Dog as a Model for Osteoarthritis: The FGF4 Retrogene Insertion May Matter

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ABSTRACT: Osteoarthritis (OA) is a degenerative joint disease associated with chronic pain and disability in humans and companion animals. The canine species can be subdivided into non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs, the latter having disproportionally short limbs due to disturbance in endochondral ossification of long bones. This phenotype is associated with retrogene insertions of the fibroblast growth factor 4 (*FGF4*) gene, resulting in enhanced fibroblast growth factor receptor 3 (FGFR3) signaling. The effect on cartilage is unknown and in experimental studies with dogs, breeds are seemingly employed randomly. The aim of this study was to determine whether CD- and NCD-derived cartilage differs on a structural and biochemical level, and to explore the relationship between *FGF4* associated chondrodystrophy and OA. Cartilage explants from CD and NCD dogs were cultured for 21 days. Activation of canonical Wnt signaling was assessed in primary canine chondrocytes. OA and synovitis severity from an experimental OA model were compared between healthy and OA samples from CD and NCD dogs. Release of glycosaminoglycans, DNA content, and cyclooxygenase 2 (COX-2) expression were higher in NCD cartilage explants. Healthy cartilage from NCD dogs displayed higher cartilage degeneration and synovitis scores, which was aggravated by the induction of OA. Dikkopf-3 gene expression was higher in NCD cartilage. No differences in other Wnt pathway read outs were found. To conclude, chondrodystrophy associated with the *FGF4* retrogene seems to render CD dogs less susceptible to the development of OA when compared with NCD dogs. These differences should be considered when choosing a canine model to study the pathobiology and new treatment strategies of OA. © 2019 The Authors. *Journal of Orthopaedic Research®* Published by Wiley Periodicals, Inc. J Orthop Res 37:2550–2560, 2019

Keywords: chondrodystrophy; FGF4 retrogene; osteoarthritis; synovial inflammation; Wnt pathway.

Osteoarthritis (OA) is one of the leading causes of chronic pain and disability and is accompanied by socioeconomic and psychological burdens.[1] Treatment remains symptomatic and current efforts within the scientific community focus on a better understanding of OA pathobiology in order to develop effective treatment strategies. The similarities between human and canine joint anatomy and OA pathogenesis, together with the possibility to undergo diagnostic and therapeutic procedures similar to man, contribute to the fact that the dog has often been employed as a large animal model.[2,3] Dogs not only serve as a model to human medicine but also function as a model for its own species. OA causes clinical signs in over 20% of dogs older than 1 year of age and 80% of geriatric dogs.[4] OA can occur in all dog breeds although large breeds are affected more frequently.[5] In experimental studies with dogs, breeds are seemingly employed randomly, the majority being either purpose-bred hound-type dogs or Beagles, which have a considerably different body conformation.

In this respect, the domestic dog (*Canis familiaris*) is undoubtedly the most morphologically diverse mammalian species. One aspect of variation is leg length, also known as chondrodystrophy. This is defined by dysplastic, shortened long bones and is characteristic for breeds such as the Dachshund and Beagle. From a histological point of view, chondrodystrophic (CD) dogs show shortening of the long bones primarily by calcification of the growth plates early in development. While it cannot be excluded that histology may even differ between CD breeds, there is evidence that the growth plates of the long bones of CD dogs show disorganization of the proliferative zone and reduction in depth of the maturation zone in comparison with non-chondrodystrophic (NCD) dogs.[6] Expressed fibroblast growth factor 4 (*FGF4*) retrogenes on CFA12 or CFA18, leading to over-activation of fibroblast growth factor receptor 3 (FGFR3), have been agreed on as the cause of chondrodystrophy in dogs.[7,8] In a similar fashion, several mutations causing enhanced FGFR3 activity can lead to achondroplasia in humans.[9,10] In mice, *FGFR3* overexpression led to disruption of growth plate architecture and enhanced terminal chondrocyte differentiation, whereas inhibition of FGFR3 signaling led to skeletal overgrowth and disruption of chondrocyte homeostasis.[11] Interestingly, *FGFR3* signaling also delayed subchondral bone sclerosis and OA progression in murine femorotibial joints,[12,13] while deletion of *FGFR3* induced

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OA-like defects in temporomandibular joints in adult mice.[14] These findings suggest that chondrodystrophy associated with the *FGF4* retrogene(s) may alter cartilage (patho)physiology and this may even have a protective effect against OA.

FGFR3 signaling was found to activate canonical Wnt signaling in a rat chondrosarcoma cell line and mouse limb bud micromass cultures.[15] Furthermore, studies in human and experimental animals also imply multiple roles for Wnt in OA. An increase in Wnt-related molecules has been found in osteoarthritic cartilage,[16,17] accompanied by an increase in Wnt antagonists such as members of the Dickkopf family.[18] Both gain- and loss-of-function of β-catenin in cartilage induced osteoarthritic changes in mice.[19,20] Genetic studies in humans suggest that the canonical Wnt pathway participates in the pathogenesis of OA in at least a subset of patients.[21,22] It is therefore hypothesized that excessive activation of Wnt/β-catenin signaling enhances articular cartilage destruction. A basal level of Wnt/β-catenin signaling may nevertheless be required to promote regenerative potential of articular cartilage.[18]

Wnt signaling was shown to differ between CD and NCD dogs at least at the intervertebral disc level, concurring with a different clinical representation of disc disease.[23] Hence the use of dogs as a model in OA, that is, Beagles (CD) versus purpose-bred hound-type dogs (NCD) may be obscured by an important confounder involved in OA susceptibility and progression. To our knowledge, articular joint pathobiology related to canonical Wnt signaling has not been described for the canine species. Therefore, considering the key role of dogs as an OA model, the aim of this study was to identify possible variations in cartilage and synovium homeostasis associated with the presence of the *FGF4* retrogene(s), and possible inherent differences in the canonical Wnt signaling pathway, between CD and NCD dogs. This was performed in an ex vivo explant culture under basal conditions or in the presence of a pro-inflammatory stimulus to mimic the OA environment. To assess possible translational implications, previously obtained biochemical data and histological sections of healthy and osteoarthritic cartilage and synovial tissues generated by standardized experimental protocols in previously performed studies, were compared between CD and NCD experimental dogs. OA was induced by application of a surgical groove on the femoral condyle.[24] This validated canine OA model makes use of a one-time trigger, leading to slowly progressing cartilage degeneration in the absence of permanent joint instability.[24]

