Fw GCCGGAAGGTTGTAGTCGT GGAGGAAGGCCAGGTAATTC	87	61
	RPS19	Fw CCTTCCTCAAAAAGTCTGGG Rv GTTCTCATCGTAGGGAGCAAG	95	61-63
	SRPR	Fw GCTTCAGGATCTGGACTGC Rv GTTCCCTTGGTAGCACTGG	81	61.5
	YWHAZ	Fw CGAAGTTGCTGCTGGTGA Rv TTGCATTTCCTTTTTGCTGA	94	58

Table 2.6. Gene-specific primer sequences with associated amplification temperatures.

In vitro study of the controlled release of celecoxib on articular cartilage cells

Abstract

Background. The treatment of osteoarthritis, a degenerative joint disease, remains symptomatically and ways to optimize treatments are, therefore, crucial. In this study, the potential of celecoxib-loaded polyester amide (PEA) microspheres was tested *in vitro* as a drug delivery system to apply as a minimally invasive treatment of osteoarthritis.

Materials and method. Healthy canine articular chondrocytes were cultured in a monolayer culture system. This was in a batch-wise manner for 3-4 days to prevent cell confluency and changes in cell behaviour and phenotype. Prostaglandin E_2 (PGE_2) production, stimulated by tumour necrosis factor alpha (TNF-α), was used as a read-out parameter for inflammation. PEA microspheres loaded with celecoxib were added to transwell baskets overlying the cell monolayer in two concentrations (10^{-4} M and 10^{-7} M). Free added celecoxib (CXB, 10^{-6} M) served as a control. These transwell baskets were transferred accordingly to new cell cultures every 3-4 days for a total period of 28 days. Celecoxib release, inhibition of PGE_2 production, and DNA content were determined over the course of 4 weeks. Furthermore, the gene expression profile of cartilage cells in all culture conditions was determined at one representative time point (day 4).

Results. The controlled release of celecoxib by PEA microspheres was successfully demonstrated in 10^{-4} M and 10^{-7} M microspheres loaded with celecoxib. After an initial burst release, a slow continuous release was observed. The cumulative celecoxib release was approximately 40% (10^{-4} M celecoxib-loaded microspheres) and 39% (10^{-7} M celecoxib-loaded microspheres) after 28 days of culturing.

Conclusions. TNF-α did not appear to stimulate marked PGE_2 production. However, the high dose (10^{-4} M) PEA microspheres loaded with celecoxib were still able to show PGE_2 suppression comparable to that of the free added celecoxib (10^{-6} M). Overall, the DNA content increased compared to the initial DNA content at the start of every time point. Given that, generally, the genes remained unaffected by the presence of the (loaded) microspheres, it seems that the celecoxib-loaded microspheres do not negatively influence the matrix quality. The *in vitro* potency of PEA microspheres as a safe drug delivery system for celecoxib was demonstrated. This underlines the benefits of using a controlled release as opposed to currently available intra-articular injection formulations with only short-term effects. The next step is to investigate the efficacy of celecoxib-loaded PEA microspheres in canine patients suffering from osteoarthritis.

Keywords: PEA microspheres, controlled celecoxib release, monolayers, canine, chondrocytes, osteoarthritis.

Introduction

The prevalence of osteoarthritis (OA) is still increasing due to population ageing and increases of related factors, such as obesity and inactivity[1]. By 2050, it is estimated that 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease[126,127]. OA is not only a problem among humans, but also affects our canine companions. It is estimated that 20% of the dogs over one year of age exhibit clinical signs of OA[2].

The widespread use of anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) in degenerative disease of the joint cartilage is justifiable, as the disease process has an inflammatory character[15,128,129]. NSAIDs exert their pharmacological action by inhibiting cyclooxygenase (COX) enzymes, which catalyse the conversion of arachidonic acid to prostaglandins (PGs), prostacyclins, and thromboxanes[130]. Selective COX2 inhibitors have been found to be favourable in osteoarthritis (OA) in humans, in late-stages but also early stages of the disease[117]. However, the application of selective COX2 inhibitors is still accompanied by several drawbacks, including cardiac[131] and renal[67,68] adverse effects. This limits its potential for long-term oral use. Moreover, the clearance of the systemically administered drugs is fast, requiring continuous administration at high doses[69].

Therefore, local administration seems a sensible approach. Single intra-articular administration with currently available formulations unfortunately only has a temporary effect[132,133]. A solution to this problem is provided by the use of biomaterial-based controlled release systems for the continuous local delivery of anti-inflammatory therapeutics over prolonged periods of time[134,135]. Petit *et al.*[135] studied the release behaviour and intra-articular biocompatibility of celecoxib-loaded acetyl-capped PCLA-PEG-PCLA thermogels *in vitro* and *in vivo*. *In vitro* experiments showed an initial burst release of approximately 10 days of celecoxib, a selective COX2 inhibitor, followed by a sustained release for about 90 days. *In vivo*, celecoxib release of 30% was seen during the first 3 days followed by a sustained release of 4-8 weeks. The safety of celecoxib-loaded hydrogels has already been tested in healthy rats. No cartilage or bone changes were observed following injection into the knee joints of rats[135] or after injection in the intervertebral disc in dogs[71].

Earlier research of Petit *et al.*[135] already showed sustained release of anti-inflammatory drugs via microspheres (nanostructures biomaterials) in rat models for approximately 8 weeks. Celecoxib-loaded poly lactic-co-glycolic acid (PLGA) based microspheres have been shown to have an efficient anti-inflammatory effect over a period of at least 3 weeks. PGE_2 levels were reduced to an average of 30% using microspheres loaded with 0.1 nmol celecoxib[136]. Moreover, celecoxib-loaded PCLA-PEG-PCLA hydrogel has been effectively applied in client-owned dogs for the treatment of back pain[71].

Another suitable candidate for an intra-articular drug delivery system with auto regulatory behaviour is a polyester amide (PEA) based injectable microsphere formulation. PEA polymers are based on α-amino acids, aliphatic dicarboxylic acids, and aliphatic α-ω diols[137]. PEA microspheres have some advantages over the widely used PLGA microspheres. The incorporation of amino acid-based building blocks in PEA microspheres provides: 1) imparting chemical functionality, which improves hydrophilicity and possible interactions with proteins and genes, as well as facilitating further modification with bioactive molecules; 2) possibly the improvement of biological properties of materials (including cell-materials interactions); 3) enhancing thermal and mechanical properties; 4) providing metabolizable buildings blocks[73]. Contrary to the degradation of PLGA microspheres, the local pH is not affected by the degradation of the polymer backbone of PEA microspheres, as this degradation is via a unique surface erosion mechanism and by

enzymatic degradation. In PLGA microspheres the pH may drop to 4.7 or lower[138,139], which may irritate the tissues. *In vivo* safety of PEA microspheres has already been demonstrated in a canine model of intervertebral discs[140]. PEA microspheres loaded with celecoxib could provide a suitable way in treating OA in both canine and human patients over a prolonged period of time.

Additionally to being a selective COX2 inhibitor, there is evidence that celecoxib possesses chondroprotective and disease-modifying effects as well, on both human chondrocytes and articular cartilage[116,117] (**Figure 3.1**). These chondroprotective effects include prevention of synovial hyperplasia and inhibition of bone destruction and have been demonstrated *in vitro* and *in vivo* specifically in animal models[116], as well as improvement of cartilage matrix integrity in OA already in an early phase[117].

The purpose of the present study is threefold: 1) investigating the controlled release of celecoxib-loaded PEA microspheres in a pro-inflammatory environment; 2) evaluating the anti-inflammatory effect of the celecoxib-loaded PEA microspheres over time; and 3) assessing a possible dose-dependent effect from the celecoxib-loaded PEA microspheres. This is approached with the aid of a monolayer culture system based on canine cartilage cells receiving a pro-inflammatory stimulus with a total follow up of 4 weeks.

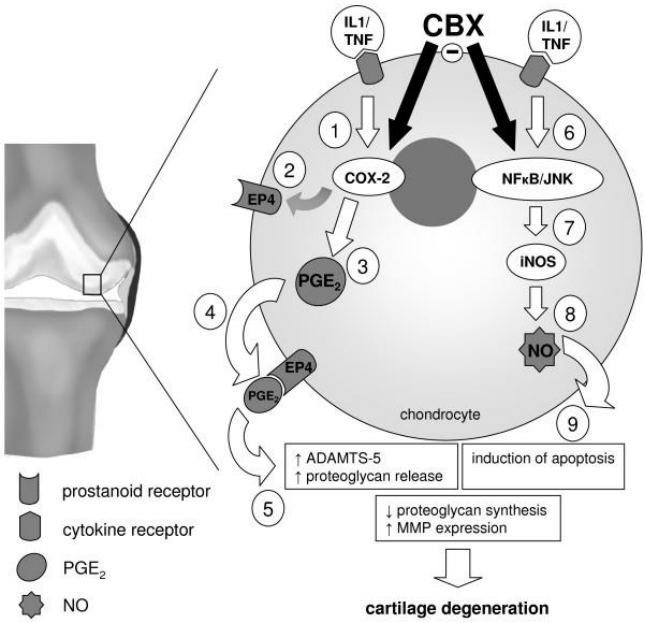

Figure 3.1. Schematic overview of the proposed effects of celecoxib (CBX) on cartilage degeneration. *IL* = interleukin. *TNF* = tumour necrosis factor. *EP* = endogenous pyrogens. *COX* = cyclooxygenase. *NFκB* = nuclear factor kappa-light-chain-enhancer of activated B cells. *JNK* = c-Jun N-terminal kinase. *PG* = prostaglandin. *iNOS* = inducible isoform nitric oxide synthase. *NO* = nitric oxide. *ADAMTS* = a disintegrin and metalloproteinase with thrombospondin motif. *MMP* = matrix metalloproteinase. Reprinted from Zweers *et al.* (2011)[116].