METHODS

Ex Vivo Explant Culture of Canine Articular Cartilage and Synovial Tissue

Cartilage and synovial tissue from the weight-bearing surfaces of femorotibial joints of healthy NCD (Fig. 1A; $n = 11$) and CD (Fig. 1B; $n = 10$) donors were collected *post-mortem* complying with the 3R principles: all dogs had been euthanized as part of unrelated studies (approved by the Utrecht University Animal Ethics Committee, approval numbers #2016.II.529.002 and #2014.II.06.048). Of the CD dogs, eight were Beagles and two were Beagle/Bedlington crosses with a median age of 48.5 months (range: 21–62 months), four males and six females. NCD donors were purpose-bred hound-type dogs ($n = 11$, median age 24 months (range: 19–36 months, all female) (Table 1). Within 10–60 min of death, the joint cavity was opened under aseptic conditions in order to collect the non-calcified cartilage layer and synovial tissue. The obtained tissue was collected in 50 ml tubes

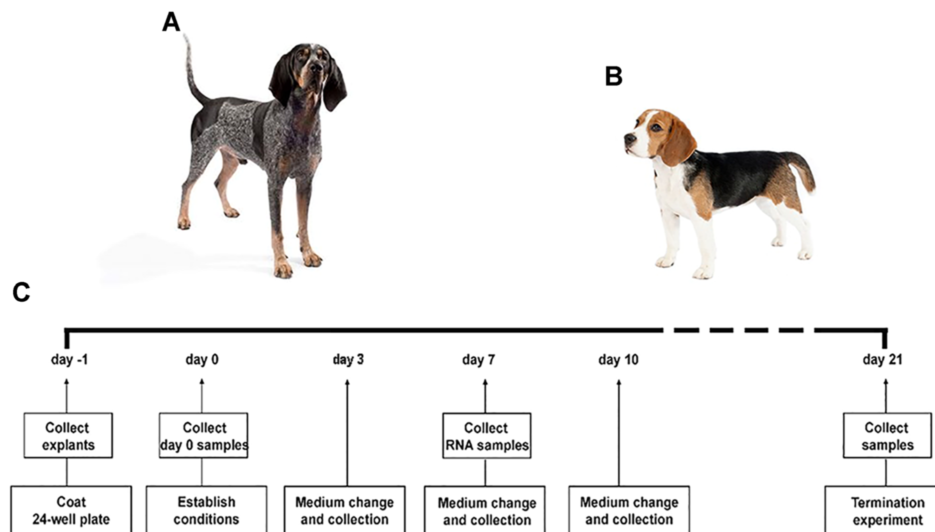

Figure 1. Typical example of an experimental non-chondrodystrophic dog (A; Hound-type dog) and a chondrodystrophic dog (B; Beagle). (C) Setup of the cartilage and synovium explant culture. On day 7, samples for RNA isolation were collected. On day 0 and 21, samples for glycosaminoglycan (GAG) and DNA content, and histology were collected. The experiment was terminated after 21 days of culturing. Immunohistochemistry for collagen type I and II, cyclooxygenase 2 (COX-2), and β-catenin was performed on day 0 samples, to evaluate the tissues in their native state. Osteoarthritis Research Society International and synovitis scores were performed on tissue explants on day 0 and after 21 days of culturing, to assess the severity of cartilage degeneration and synovitis, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Characteristics of the canine donors used in the in vitro cartilage explant experiment

Breed	CD/NCD	Age (months)	Body weight (kg)	Sex
Beagle	CD	45	10	Female
Beagle	CD	48	11	Female
Beagle	CD	26	10	Female
Beagle	CD	54	10	Male
Beagle	CD	62	10	Male
Beagle	CD	50	10	Male
Beagle	CD	48	10	Male
Beagle	CD	21	10	Female
Beagle × Bedlington terrier	CD	49	15	Female
Beagle × Bedlington terrier	CD	49	16	Female
Hound-type dog	NCD	29	25	Female
Hound-type dog	NCD	20	23	Female
Hound-type dog	NCD	22	22	Female
Hound-type dog	NCD	29	28	Female
Hound-type dog	NCD	24	24	Female
Hound-type dog	NCD	26	24	Female
Hound-type dog	NCD	24	32	Female
Hound-type dog	NCD	36	23	Female
Hound-type dog	NCD	19	22	Female
Hound-type dog	NCD	19	21	Female
Hound-type dog	NCD	21	23	Female

CD, Chondrodystrophic; NCD, non-chondrodystrophic.
The *FGF4* retrogene insertion on CFA12 was present in all CD dogs. The *FGF4* retrogene insertion on CFA18 was present in none of the tested CD or NCD dogs.

with 25 ml hgDMEM + Glutamax (31966; Invitrogen, Paisley, UK) + 1% v/v Penicillin/Streptomycin (p/s, P11-010; PAA Laboratories, Cölbe, Germany) and was kept on ice until further processing. After washing with hgDMEM + p/s, an overnight rest at 37°C in a Petri dish (353803; Corning, Amsterdam, the Netherlands) with 25 ml hgDMEM + p/s was included. Thereafter, cartilage originating from the tibial plateaus and synovium were cut into pieces with mean ± standard deviation wet weights of 10.4 ± 4.9 mg per cartilage explant and 47.5 ± 28.6 mg per synovial explant. Two cartilage or synovial explants from each donor were cultured per well of a 24-well plate (662160; Greiner Bio-one, Alphen a/d Rijn, the Netherlands). Plates were coated with 1% agarose (V3121; Promega, Madison, Wisconsin) to prevent adhesion. Samples were cultured for 21 days in culture medium alone or in combination with 10 ng/ml of tumor necrosis factor-α (TNF-α) to mimic the arthritic environment (210-TA; R&D Systems, Minneapolis, MN): hgDMEM, 1% v/v ITS + premix (354352; Corning), 0.04 mg/mL L-Proline (P5607; Sigma), 1% v/v p/s, 0.05% v/v fungizone (15290-018; Invitrogen), 0.1 mM ascorbic acid 2-phosphate (A8960; Sigma-Aldric, Saint Louis, MO), and 1 ng/ml bovine serum albumin (A9418; Sigma-Aldrich). To assess the response of CD and NCD joint tissue to an anti-inflammatory drug, celecoxib (10^{-6}M) was added. The medium was changed and collected on days 3, 7, 10, 14, 17, and 21 (Fig. 1C) and stored at −20°C until further use. Cartilage and synovial explants were collected at days 0, 7, and 21 of culturing ($n = 2$ per donor per condition). The pooled explants per condition per donor were lyophilized to obtain dry weights of the explants.

Gene expression analysis

Cartilage explants ($n = 2$ per donor) were collected, snap frozen in liquid nitrogen and stored at −80°C after seven days of culturing. To assess cartilage homeostasis, gene expression levels of aggrecan (*ACAN*), collagen1α1 (*COL1A1*), collagen2α1 (*COL2A1*), a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*) and metalloproteinase 13 (*MMP13*) were measured. Inflammatory mediators (*IL1β, IL6, PTGES1*) and canonical Wnt signaling (*AXIN2, CCND1, DKK3, FZD1, LRP5A*, and *WIF1*) were also assessed. A subset of donors were assayed for the *FGF4* retrogene insertion on CFA12 and 18 using a PCR-based assay, as described by Brown et al.,[7] with some minor modifications. Details on *FGF4* genotyping and RT-qPCR methods and primers are provided in Supplementary File 1.

Biochemistry

Cartilage explants were digested overnight in 600 μl of papain digestion solution (papain buffer (200 mM $H_2NaPO_4{\cdot}2\ H_2O$ (21254; Boom BV, Meppel, the Netherlands), pH 6) , 10 mM EDTA (100944; Merck Millipore, pH 6.0), 10 mM Cysteine HCL (C7880; Sigma-Aldrich), and 10 mM papain (P3125; Sigma-Aldrich). DNA content was measured according to the manufacturer's instructions (Q32851; Invitrogen) and dimethylmethylene blue (DMMB) assay was performed to measure glycosaminoglycan (GAG) levels.[25] Prostaglandin E_2 (PGE_2) levels were determined in culture medium by ELISA (514010; Cayman Chemical) following the manufacturer's instructions and subsequently corrected for explant dry weight. Cumulative GAG and PGE_2 release were calculated over the 21 day culture period.

Histology

Cartilage and synovial explants were fixed in neutral buffered formalin (NBF 4%, 4286, Klinipath, Duiven, the Netherlands) for at least 24 hours. The samples were embedded in paraffin, and 5 μm sections were stained with Safranin O/Fast Green staining on cartilage and hematoxylin and eosin (H&E) staining on synovial tissue. Sections were blindly scored for OA severity according to the protocol of the Osteoarthritis Research Society

International (OARSI) scoring for the assessment for OA for the dog (K.H., A.M.-B.).[3] In this protocol, the severity of cartilage, chondrocyte, and proteoglycan pathology were assessed. H&E stained sections were scored for the severity of synovitis in a blinded fashion by a scheme proposed by Krenn et al. (K.H., A.R.T.).[26] Immunohistochemistry for collagen type I and II (cartilage quality), β-catenin (canonical Wnt pathway),[27] and cyclooxygenase 2 (COX-2) (inflammation marker)[28] was performed on explants collected on day 0 (Supplementary File 2). Isotype controls and normal mouse IgG1 (3877; Santa Cruz Biotechnology, Heidelberg, Germany) showed no specific staining. To quantify COX-2 and β-catenin immunopositivity, the percentage of positive cells over total amount of cells was calculated in ImageJ. Cytoplasmic or nuclear staining for β-catenin were scored separately.

Canonical Wnt Signaling Activity at the Cellular Level

In order to determine canonical Wnt signaling activity at the cellular level, chondrocytes were isolated from healthy CD and NCD cartilage tissue, and the level of Wnt activity was measured by using a TCF-reporter assay (Supplementary File 3).

OA and Synovitis Severity of Canine Joints With Experimentally Induced OA

In order to explore differences in OA susceptibility between CD and NCD dogs, articular cartilage sections and synovial tissues were analyzed from young-adult dogs that were employed in previous experiments where OA was induced and followed up in a standardized manner (Table 2). These studies were approved by the Utrecht University Animal Ethics Committee, approval numbers 01065, 02070707, and 99029. OA had been induced by applying standardized grooves on the lateral and medial femoral condyles with a 1.5-mm diameter Kirschner-wire [30,31] ($n = 19$ Beagles; $n = 12$ Labrador Retrievers).[29] While Labrador Retrievers are predisposed to the development of secondary OA to elbow and hip dysplasia,[32] the femorotibial joint employed in the present study has not been reported to relate to hereditary dysplasia. Cartilage was collected 10 weeks after OA induction, as described previously.[33] Cartilage sections ($n = 2$ per region) collected from the medial and lateral tibial plateaus of healthy contralateral and OA joints were assessed histologically. Sections were assessed blinded for OA and synovitis (AT) [24,29,30] and average values calculated for each joint.[3] Furthermore, synovitis severity was scored in the synovial tissue collected from three locations in the joint (medial, middle, and lateral infrapatellar).[26,33] For biochemical analysis, the cartilage samples were assayed, as described previously.[34] Total cartilage GAG content (μg/mg wet weight), GAG synthesis, GAG release and retention of newly formed GAGs were determined and averaged for tibial plateaus for eight explants per donor per parameter.[35] For statistical meta-analysis, the dog individual data was employed.