Materials and methods

In vitro monolayer culture of articular cartilage cells

Sources of canine tissue and passaging

Canine articular cartilage cells (ACs) were collected and passaged as earlier described (Chapter 1). For this purpose, ACs from non-chondrodystrophic dogs were employed on the basis that primarily medium to large breed dogs develop clinical OA. At passage 1 (P1), cells were frozen in aliquots per 1 million (counted with TC20™ Automated Cell Counter (1450102, Bio-Rad, Veenendaal, the Netherlands) and 0.40% Trypan Blue Dye (1450021, Bio-Rad, Veenendaal, the Netherlands)) in freezing medium, containing 100 µL/mL dimethyl sulfoxide (DMSO, 20-139, EMD Millipore corporation, Billerica, USA) and 100 µL/mL foetal bovine serum (FBS, Gibco, 16000-044) in hgDMEM (high glucose, GlutaMAX, pyruvate (Invitrogen, 31966)). For the successive time intervals one vial per donor was used. Six different non-chondrodystrophic canine articular cartilage cell donors (**Table 3.1)** were used.

	Donor	Breed	Gender (M/F)	Age (months)
1	1208447	Labrador Retriever	M	4.3
2	1208446	Labrador Retriever	F	4.3
3	938017	Mixed Breed	F	20
4	940232	Mixed Breed	F	20
5	1208445	Labrador Retriever	F	4.3
6	955175	Mixed Breed	F	20

Table 3.1. Donor characteristics of the canine cartilage cells.

Release and bioactivity in TNFα-stimulated chondrocyte monolayer culture

For each time point AC cells (P2) from each donor were expanded for three to four days in a T75 flask (658175 Cellstar®, Greiner Bio-one, Alphen aan de Rijn, the Netherlands) in expansion medium under normoxic conditions. The expansion medium consisted of hgDMEM, 100 µL/mL FBS, 10 µL/mL penicillin/streptomycin (p/s, PAA laboratories, P11-010), 5 µL/mL ascorbic acid 2-phosphate (Sigma, A8960), 1 µL/mL dexamethasone (Sigma, D1756), 1 µL/mL basic fibroblast growth factor (AbD Serotec, PHP105), and 5 µL/mL fungizone (15290-018, Invitrogen). After the expansion, cells were counted and monolayers of approximately 60.000 cells were seeded (n=2 per condition) in a Cellstar 24-wells plate (662160, Greiner bio-one, Germany) in chondrogenic medium: hgDMEM, ITS+ premix (10 µL/mL (Corning 354352)), L-Proline (2 µL/mL (Sigma, P5607)), p/s (10 µL/mL), fungizone (5 µL/mL), ascorbic acid 2-phosphate (5 µL/mL), and bovine serum albumin (10 µL/mL). Loaded PEA microspheres with celecoxib were dispersed in culture medium and placed in Transwell® baskets (pore size 0.4 µm, polycarbonate membrane, 3413 Costar Corning, USA). Two concentrations, a low dose and a high dose, of celecoxib-loaded microspheres were utilized: 10^{-7} M (LD-MS) and 10^{-4} M (HD-MS). To study the effect of microspheres on modulating cellular response when stimulated with the cytokine tumour necrosis factor alpha (TNF-α, control), 0.133 mg of unloaded microspheres (U-MS) were used. Moreover, unloaded and loaded microspheres were cultured similarly as described before, but in the absence of cells (NC, n=2 per condition per time point) to rule out any interference of the microspheres with analyses and to compare the release of celecoxib. Cells treated with free added 10^{-6} M celecoxib (CXB), as a single dose, were included as comparisons at each time interval. As 10^{-6} M celecoxib is dissolved in alcohol (EtOH, 2%), cells were also cultured in chondrogenic medium supplemented with a similar concentration of EtOH (10^{-6} M). Cells were incubated

for 4 hours at 37 °C, at 5% CO_2. Thereafter, TNF-α was added at a final concentration of 10 ng/mL to the culture medium. Cells cultured in plain chondrogenic medium without TNF-α served as negative controls (**Figure 3.2**).

For bioactivity determination, cells were incubated for 72-96 hours before the microspheres were transferred to another 24-wells culture plate containing cells seeded according to the procedure described above. This procedure was performed 8 times, resulting in a release period of 28 days. Cell confluency was confirmed and recorded with the Leica DFC425 C (Leica microsystems, Wetzlar, Germany) mounted on a CKX41 microscope (Olympus, Zoeterwoude, the Netherlands) for every time point after adding and later transferring the transwell baskets.

At every 72-96 hour time point medium was collected and either 200 µL Hanks balanced salt solution (HBSS, 14025-050, Gibco® Life Technologies™, Bleiswijk, the Netherlands) was added to later measure DNA content or 200 µL RLT lysis buffer (74004, Qiagen, Hilden, Germany) for RNA isolation. Samples were stored at -70 °C until further analysis if not used immediately. DNA content (n=1 per condition for 6 donors per time point), glycosaminoglycan (GAG) release (n=1 per condition for 6 donors per time point), PGE_2 release (n=1 per condition for donors 1 to 3, per time point), and celecoxib release (n=1 per condition for 3 donors per time point) were determined.

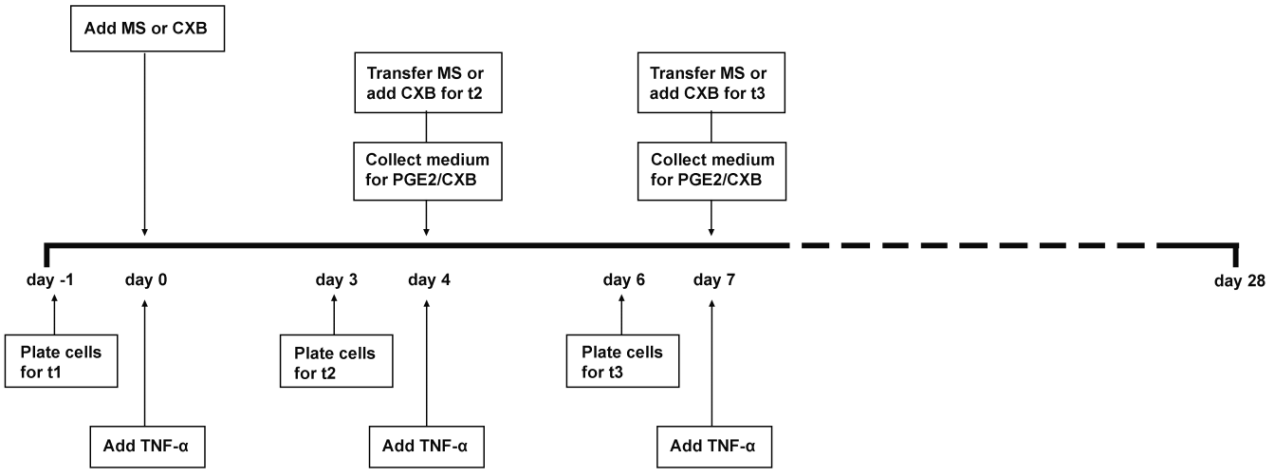

Figure 3.2. Schematic overview of the bioassays used for evaluating bioactivity of released celecoxib and in inhibiting prostaglandin E_2 (PGE_2) production in tumour necrosis factor alpha (TNF-α) stimulated chondrocytes over a culture period of 28 days. TNF-α was added four hours after the microspheres and free added celecoxib. *MS* = microspheres (unloaded and two concentrations of celecoxib-loaded microspheres). *CXB* = celecoxib.

Cell proliferation and extracellular matrix production

GAG release

After the 72-96-hour time point medium was collected and stored at -20 °C until further measurements. A DMMB assay was performed on the medium as described earlier (Chapter 1). Undiluted samples were analysed. To correct for the interference of the colorimetric intensity by the medium colour the standard curve of shark chondroitin sulphate was diluted in plain hgDMEM and plain hgDMEM was included in the assay.