Statistical Analysis

Data were statistically analyzed using R Studio v3.3.1. A normality check was performed using a bootstrapped Shapiro Wilks test. Data that were not normally distributed were subjected to the Kruskal Wallis and post-hoc Mann–Whitney *U* test. Normally distributed data were subjected to the analysis of variance and post-hoc tests (Benjamini and Hochberg) for multiple comparisons. Since multiple factors (donor, breed type, age, body weight, sex, culture condition, experiment, and day of culturing) could influence the outcome of the present results, a multivariate regression model, the COX proportional hazard model, was used, when necessary. For this purpose, donor, age, weight, sex, experiment, and day of culturing as random effects, and culture condition and breed as fixed effects, were tested for the *goodness of fit* where any of these factors appeared. Then, the model that retrieved the lowest Akaike information criterion (AIC) values, assessed by a stepwise regression method (MASS R package), was chosen for the analysis of the corresponding data set. The statistical model used for each comparison is listed in Supplementary File 4. The effect size (ES) and ESs confidence intervals (CI set at 95%) were also taken into consideration to evaluate the significances. Effect sizes were retrieved as *Hedge's g* for parametric data (medium, 0.5–0.8; large, 0.8–1.2; very large, 1.2–2; and huge, >2[36]) and, for non-parametric data, Cliff's delta was assessed ($0.28 < ES < 0.43$, medium; $0.43 \leq ES < 0.7$, large; $ES \geq 0.7$, very large [37]). Differences were considered significant when $p < 0.05$, or when $0.05 < p\text{-value} < 0.1$ and ES was medium or larger.

RESULTS

Native Cartilage From CD Dogs Differed From NCD Cartilage

The *FGF4* retrogene insertion on CFA12 was present in samples of all CD dogs, but not in NCD dogs. The *FGF4* retrogene insertion on CFA18 was not present in any of the used donors (Supplementary File 1). CD donors were older and weighed less than their NCD counterparts ($p < 0.001$). Cartilage explants retrieved from healthy femorotibial joints of NCD dogs showed a significantly higher OARSI score (Fig. 2A; $p < 0.001$) than CD dogs (day 0). Protein expression of COX-2 was higher in NCD versus CD cartilage at the same initial day (Fig. 2C; $p < 0.001$), in line with histological changes, but no differences were found in synovitis score and COX-2 immunopositivity in the synovium (Fig. 2B and D; $p > 0.15$). Total DNA was higher in

Table 2. Overview of canine donors used for retrospective analysis of joint histology in experimental OA (i.e., groove model)

No. of joints	Period (weeks)	Breed	Age (years)	Body weight (kg)	Sex	Reference
12 healthy, 6 OA	10	Labrador retriever (NCD)	2.5 ± 1.2	21–26	Female	Frost-Christensen et al.[29]
9 healthy, 4 OA	10	Beagle (CD)	1.5 ± 3	10–15	Female	Sniekers et al.[30]
10 healthy, 5 OA	10	Beagle (CD)	2.4 ± 0.3	10–15	Female	Mastbergen et al.[24]

CD, chondrodystrophic; NCD, non-chondrodystrophic; OA, osteoarthritis.