DNA content

The DNA content from one replicate per condition per time point per donor (n=6, with the exception of day 28 with n=5) was measured using Qubit™ dsDNA high sensitivity assay kit

(Q32851, Invitrogen, Eugene, USA). For this, 200 µL of HBSS was added to the cells after removal of the medium. Subsequently, the plates with monolayers were frozen (at -70 °C) and thawed 3 times. Thereafter, wells containing the monolayers were scratched with a pipette tip. The suspension was collected and DNA was measured according to the manufacturer's instructions.

Gene expression

One replicate per condition per donor (n=3) for the first time point (day 4) was collected for RNA isolation. For this 200 µL RLT was added to the cells after removal of the medium. Subsequently, the plates with monolayers were frozen until further use. After this 150 µL of RLT with 1% β-mercaptoethanol (101726966, Sigma-Aldrich Chemie GMBH Steinheim, Germany) was added and the RNAeasy® Micro Kit (74004, Qiagen, Hilden, Germany) was used according to the guidelines of the manufacturer. DNA removal was safeguarded in this process by performing an on column DNase step. The iScript™ cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands) was used according to the guidelines of the manufacturer. Six reference genes were chosen to normalise for the target gene expression of ECM anabolism, ECM catabolism, PGE_2 pathway enzymes, inflammatory mediators and apoptotic markers (**Table 3.4**). IQT™ SYBR Green Supermix Kit (Bio-Rad, Veenendaal, the Netherlands) and the CFX384 Connect™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands) were used to perform qRT-PCR. Relative quantitative gene expression was determined by the Normfirst method. The mean Ct-values of the reference genes normalized the mean Ct-values of the genes of interest (GOI): $\Delta Ct = Ct_{mean\ ref} - Ct_{GOI}$. Thereafter, $E^{\Delta Ct}$ were calculated whereby E indicates the efficiency of amplification from the GOI/reference gene.

Controlled release

Celecoxib release

A standard curve was generated by stepwise dilution of a celecoxib stock (1 mg/mL celecoxib in 100% ethanol). The celecoxib ELISA kit (180719, Neogen Corporation, Lansing, United States) was used according to the guidelines of the manufacturer. Samples were lyophilised undiluted for 3 hours and dissolved in 50 µL EIA buffer (2500, Neogen Corporation, Lansing, United States) overnight at 4 °C prior to analysis.

Inflammatory response

PGE_2 release

The PGE_2 measurement was performed on the collected medium as described previously with the competitive colorimetric ELISA (Prostaglandin E2 monoclonal ELISA kit, 514010, Cayman Chemical, Ann Arbor, USA) (Chapter 1). Samples were analysed undiluted. One replicate per condition for donors 1 to 3 from each time point were used due to financial limitations.

Statistics

All data were statistically analysed using IBM SPSS statistics 24. A normality check was performed to determine normal distribution of the data, using a Shapiro Wilks test. Data that were not normally distributed were subjected to the Kruskal Wallis and Mann Whitney test. Normally distributed data was subjected to the ANOVA or a mixed model. Post-hoc tests (Benjamini & Hochberg) were performed for multiple comparisons. The TNF-α-stimulated culture without the presence of celecoxib or microspheres was assigned as the control.

The effect size (ES) and ES's confident intervals (CI set at 95%) were also taken into consideration to evaluate the significances. The definitions of the effect sizes, provided by

Sawolowsky (2009)[111], are illustrated in **Table 3.2**. When data was normally distributed, ES were calculated and subsequently provided as Hedge's g (Cohen's delta correction for a low sample number[112], **Table 3.2**) and when not normally distributed provided as a Cliff's delta[113] in the present study. Every *p*-value with a lower ES than described in **Table 3.3** was considered not relevant or uncertain[114,115].

Effect size (ES)	Hedge's g	Cliff's delta
None	ES<0.01	
Very Small	0.01≤ES<0.2	ES<0.11
Small	0.2≤ES<0.5	0.11≤ES<0.28
Medium	0.5≤ES<0.8	0.28≤ES<0.43
Large	0.8≤ES<1.2	0.43≤ES<0.7
Very Large	1.2≤ES<2	ES≥0.7
Huge	ES≥2	

Table 3.2. A definition of the effect sizes (ES). Table derived from Vargha *et al.* (2000)[113], Sawilowsky (2009)[111], and Miranda-Bedate *et al.* (manuscript in preparation).

p-value	Effect size (ES)	Interpretation
Independent of p-value	None, very small, small	Uncertain
***p*≤0.05**	Medium and larger	Substantive significant
0.05<*p*≤0.1	Large and larger	Substantive significant
***p*≤0.15**	Very large and huge	Substantive significant

Table 3.3. Threshold and interpretation to determine whether or not data is significantly interpreted. Table derived from Ellis (2010)[114], Greenland *et al*. (2016)[115], and Miranda-Bedate *et al.* (manuscript in preparation).

Results

***In vitro* monolayer culture of articular cartilage cells**

Cell confluency (40-60%) appeared to be consistent for all donors at the initiation of each culture per time point (**Figure 3.3**). Unloaded and loaded PEA microspheres seemed identical by appearance (**Figure 3.3**). After 25 days of culturing the number of microspheres had visually hardly decreased. (**Figure 3.8**).

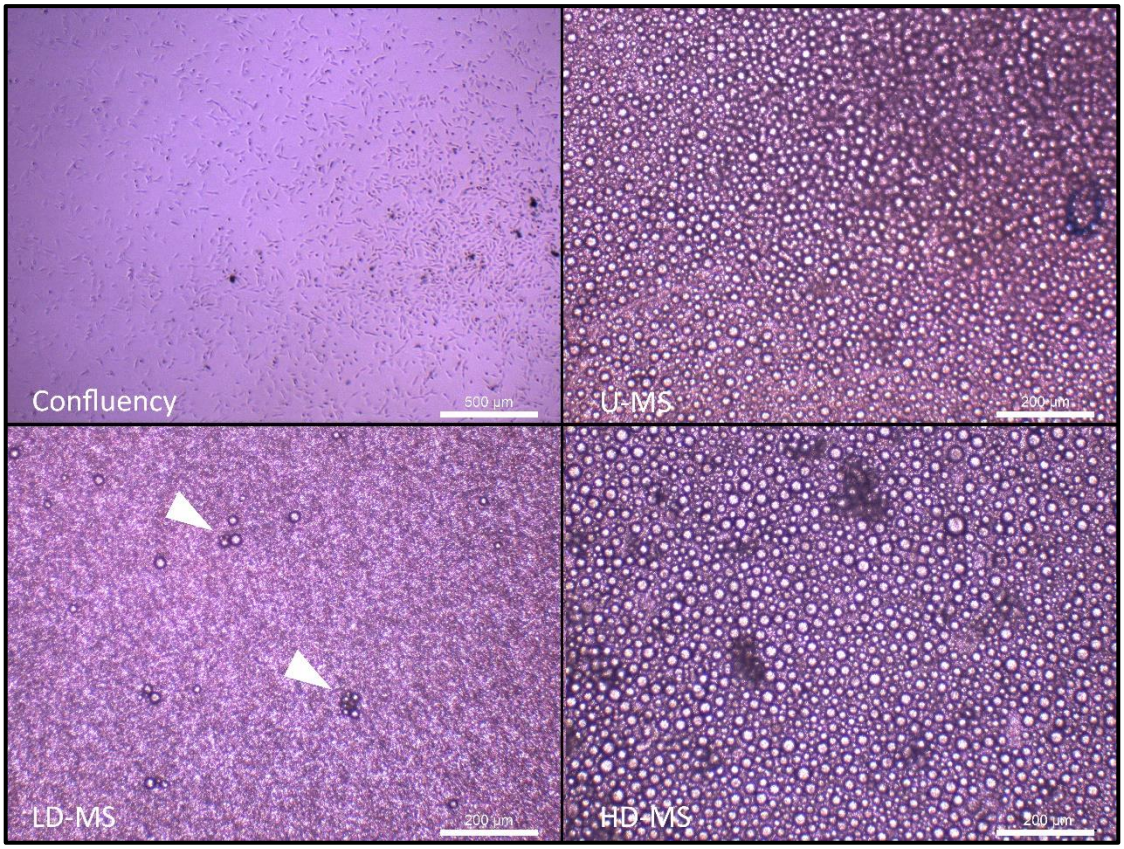

Figure 3.3. Confluency of the monolayers was confirmed by microscopic imaging. A donor and time point representative for the whole data set is displayed here, on the right side of the image the centre of the well with the monolayer expanding towards the edges. Scale bar indicates 500 µm (**top left**). Unloaded and loaded microspheres seemed identical by appearance (day 0); unloaded microspheres (U-MS) (**top right**), low dose celecoxib-loaded microspheres (LD-MS, corresponding with 10^{-4} M celecoxib) (**bottom left**), and high dose celecoxib-loaded microspheres (HD-MS, corresponding with 10^{-7} M celecoxib) (**bottom right**). Note that in LD-MS (**arrowheads**) distinctly less MS are visible. Scale bar indicates 200 µm.

Cell proliferation and extracellular matrix production

The culturing period of three to four days per time point turned out to be too short for the GAG release to be measurable, as the measured optical density (OD) was comparable to the OD of cell-free samples.