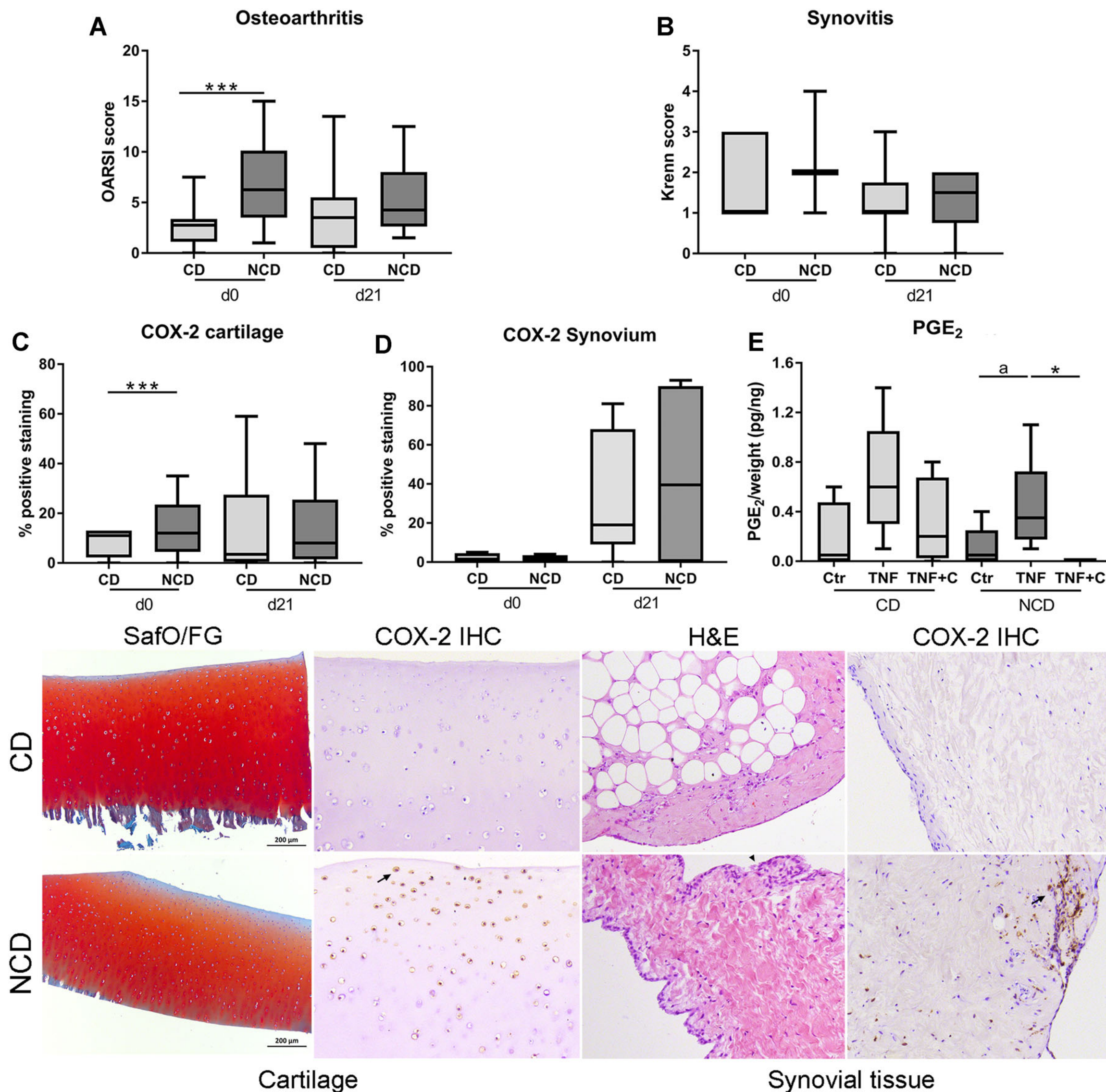

Figure 2. Histological scoring and cyclooxygenase 2 (COX-2) immunohistochemistry of cartilage and synovium explants obtained from healthy experimental non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs. Top: the OARSI score of native explants was higher in NCD versus CD cartilage at day 0 (A), while no differences were found with regard to synovial inflammation (B). NCD cartilage contained significantly more COX-2 expressing cells than CD cartilage explants at day 0 (C). Although the overall COX-2 expression increased during the 21-day culture period, no differences were found between CD and NCD synovial tissue (D). Total prostaglandin E_2 (PGE_2) levels only increased in NCD explants in the presence of a pro-inflammatory stimulus (TNF-α) and were significantly suppressed by celecoxib (E). Data depicted as boxplots with mean and 5–95 percentile. $n = 11$, NCD; $n = 10$, CD. $n = 2$ per condition per donor. $^{*}p < 0.05$; $^{***}p < 0.001$; a, small effect size. Bottom: Representative examples of Safranin O/Fast Green stained cartilage, hematoxylin and eosin stained synovial tissue, and cartilage and synovial sections stained for COX-2. All samples were obtained at day 0 representing the state of native tissue. Arrows indicate immunopositive cells. [Color figure can be viewed at wileyonlinelibrary.com]

NCD versus CD cartilage (Fig. 3A; $p = 0.028$), suggesting higher cellularity. GAG/DNA and GAG/dry weight did not differ at day 0 (Fig. 3C and D; $p > 0.15$). In both CD and NCD cartilage, collagen I protein expression was very low to absent, but abundant immunopositivity for collagen II was noted (Supplementary File 2).

Cartilage Explants of CD Dogs Were Better Capable of GAG Retention During the 21-Day Culture Period

The DNA content remained significantly higher in NCD versus CD cartilage after 21 days of culturing (Fig. 3A; $p = 0.007$). During the entire culture period, GAG release was higher in NCD versus CD cartilage (Fig. 3B; $p < 0.001$). This led to a borderline significant lower GAG/dry weight and GAG/DNA in NCD cartilage at day 21 (Fig. 3C,D; $p = 0.065$, small ES; $p = 0.007$, respectively). PGE_2 production tended to increase with TNF-α stimulation only in NCD cartilage (Fig. 2E; $p = 0.068$, small ES), and was only effectively suppressed in NCD cartilage by the celecoxib ($p = 0.032$). Although COX-2 protein expression in the synovial tissue was increased in both CD and NCD cartilage after the 21-day culture period compared with day 0 ($p < 0.05$), OARSI score, synovitis score and COX-2 expression in cartilage explants did not differ between CD and NCD tissue (Fig. 2A and D). On the

same lines, gene expression levels of matrix genes *ACAN*, *COL1A1*, *COL2A1*, ADAMTS5, and *MMP13* were not significantly different between CD and NCD cartilage after 1 week of culture (Supplementary File 1). Gene expression of *FZD1*, *IL1β*, *IL6*, *LRP5*, *PTGES1*, and *WIF1* was undetectable.

Canonical Wnt Signaling Did Not Differ in Healthy Cartilage Explants of CD and NCD Dogs

No differences were found in gene expression of downstream targets *AXIN2* and *CCND1* messenger RNA (mRNA) expression (Fig. 4A and B; $p = 0.17$, $p = 0.16$, respectively), although mRNA expression of the Wnt antagonist *DDK3* was increased ($p = 0.01$; Fig. 4C) in NCD versus CD cartilage. There were no differences in β-catenin immunopositivity in the nuclear and/ or cytoplasmic regions (Fig. 4D, $p > 0.15$). Considering complex canonical Wnt signaling regulation, a TCF-reporter assay was performed to explore Wnt signaling in CD and NCD chondrocytes cultured either in basic conditions alone or in the presence of a pro-inflammatory OA-like stimulus. There was a slightly higher canonical Wnt signaling in CD versus NCD articular cartilage cells in chondrogenic medium only in the presence of TNF-α (Fig. 4E; $p = 0.09$, medium ES). There also was a higher canonical Wnt activity in chondrocytes retrieved from healthy female CD versus NCD donors (Supplementary File 3).

Before and After experimental OA Induction, Joints of NCD Dogs Showed More Severe Histological Cartilage Degeneration and Synovitis

Macroscopically, there was evidence of cartilage degeneration in the experimental but not the control joints, as reported previously.[24,29,30] OA severity, as indicated by the OARSI score, was significantly higher in NCD versus CD control joints (Fig. 5A; $p = 0.007$) and was also significantly higher in femorotibial joints from NCD versus CD dogs 10 weeks after OA induction (Fig. 5A; $p < 0.0001$), which was in line with the explant study with cartilage from healthy joints. In both CD and NCD dogs, OARSI scores were significantly higher in OA joints compared with healthy control joints ($p = 0.02$; $p < 0.001$ respectively), as expected.

Synovial inflammation increased as well after OA induction in both CD and NCD dogs (Fig. 5B; $p < 0.001$, $p = 0.008$ respectively). Synovial inflammation was also higher in NCD vs CD joints with induced OA (Fig. 5B; $p = 0.11$, large ES, respectively).