DNA was used to determine cell content after being exposed to (loaded) microspheres for 72-96 hours as a measure of cell viability. On the first day of every time point approximately 60.000 chondrocytes were seeded as a monolayer. In the first time point (day 4) a lower DNA content was measured for the U-MS, LD-MS, and HD-MS conditions, while in the control condition (TNF-α stimulated) DNA content had increased. Overall, the MS conditions were associated with an increase of DNA content at every time

point, with day 25 being the exception with a slightly decreased DNA content. No significant differences between the control, U-MS, CXB, LD-MS, and HD-MS were found with regard to DNA content over the whole culturing period (**Figure 3.4**).

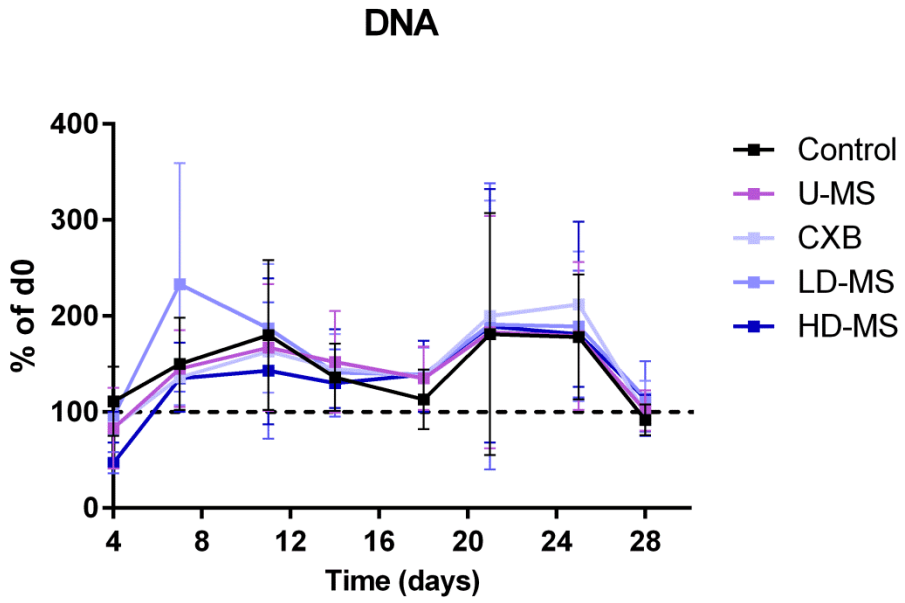

Figure 3.4. DNA content (µg) (mean±SD) as an indication for the cell viability after culturing in the presence of (loaded) microspheres for 3-4 consecutive days. The averages of the day 0 DNA content of all six donors for the corresponding time points was set to 100%. N=6 per condition per time point, with the exception of time point 8 (day 28) with n=5. No significant differences were found between the conditions. *Control* = chondrocytes stimulated with tumour necrosis factor alpha (TNF-α). *U-MS* = unloaded microspheres. *CXB* = free added celecoxib (10^{-6} M). *LD-MS* = low dose (10^{-7} M) celecoxib-loaded microspheres. *HD-MS* = high dose (10^{-4} M) celecoxib-loaded microspheres.

Gene expression

Gene expression levels of *IL1ß, CASP3,* and *FASL* could not be detected. *IL10* was only expressed in the control (TNF-α stimulated). Gene expression of all genes of interest was comparable between all culture conditions. Only *COL1A1* was significantly increased (*p*-value <0.05, huge ES) by 21-fold in HD-MS compared to CXB, however, this was not the case when compared to the control. Though not significant (*p*-value >0.05), large effect sizes were still found in some other genes. In *ACAN* a large ES was found for the upregulation of HD-MS compared to the control, as was the case in *COL2A1* with a very large ES. A huge ES was found for the downregulation of *PTGES1* in HD-MS compared to the CXB. Lastly, *IL6* was downregulated in HD-MS compared to CXB with a very large ES (**Figure 3.5**).

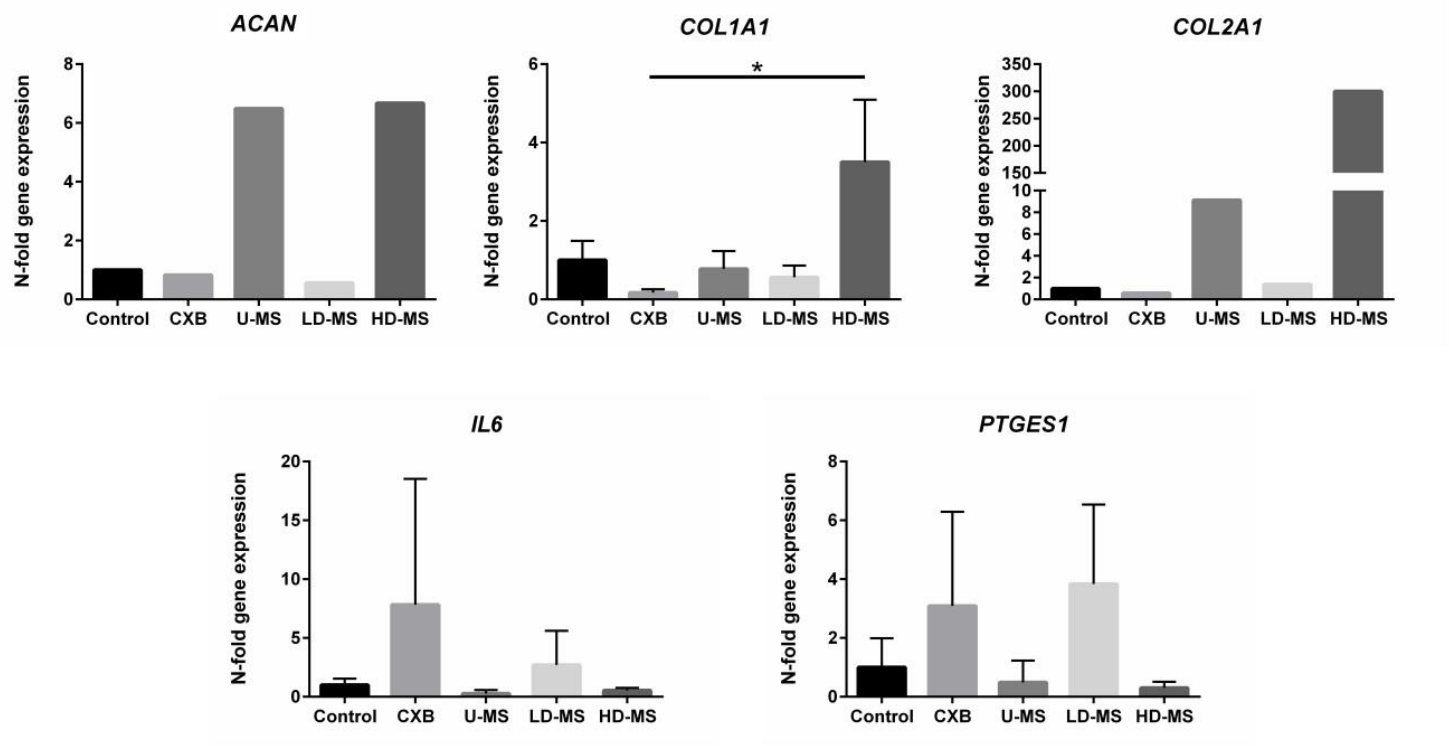

Figure 3.5. N-fold gene expression (relative to normal±SE) of *ACAN, COL1A1, COL2A1, IL6,* and *PTGES1*. Only the upregulation of the high dose (10^{-4} M) celecoxib-loaded microspheres (HD-MS) compared to the single dose free added celecoxib (CXB) in *COL1A1* was significantly increased (*p*-value <0.05, huge effect size (ES). HD-MS did, however, not significantly differ from the control (tumour necrosis factor alpha (TNF-α) stimulated). In *COL2A1, ACAC, PTGES1,* and *IL6* no significances were found (*p*-value >0.05), though ES was large to huge in some conditions. *LD-MS* = low dose (10^{-7} M) celecoxib-loaded microspheres. ***** = *p*-value <0.05.

Controlled release of celecoxib

From both LD-MS and HD-MS celecoxib was gradually released throughout time after an initial burst release the first 11 days (32% in HD-MS and 20% in LD-MS). It also seemed to be unaffected by the presence of cells (NC, 21% in HD-MS and 21% in LD-MS) and cell donor (**Figure 3.7**). After 14 days of culturing celecoxib release in the LD-MS was too low to be detected. Even though, microspheres could still be detected microscopically (**Figure 3.6**).

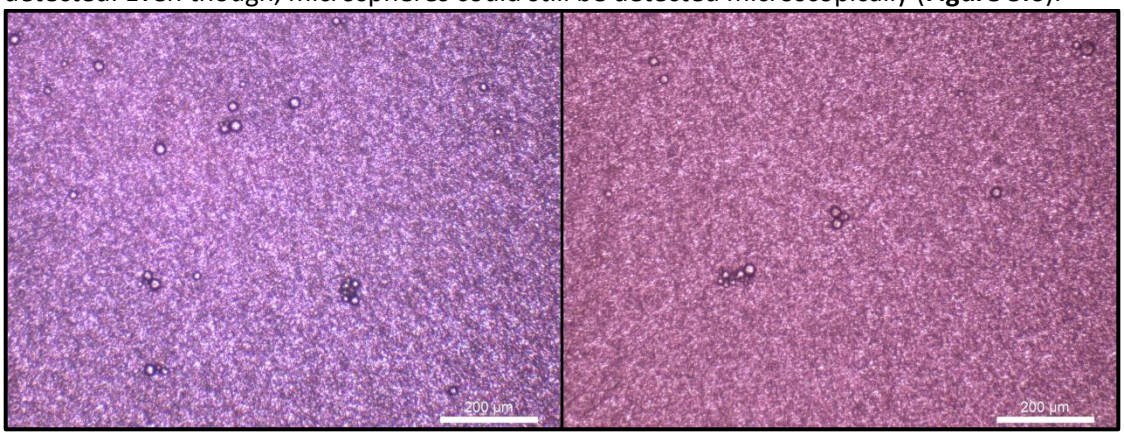

Figure 3.6. Low dose (10^{-7} M) celecoxib-loaded microspheres on day 0 (**left**) and after 25 days of culturing (start of time point 8) (**right**). Even though celecoxib release was undetectable after 14 days of culturing, microspheres were still present after 25 days of culturing. Scale bar indicates 200 µm.

At the end of the 28-day culturing period, celecoxib was still being released in the HD-MS condition (**Figure 3.7**). Cumulative, 39% in de LD-MS and 40% in the HD-MS had been released after 28 days of culturing. Interestingly, the presence of chondrocytes did seem to accelerate the celecoxib release in LD-MS (41% after 28 days of culturing in the absence of cells), but the contrary was the case for HD-MS (25% after 28 days of culturing in the absence of cells) (**Figure 3.7, bottom**). Microscopic imaging confirmed that microspheres were still abundantly present in the HD-MS condition after 28 days of culturing (**Figure 3.8**).

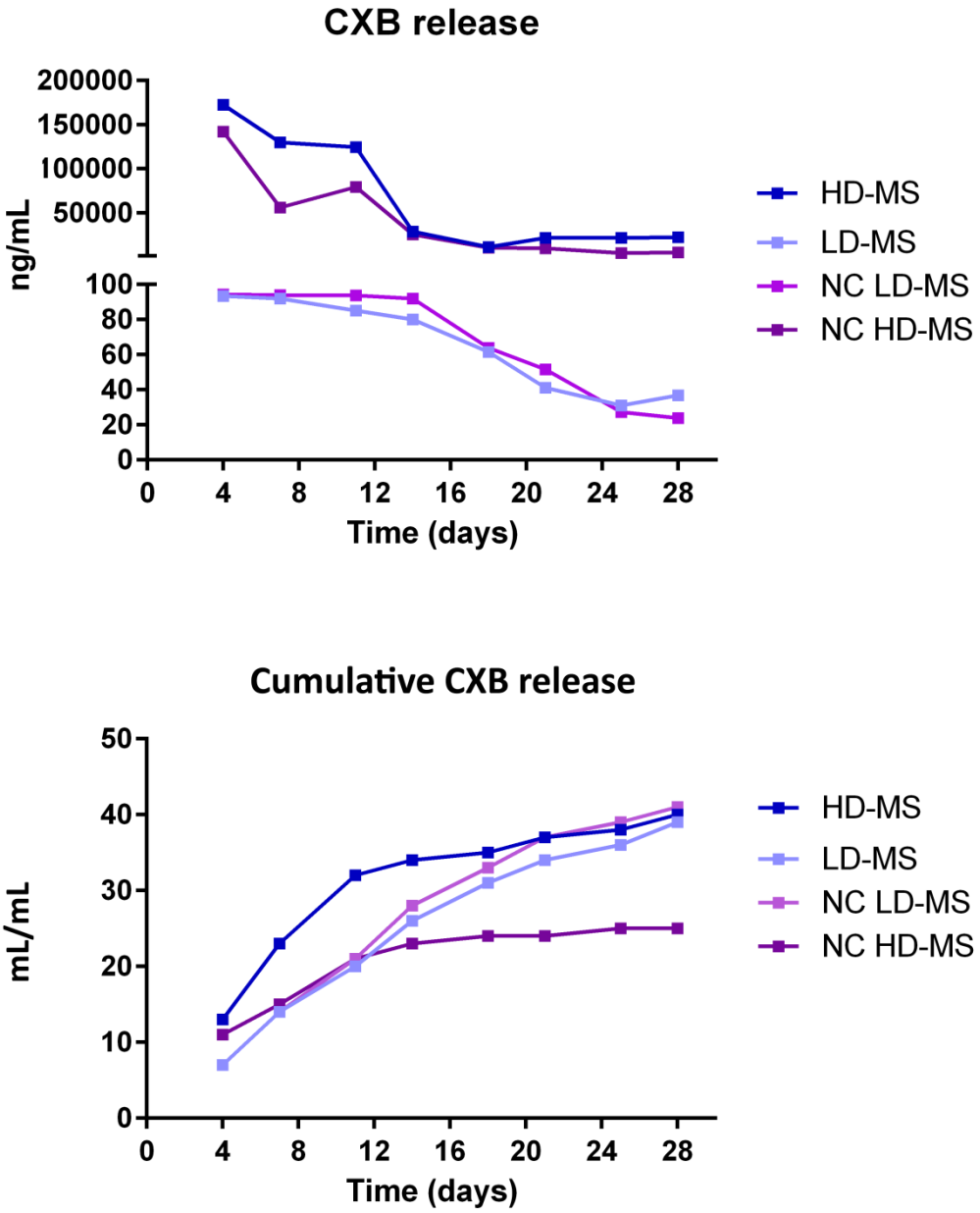

Figure 3.7. *In vitro* absolute (ng/mL) (mean) (**top**) and cumulative (mL/mL) (mean) (**bottom**) celecoxib (CXB) release from the celecoxib-loaded microspheres, low dose (LD-MS, corresponding with 10^{-7} M CXB) and high dose (HD-MS, corresponding with 10^{-4} M CXB) in the presence of articular cartilage cells (in monolayers) and in the absence of articular cartilage cells (NC) over a period of 28 days. Celecoxib is released in slow and gradual manner after an initial burst release in the first 11 days. After 28 days of culturing HD-MS were still releasing celecoxib, indicating a longer release period than the measured 28 days. Interestingly, the presence of chondrocytes did seem to accelerate the celecoxib release in HD-MS, but the contrary was the case for LD-MS. N=3 for MS and n=1 for NC.

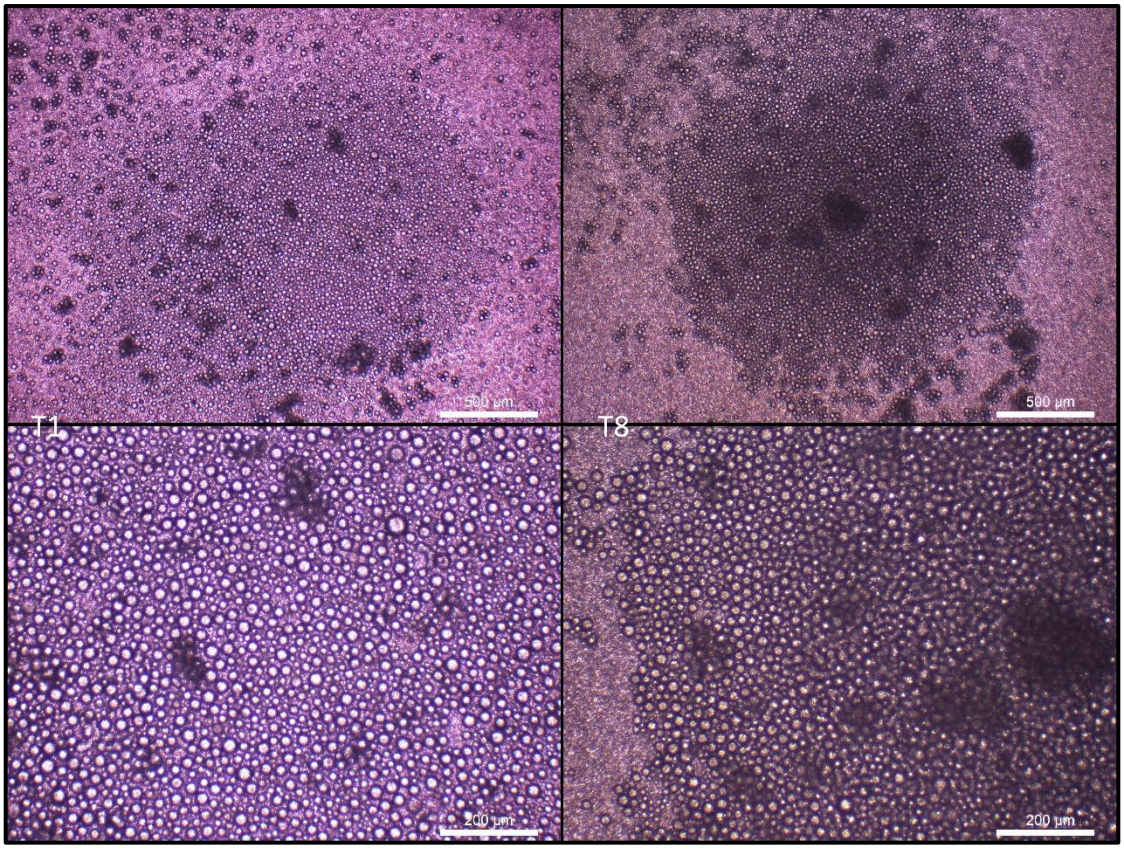

Figure 3.8. High dose (10^{-4} M) celecoxib-loaded microspheres (HD-MS) on day 0 (time point 1 (T1)) (**left**) and after 25 days of culturing (start of T8) (**right**). The amount of microspheres seems to have hardly decreased. Scale bar indicates 500 µm (**top**) and 200 µm (**bottom**).