A meta-analysis was conducted on GAG (glycosaminoglycan) content and synthesis of freshly collected cartilage explants from these experimental studies. The total GAG content (Fig. 5C) was higher in healthy and OA cartilage of CD versus NCD donors ($p < 0.0001$, $p = 0.1$, large ES, respectively) and declined only in NCD dogs after OA induction ($p = 0.002$). The rate of GAG synthesis (Fig. 5D) in healthy cartilage was not different, although it was higher in CD versus NCD OA cartilage ($p = 0.018$). The rate of GAG synthesis significantly increased in CD

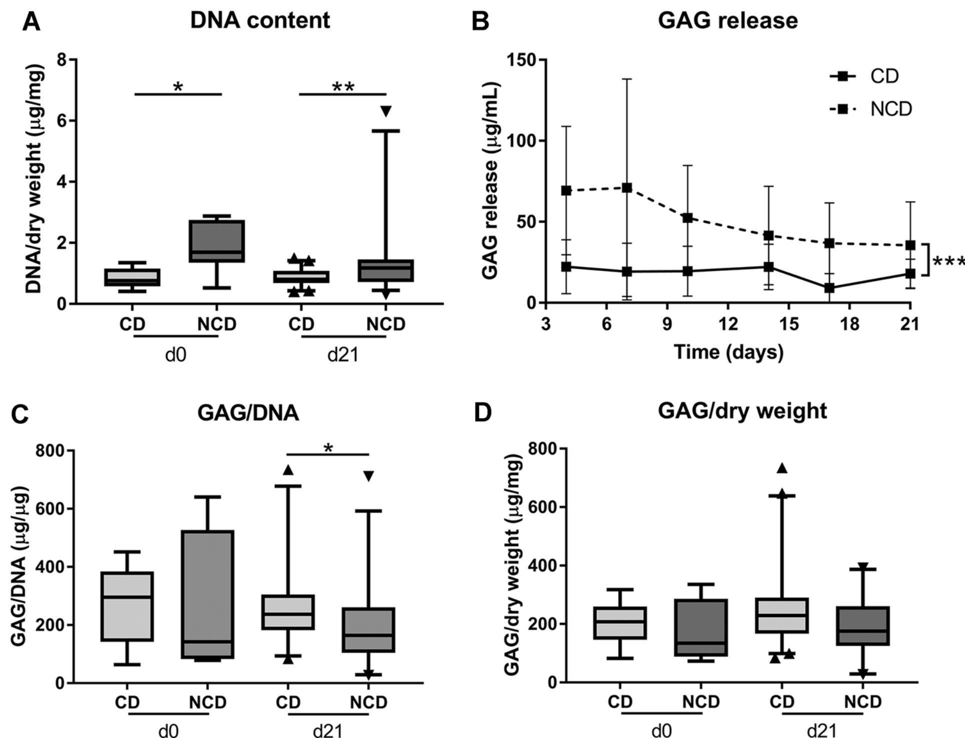

Figure 3. Biochemical analysis of cartilage explants derived from non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs. The DNA content (A) of NCD cartilage was higher at day 0 and 21 of culturing. The glycosaminoglycan (GAG) release over the total culture period was higher in NCD cartilage (B). Although GAG/DNA content was lower in NCD versus CD cartilage after the 21 day culture period (C), there was no difference in GAG/dry weight at day 0 and 21 (D). Data depicted as boxplots with mean and 5–95 percentile. $n = 11$, NCD; $n = 10$, CD. $n = 2$ per condition per donor. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

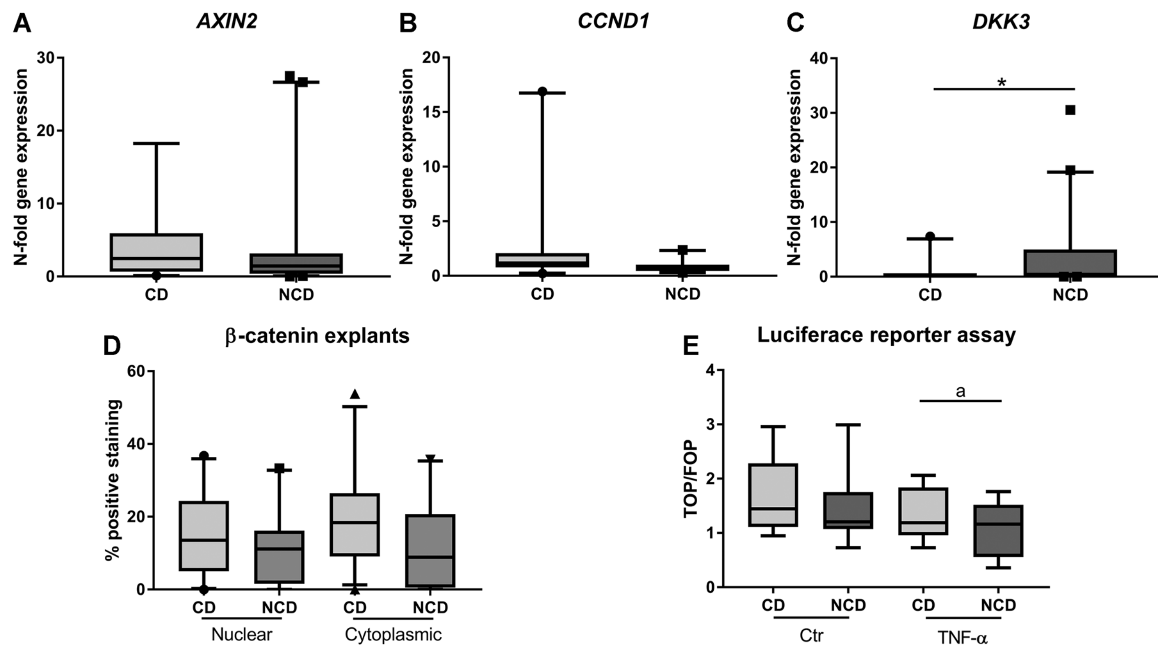

Figure 4. Analysis of the canonical Wnt signaling in cartilage of non-chondrodystrophic (NCD) and chondrodystrophic (CD) dogs. No differences were found in *AXIN2* and cyclin-D1 (*CCND1*) messenger RNA (mRNA) expression (A and B), while dikkopf-3 (*DKK3*) mRNA expression was higher in NCD cartilage (C). B-catenin immunopositivity did not differ between NCD and CD donors (D). $n = 11$, NCD; $n = 10$, CD. $n = 2$ per condition per donor. Wnt activity measured by the TCF-reporter assay was slightly lower in NCD cartilage explants stimulated with tumor necrosis factor-α (TNF-α) (E). Data depicted as boxplots with mean and 5–95 percentile. $n = 8$, CD; $n = 8$, NCD. $n = 2$ per condition per donor. $n = 2$ for each control condition. $^{*}p < 0.05$; a, medium ES.