Inhibition of PGE_2 production in TNFα-stimulated chondrocytes by sustained release of celecoxib

Passage 3 (P3) chondrocytes produced basal levels of PGE_2 without the stimulation of TNF-α (data not shown). The used TNF-α did not appear to further stimulate PGE_2 production. Over all time points, PGE_2 release was significantly downregulated in HD-MS and CXB compared to the control (**Figure 3.9, top** and **middle**). The effect of LD-MS on the PGE_2 production was similar to the U-MS, namely that PGE_2 production was hardly suppressed. When corrected for DNA, PGE_2 over all time points was even significantly downregulated in both the LD-MS and the HD-MS, and also in CXB (**Figure 3.9, bottom**). On each time point, the effect of the celecoxib released from the HD-MS was comparable to the free CXB (10^{-6} M) directly added to the culture medium at each 3-4-day interval.

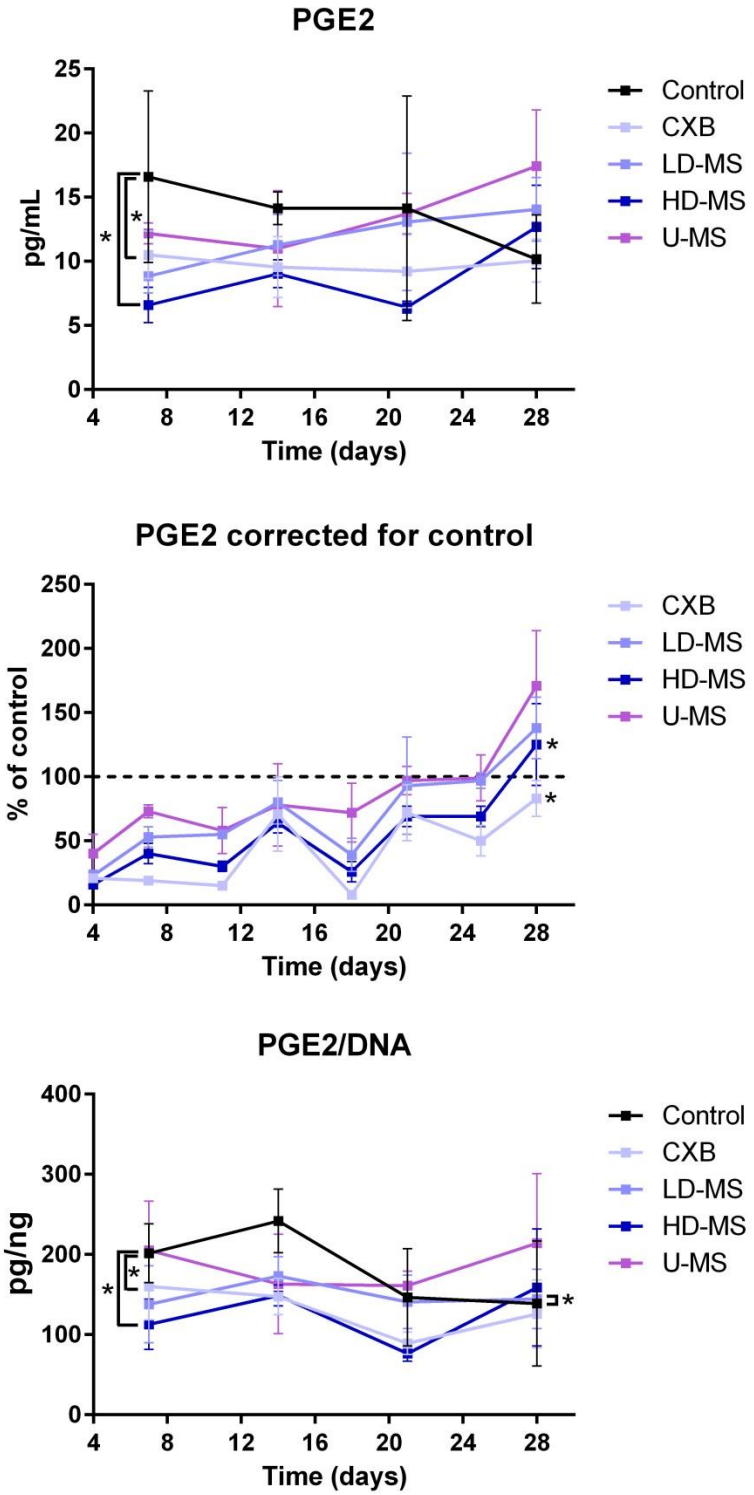

Figure 3.9. Prostaglandin E_2 (PGE_2) release levels (pg/mL) (mean±SD) weekly (**top**), PGE_2 corrected for the release by the control (tumour necrosis factor alpha (TNF-α) stimulated) (%) (mean±SD) (**middle**), and the PGE_2 corrected for DNA (pg/mL) (mean±SD) (**bottom**) in chondrocyte monolayers stimulated with TNF-α. The inhibitory effects of celecoxib-loaded PEA microspheres are displayed in a low dose (LD-MS) and high dose (HD-MS) compared to cells treated with the direct addition of celecoxib (CXB) as a single dose to the medium, unloaded microspheres (U-MS), and the control (TNF-α-stimulated). Over all time points, PGE_2 release was significantly downregulated in HD-MS compared to the control. In PGE_2 corrected for DNA both the LD-MS and the HD-MS significantly downregulated. ***** = *p*-value <0.05.

Discussion

In the current study, we assessed the bioactivity of celecoxib released over time from a biomaterial-based delivery system, PEA microspheres, in the presence of TNF-α-stimulated canine chondrocytes. We demonstrated the controlled release pattern of celecoxib-loaded PEA microspheres, giving an impression of the release pattern *in vivo*. Previous work based on PCLA-PEG-PCLA hydrogels has also demonstrated sustained release of celecoxib both *in vitro* and *in vivo* in a rat model[135], as well as *in vivo* in an equine model of arthritis[141]. Unpublished work in an osteoarthritis rat model revealed disease modifying effects due to celecoxib-loaded PEA microspheres; reduction of osteophyte formation, bone cysts, and loose bodies on micro computed tomography (μCT)[142]. Altogether, this data supports the potency of celecoxib-loaded PEA microspheres in a clinical setting.

The PEA microspheres provide a suitable platform for the sustained release of small molecule drugs, such as celecoxib. In the current study, release profiles of celecoxib in the culture medium showed a slow gradual release for over 28 days with a cumulative release of 39% in the LD-MS and 40% in the HD-MS. This indicates that the release pattern of celecoxib-loaded on PEA microspheres is similar regardless of the concentration of the microspheres within the *in vitro* culture system. Sustained release of celecoxib from PEA microspheres has already been demonstrated in phosphate buffered saline (PBS) for 80 days, in which a short initial burst release of ~15% in the first days[70]. This had, however, not yet been demonstrated in the presence of (canine) chondrocytes. Degradation of the microspheres is by a surface erosion mechanism, but more importantly, by enzymatic degradation, which could provide drug release reactive to the disease process[70]. Enzymatic degradation may be accelerated by the inflammatory response and synovial membrane entrapment *in vivo*[70]. Nonetheless, the HD-MS released only a part of the loaded celecoxib over the period of 28 days, while the celecoxib levels were sustained throughout the culture period after the initial burst release of celecoxib and were not influence by the presence of cells. This, altogether, indicates that celecoxib would still be released after the 28-day culturing period. Especially since both the LD-MS and HD-MS have a similar release. This is further substantiated by the substantial presence of microspheres at termination of the culture period.

Contrary to previous reports[136], in human OA chondrocytes, TNF-α did not seem to provide a proper pro-inflammatory stimulus to P3 canine chondrocytes as indicated by the stable PGE_2 levels. The lack of response could be attributed either to inactivity of the human TNF-α due to species differences or unresponsiveness of P3 healthy chondrocytes. Regardless of the underlying cause, we most probably did not establish a pro-inflammatory environment and as such cannot comment on the effect of degradation of microspheres on celecoxib release as such. Janssen *et al*. (2016)[70] reported an 80% reduction of PEA microspheres 12 weeks after injection in rat knees compared to 3 weeks after injection. Furthermore, degradation in OA(-induced) knees is greater than in healthy knees. This is most probably related to inflammation-related serine protease that drives PEA degradation. For future research, it would be interesting to compare the release behaviour of celecoxib from microspheres between cells with basal PGE_2 production, and cells with an established pro-inflammatory environment.

Sustained release of celecoxib from celecoxib-loaded PEA microspheres effectively reduced PGE_2 production by canine chondrocytes over the whole culture period. Hydrophobic small molecules, like celecoxib, are known to bind to proteins, including serum proteins such as albumin, the most abundant protein in plasma[143,144], thus altering their bioavailability. Celecoxib concentrations effectuated by the controlled release system could, therefore, not correlate with activity. Therefore, in a bioassay we determined the PGE_2 suppression. Indeed, the HD-MS resulted in sustained decrease of PGE_2 comparable to free

added CXB at a concentration of 10^{-6} M, while LD-MS most probably released insufficient celecoxib levels for effective reduction of PGE_2 production. Notably, the presence of celecoxib did not affect DNA content, nor did it significantly affect gene expression levels of genes determined as markers for matrix anabolism, catabolism, apoptosis, inflammation, and the cell cycle. *IL10*, for instance, was only expressed in the control (TNF-α stimulated). This altogether, indicates that celecoxib does not affect chondrocyte health, is not cytotoxic, and does not induce an inflammatory response. Gene expression only revealed a significant difference in *COL1A1* in which the HD-MS was upregulated compared to the CXB. HD-MS was, however, not significantly upregulated compared to the control. Upregulated *COL1A1* gene expression could indicate a fibroblastic phenotype and a significant increase has been observed due to passaging, as early as P1[145]. However, there are indications towards a favourable effect of celecoxib released from HD-MS on matrix quality. Though not significant (*p*-value >0.05), very large and large ES were observed in *COL2A1* and *ACAN*, respectively, for an upregulation in the HD-MS compared to the control. Moreover, *IL6* (very large ES) and *PTGES1* (huge ES) were downregulated in the HD-MS compared to the CXB, indicating that the HD-MS was better capable of suppressing inflammation.

Despite the promising advances of celecoxib-loaded PEA microspheres *in vitro* and *in vivo* in experimental animals, application of this platform will need to be further evaluated in a clinical trial with client-owned dogs. Such a study setup should shed more light on clinical efficiency and possible adverse events after intra-articular application. This would also be a final step in translating this treatment strategy to human OA patients. A placebo-controlled, double-blind prospective clinical trial in client-owned dogs with clinically relevant OA is currently ongoing at the University Clinic for Companion Animals, at the Faculty of Veterinary Medicine in Utrecht.

Conclusions

In the current study, we report the application of PEA microspheres for delivery of celecoxib and a bioassay for determining their bioactivity, with the aim of eventually treating canine and human patients with inflammatory joint diseases, such as OA. We demonstrated no cytotoxicity, gradual celecoxib release, and a PGE_2 suppression by the 10^{-4} M celecoxib-loaded microspheres for 28 days. This application could also be promising for the treatment of canine and human OA patients.

Gene category	Gene	Primer sequence (5'-'3)	Product size (bp)	qRT-PCR temperature condition (°C)
Anabolic	*ACAN*	Fw GGACACTCCTTGCAATTTGAG Rv GTCATTCCACTCTCCCTTCTC	110	61-62
	COL1A1	Fw GTGTGTACAGAACGGCCTCA Rv TCGCAAATCACGTCATCG	109	61
	COL2A1	Fw GCAGCAAGAGCAAGGAC Rv TTCTGAGAGCCCTCGGT	150	60.5-65
	SOX9	Fw CGCTCGCAGTACGACTACAC Rv GGGGTTCATGTAGGTGAAGG	105	62-63
Catabolic	*ADAMTS5*	Fw CTACTGCACAGGGAAGAG Rv GAACCCATTCCACAAATGTC	148	61
	MMP13	Fw CTGAGGAAGACTTCCAGCTT Rv -TTGGACCACTTGAGAGTTCG	250	65
Anti-catabolic	*TIMP1*	Fw GGCGTTATGAGATCAAGATGAC Rv ACCTGTGCAAGTATCCGC	120	66
Apoptotic marker	*BAX*	Fw CCTTTTGCTTCAGGGTTTCA Rv CTCAGCTTCTTGGTGGATGC	108	58-59
	BCL2	Fw GGATGACTGAGTACCTGAACC Rv CGTACAGTTCCACAAAGGC	80	61.5-63
Cell cycle	*AXIN2*	Fw GGACAAATGCGTGGATACCT Rv TGCTTGGAGACAATGCTGTT	128	60
PGE_2 pathway	*PTGES1*	Fw CCAGTATTGCCGGAGTGACCAG Rv AAACGAAGCCCAGGAACAGGA	97	68
Inflammatory mediator	*IL1β*	Fw TGCTGCCAAGACCTGAACCAC Rv TCCAAAGCTACAATGACTGACACG	115	68
	IL6	Fw GAGCCCACCAGGAACGAAAGAGA Rv CCGGGGTAGGGAAAGCAGTAGC	123	65
	IL10	Fw CCCGGGCTGAGAACCACGAC Rv AAATGCGCTCTTCACCTGCTCCAC	91	63
Reference gene	*GAPDH*	Fw TGTCCCCACCCCCAATGTATC Rv CTCCGATGCCTGCTTCACTACCTT	100	58
	HNRPH	Fw CTCACTATGATCCACCACG Rv TAGCCTCCATAACCTCCAC	151	61
	RPL13	Fw GCCGGAAGGTTGTAGTCGT GGAGGAAGGCCAGGTAATTC	87	61
	RPS19	Fw CCTTCCTCAAAAAGTCTGGG Rv GTTCTCATCGTAGGGAGCAAG	95	61-63
	SRPR	Fw GCTTCAGGATCTGGACTGC Rv GTTCCCTTGGTAGCACTGG	81	61.5
	YWHAZ	Fw CGAAGTTGCTGCTGGTGA Rv TTGCATTTCCTTTTTGCTGA	94	58

Table 3.4. Gene-specific primer sequences with associated amplification temperatures.

General discussion and conclusions

In this HP thesis, we looked into the differences of non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs at the articular cartilage level and how this could affect the response of these two canine breeds to an inflammatory stimulus and anti-inflammatory treatment *in vitro*. It has already been established that NCD and CD dogs are not only different by appearance, but also on a tissue level, namely in intervertebral discs (IVDs)[74,78,85]. Based on this, the influence of chondrodystrophy on cartilage metabolism was further investigated.

The genetic basis of chondrodystrophy presumably lays in an insertion in the *FGF4* gene, either on CFA12 *FGF4* or CFA18 *FGF4*, or even both[74,76]. An insertion on CFA18 *FGF4* possibly only impacts limb length, while an insertion on CFA12 *FGF4* has also been directly linked to IVD degeneration and explains the chondrodystrophic phenotype[74]. Breeds that have both *FGF4* insertions have the most dramatic decrease in height: Basset Hound, Cardigan Welsh Corgi, and Dachshund[74]. We have found evidence that support the hypothesis that NCD and CD dogs differ on a cartilage level. FGF ligands exert their effect through FGFRs[146]. Gain of function mutations in *FGFR3* result in chondrodysplasia[147] and FGFR3 seems to prevent articular cartilage degeneration[83,148]. FGFR3-deficient mice show disruption of the cartilage homeostasis and chondrocyte-specific deletion of *FGFR3* accelerated articular cartilage destruction, while chondrocyte-specific FGFR3 activation in adult mice delays articular cartilage destruction. Based on this, targeting FGFR3 has been opposed as a treatment strategy for osteoarthritis (OA)[83]. In humans, patients with achondroplasia, a gain-of-function mutation in *FGFR3*, exhibit severe bowleg deformity and a higher incidence of obesity, but still they rarely develop OA[149]. Possibly because the activation of FGFR3 inhibits chondrocyte hypertrophy in articular cartilage in a similar manner as it inhibits terminal chondrocyte differentiation in growth plates[83]. Therefore, we hypothesise that chondrodystrophy in canines may also have a protective effect against OA development. We found that CD cartilage was better capable of retaining glycosaminoglycans (GAGs) in an inflammatory environment, while NCD was less potent to respond to an inflammatory stimulus but was more responsive for non-steroidal anti-inflammatory drugs (NSAIDs).