OA cartilage compared with healthy cartilage ($p = 0.002$). The release of newly formed GAGs (Fig. 5E), as a measure of retention or degradation of newly formed GAGs in the cartilage, significantly increased after OA induction in both CD and NCD cartilage compared with healthy controls ($p = 0.002$, $p < 0.001$ respectively). The total GAG release (Fig. 5F) also increased after OA induction in both dog breeds ($p < 0.03$, $p = 0.1$ with large ES) but did not differ between them ($p > 0.1$).

DISCUSSION

To the authors' knowledge, this is the first study reporting on intrinsic differences between cartilage and synovium of specific dog breeds often employed in experimental OA models, that is, with (Beagles, CD dogs) and without chondrodysplasia (purpose-bred Hounds and Labrador Retrievers, NCD dogs) based on genetic background leading to aberrant FGFR3 activation. Humans suffering from chondrodysplasia due to increased FGFR3 signaling often present with orthopedic conditions, caused by bone and joint deformities, joint laxity, brittle bones, and limb length inequality.[38] These abnormalities predispose patients to degenerative joint diseases and spinal conditions, but they surprisingly exhibit a lower incidence of OA.[39] In chondrodystrophic dogs, these bony deformities are seen as well,[6] and are also accompanied by lower conventional OA rates.[40] These clinical observations match with the experimental results in the current study. Histological and biochemical analysis of both untreated joints of young-adult dogs, and after standardized induction of OA, revealed more severe OA changes and synovitis in NCD cartilage than in CD cartilage.

These intrinsic differences are even present in healthy young-adult dogs. CD-derived cartilage contained less DNA than NCD-derived cartilage, which is in line with the observation that gain-of-function of FGFR3 caused decreased proliferation of chondrocytes.[9,10] The total GAG release into the culture medium from healthy CD cartilage was lower. This could indicate higher degradation or a higher capability of CD dogs in retaining GAGs inside the cartilage,[41] but could also reflect a lower total GAG production because of activated FGFR3 signaling.[9,10] Indeed, after 21 days of culturing, total GAG production (content + release) was lower in CD cartilage explants. However, focussing on GAG content alone, it is higher in CD dogs, which could be one of the protective mechanisms against OA.[42] Similar results were found in the meta-analysis of the GAG incorporation assay of cartilage explants collected from experiments on models of induced OA. While there was no significant difference in GAG synthesis in healthy cartilage, cartilage of CD donors contained more GAGs compared with NCD donors, regardless of OA status. Altogether, these observations indicate a differential cartilage physiology between CD and NCD dogs, pointing toward protective effects of carrying a *FGFR3* polymorphism at CFA12 (chondrodystrophy). Other differences in the genetic background of the studied breeds besides the *FGF4* retrogene insertion may confer differences in cartilage (patho)physiology. This remains to be determined with in depth fundamental