There are many signalling pathways implicated in OA development. One of the well accepted pathways is the Wnt signalling pathway. The Wnt signalling pathway seems to play a role in the differences observed between NCD and CD derived cartilage at the biochemical level and their inherent response to a pro-inflammatory stimulus. Wnt signalling pathway has multiple roles in the regulation of cartilage development, growth, and maintenance[75]. Interestingly, Wnt signalling is required for progression of endochondral ossification[108] and growth of the axial and appendicular skeleton[108,109]. This indicates a possible interplay between chondrodystrophy and Wnt activity. In IVDs, gene expression analysis has revealed that Wnt activity is upregulated in CD dogs, while *AXIN2* downregulation is observed in IVD degeneration[85]. The gene expression in cartilage explants revealed a similar pattern; *AXIN2* was significantly upregulated in CD compared to NCD derived healthy cartilage explants. Moreover, *DKK3* was also significantly more expressed in CD compared to NCD cartilage. *DKK3* upregulation has been suggested as a defence mechanism to counteract OA disease-related dysregulation of cell signalling pathways. In this way, pro-inflammatory cytokine effects, like those of tumour necrosis factor alpha (TNF-α), on cartilage degradation would be reversed and transforming growth factor beta (TGF-β) signalling enhanced, whilst activation of Wnt signalling is an attempt to counteract the changes in the Wnt pathway[125].

Interestingly, inactivation of *β-catenin,* an intracellular signalling transducer of the canonical Wnt signalling pathway, in chondrocytes of mouse embryos results in a similar phenotype of dwarfism as seen due to *SOX9* overexpression, with decreased chondrocyte

proliferation, delayed hypertrophic chondrocyte differentiation, and endochondral bone formation. In line with this, long bone growth plates in CD dogs show disorganisation of the proliferative zone and reduction in depth of the maturation zone[150]. Furthermore, either stabilization of *β-catenin* or activation of *SOX9* in chondrocytes produces a similar phenotype of severe chondrodysplasia[151]. While *SOX9* was not differently expressed between NCD and CD derived cartilage, it supports the chondroprotective state of CD dogs acquired from Wnt activation.

There should be a fine balance in the activation of the Wnt signalling pathway to maintain cartilage homeostasis. It is likely that excessive activation of Wnt/β-catenin signalling increases articular cartilage destruction and possibly promotes subchondral bone remodelling[75]. However, there are indications that complete inhibition of Wnt/β-catenin signalling induces increased articular chondrocyte apoptosis. This process is accompanied by progressive loss of the smooth surface of the articular cartilage, eventually leading to severe loss of the entire articular cartilage surface[152]. Moreover, complete inhibition of the canonical pathway has a negative impact on articular progenitors[120]. As such, it remains to be determined whether CD and NCD dogs differ in the way Wnt signalling is regulated at the cartilage level and how their genetic make-up influences this process. For now, there are indications that Wnt is more activated in CD cartilage.

Besides the current results regarding differences between CD and NCD dogs, biomechanical differences should also be considered that can contribute to the development and progression of OA. Differences between mammal species on a cartilage level have already been looked into, but little is known about the differences within the canine species[110]. Medium- or larger-sized breeds are, for instance, more commonly affected by osteochondrosis than smaller ones[153]. Presumably because larger dog breeds have a steeper growth curve that allows for an abnormal joint conformation. Osteochondrosis could subsequently be the cause of the development of secondary OA.

In translational and preclinical studies, thus far, there are no guidelines as to which animal model is most appropriate. Most probably based on availability experimental mixed breed dogs and Beagle dogs have been interchangeably used. Considering the differences that were observed in the present Honours Programme study, distinction between CD and NCD dogs when choosing the most appropriate canine OA model should be deemed crucial. Bendele (2001)[5] observed that beagle dogs displayed very little overt cartilage degeneration over a 3-month period in an OA model based on anterior cruciate ligament transection. Bendele seemed to favour NCD dogs for canine OA models as they seem to develop more severe OA changes, however, this has not been specifically investigated yet. However, it may be of interest to choose the Beagle dog, or other CD breed, if less severe degeneration is desired or the therapy is not anti-inflammatory. Moreover, the distinction between NCD and CD dogs may also be of importance when treating osteoarthritis, as based on the *in vitro* studies conducted during this Honours Programme project, NCD dogs seem to be more susceptible for inflammation and responsive to treatment with anti-inflammatory drugs.

In vitro work is the first step in developing a novel treatment strategy for OA. Therefore, we performed a study with celecoxib-loaded polyester amide (PEA) microspheres in NCD chondrocytes in monolayers to evaluate its potency as a treatment of the degenerative joint disease. The loaded microspheres indeed proved their applicability and *in vitro* safety. Anti-inflammatory medication was released over a prolonged period of at least 28 days and was effective in supressing the prostaglandin E_2 (PGE_2) levels released by cartilage cells. Biomaterial-based controlled release of celecoxib not only holds advantages over systemic or local bolus administration by increasing efficiency[132,133] and prolonging therapeutic effects, it also reduces systemic adverse effects[154,155]. Only a normal and mild foreign body response has been demonstrated after IA injection in an OA rat knee model[70].

Currently, only intra-articular injection of corticosteroids is registered for the treatment of OA in human and veterinary patients. Besides the down side of bolus injection, the intra-articular injection of corticosteroids is not without risk[156]. In a cohort study of 16,624 intra-articular medication injections in Thoroughbred horses, 13 horses developed septic arthritis, of which 12 had been injected with a corticosteroid[157]. This work underlines why selective COX2 inhibitors seem like a more sensible choice. Celecoxib-loaded PEA microspheres in a rat model of osteoarthritis have demonstrated disease modifying effects, in the form of reducing the formation of osteophytes, bone cysts, and loose bodies on µCT. Moreover, less subchondral sclerosis was observed in the celecoxib-loaded PEA microspheres treated rats compared to the rats treated with unloaded PEA microspheres, although values were still higher than in healthy control joints[142]. PEA microspheres are ascribed auto regulatory properties, as degradation of PEA microspheres is higher in OA induced knees compared to the contralateral healthy knee joint and loading the microspheres with celecoxib significantly inhibits degradation[70]. *In vivo* the drug release may be influenced by the highly viscous synovial fluid[158]. However, osteoarthritic synovial fluid typically is less viscous. This could, however, be further evaluated with a synovial fluid co-culture such as we described. Though this synovial fluid co-culture still needs standardisation.

Taken together, CD and NCD cartilage display differences in their cartilage metabolism and susceptibility for inflammation and presumably OA. Distinctions between these breeds are not only of importance for treating these breeds for OA, but also when choosing the most appropriate canine OA model.

Future work

We established that cartilage derived from chondrodystrophic (CD) dogs seems less susceptible to an inflammatory stimulus. Non-chondrodystrophic (NCD) cartilage seems to have a tendency for a pro-inflammatory state. The Wnt signalling may play a role in this, as we have indications that, based on gene expression, Wnt signalling is more activated in CD cartilage. Follow up work should determine protein levels by Western Blot to confirm this assumption. Furthermore, Wnt signalling activity of primary cartilage cells derived from CD and NCD dogs could be studied in *vitro* with the aid of the TOP/FOP ratio where in the differential effect of pro-inflammatory stimuli could be studied. To measure Wnt signalling activity, TOP-flash assays are widely used as a Wnt reporter. The reporter can be transfected into cells by use lipofectamine, together with an appropriate control (FOP), and measure the levels of canonical Wnt signalling by luciferase activity. Furthermore, TOP and FOP activity is corrected for β-actin-promotor-renilla activity, to correct for the amount of lipofectamine that has entered the cells. Moreover, future work will consist of histologically scoring cartilage of NCD and CD dogs used for osteoarthritis (OA) models. There are several studies available employing similar models of OA in both CD and NCD dogs which can be used in a retrospective manner for this purpose.

We demonstrated in a 4-week follow-up in tumour necrosis factor alpha (TNF-α) stimulated NCD chondrocytes the application of celecoxib-loaded polyester amide (PEA) microspheres, 10^{-4} M and 10^{-7} M. The high dose loaded microspheres (10^{-4} M) were successful in suppressing prostaglandin E_2 (PGE_2) release and displayed a gradual release of the celecoxib for the whole culturing period. Indicating that, indeed, anti-inflammatory drugs are very suitable for treating NCD cartilage. Follow up steps are ongoing: a double-blind placebo controlled clinical trial with canine patients suffering from OA is currently recruiting and including patients. Eventually, and based on the results of this clinical study, this treatment strategy may have to be optimised *in vivo*, focussing on loading dose of the celecoxib and clinical efficacy.

Attended Courses

- **Presenting in English** - Babel
- **Academic writing in English** – Babel
- **Monthly manuscript meeting:** scientific evaluation of a recently published article

Attended Conferences

- **Veterinary Science Day** 2016
- **European Veterinary Conference 'Voorjaarsdagen'** 2017: attended with an oral presentation "Differences between chondrodystrophic and non-chondrodystrophic dogs on cartilage level: a genetic basis for osteoarthritis?"
- **Annual meeting Nederlandse Vereniging voor Matrix Biologie** 2017: attended with a poster presentation "Differences between chondrodystrophic and non-chondrodystrophic dogs on a cartilage level: A basis for osteoarthritis?"

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