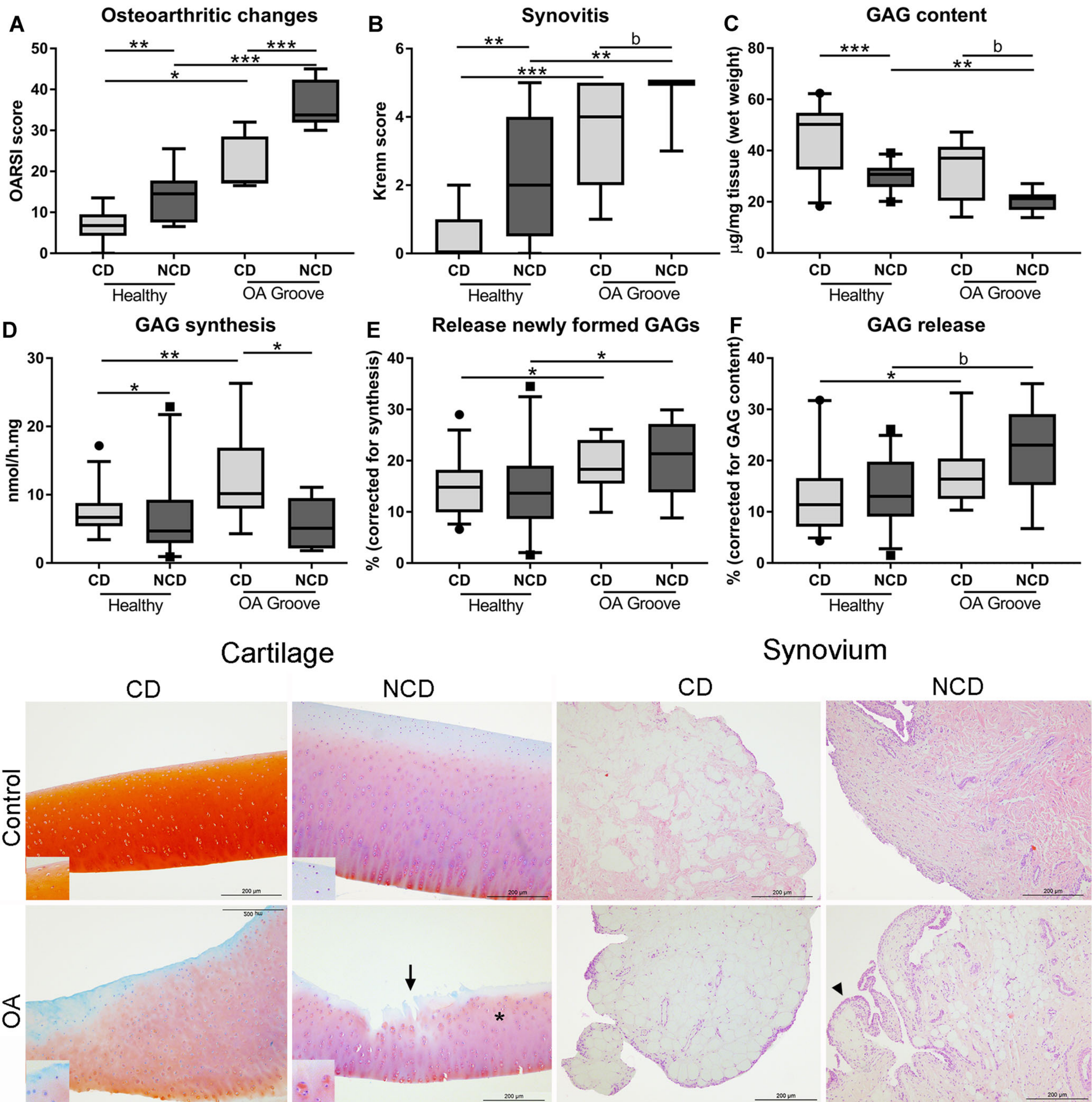

Figure 5. Top: Osteoarthritis Research Society International scores (A) and synovial inflammation (Krenn) scores (B) were significantly higher in both healthy and osteoarthritis (OA) cartilage of non-chondrodystrophic (NCD) dogs compared with chondrodystrophic (CD) cartilage. Glycosaminoglycan (GAG) content (C), changes in GAG synthesis rate (D), the percentage release of newly formed GAGs (E), and percentage total GAG release (F). $n = 11$, CD; $n = 6$, NCD. $n = 2$ replicates per region for histology (medial and lateral tibial plateau, data were pooled). $n = 8$ replicates per donor per condition for biochemical analyses. Data depicted as boxplots with mean and 5–95 percentile. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; b, large ES. Bottom: Representative examples of Safranin O/Fast Green (SafO + FG) stained cartilage sections from the medial femoral condyle and hematoxylin and eosin stained infrapatellar synovium sections (H&E) of NCD and CD joints 10 weeks after unilateral OA induction and the healthy contralateral control joint. NCD-derived cartilage shows more severe degenerative changes after OA induction: large cellular clusters (*) and fissures (arrow) are present. Synovial hyperplasia also seems more evident in NCD-derived synovial tissue after OA induction (arrowhead). [Color figure can be viewed at wileyonlinelibrary.com]

studies exploring the role of (epi)genetics of different dog breeds.

It has become increasingly clear that OA is a disease of the whole joint, with interplay between the cartilage, synovial lining and subchondral bone. The synovial tissue and cartilage can influence each other and the development and progression of OA, possible resulting in more severe OA in the case of NCD dogs. In line with this hypothesis, the present study also indicated a differential susceptibility for joint inflammation between CD and NCD donors: synovitis scores tended to be higher in synovial tissue from healthy NCD versus CD

joints in vivo and this aggravated further after OA induction. In agreement with this observation, in vitro, NCD-derived cartilage explants showed increased COX-2 immunopositivity and higher PGE_2 levels in the presence of a pro-inflammatory stimulus than CD cartilage, the main drivers of joint inflammation and degeneration.[43] It is widely known that synovial inflammation is low-grade in OA but does play an important role.[44] Synoviocytes become activated after an initial insult to the joint, after which synovial tissue drives the progression of cartilage loss and development of clinical signs, by producing inflammatory mediators, such as PGE_2 and degrading enzymes.[1] Synovial inflammation also affects in vitro cartilage metabolism by reducing GAG synthesis.[45] The tendency of NCD cartilage to show an aggravated response to degenerative or inflammatory stimuli, might predispose it to early OA.

Based on the above, we explored whether there were differences in Wnt signaling, related to the underlying possible differences in FGFR3 signaling.[15] In the current study, no differences were found in Wnt/β-catenin signaling based on IHC for β-catenin, qPCR for Wnt associated targets and Wnt activation on the cartilage level, although NCD cartilage expressed higher *DKK3* mRNA levels in culture. Several studies reported an upregulation of *DKK3* and other DKKs in OA cartilage, and its upregulation has been associated with both OA progression and chondroprotection.[46–48] Members of the *DKK* gene family demonstrated both inhibitory and potentiating actions on the Wnt signaling pathway, indicating a tissue-dependent effect.[18] Not much is known on how *DKK3* impacts Wnt signaling in cartilage, but given our findings, it could be inhibitory. It would therefore be tempting to speculate that upregulation of *DKK3* in NCD cartilage reflects early osteoarthritic changes and might be an attempt to avert further progression of degeneration. The actual involvement of the Wnt signaling pathway would need further validation at the protein and signaling level.

There are a few limitations to this study. No power analysis was performed prior to the in vitro studies, to predetermine the sample size for correct power. The experiments described in this manuscript were performed with tissues collected from dogs that were euthanized in unrelated experiments, for obvious ethical reasons. This limited the choice in donors and as such introduced a selection bias. Mostly female donors were used for both the in vitro experiments and the analysis of joint pathology in experimentally induced OA. Gene expression and biochemical analyses did not differ between female and male donors, but there was a higher canonical Wnt activity measured in chondrocytes retrieved from healthy female CD donors. It is known that females have a higher incidence of OA, although old age, obesity, and physical activity are suggested to influence this difference.[1] Whether gender affects Wnt activity, is still to be determined. Moreover, due to the setup of the study, the median age of CD donors used for characterization of joint tissues in healthy animals, was twice as high as NCD donors. Even though the CD dogs were older, their cartilage displayed less degenerative changes compatible with early OA than NCD donors, further supporting our findings. It is plausible that body weight, which is lower in CD animals, could limit OA changes by decreased biomechanical loading. However, in the human patient, body size does not correlate with OA severity.[49] Body mass index does have a major influence on OA development.[50] In this context, the dogs used in the studies presented here all had similar (optimal) body condition scores.

To conclude, the results in this study suggest a systematic physio-pathological difference in both healthy and OA cartilage between dog breeds, presumably related to the *FGF4* retrogene associated with chondrodystrophy. NCD-derived cartilage seems to be more sensitive to proinflammatory stimuli than cartilage from CD dogs, possibly predisposing NCD dogs to the development of OA. These differences should be considered when considering an in vitro or in vivo canine model to study OA, in order to avoid confounding effects. Therefore, differences in levels of factors governing cartilage homeostasis, and possibly other relevant signaling pathways influenced by the genetic background of dog breeds, have implications for the choice of dog type to investigate OA for both veterinary and human OA patients. Further research is needed to confirm these findings.

AUTHORS' CONTRIBUTION

All authors provided substantial contributions to conception and design, or analysis and interpretation of data and drafting the article or revising it critically for important intellectual content and all gave final approval of the version to be published.

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REFERENCES

1. Glyn-Jones S, Palmer AJ, Agricola R, et al. 2015. Osteoarthritis. Lancet 386:376–387.
2. Bendele AM. 2001. Animal models of osteoarthritis. J Musculoskelet Neuronal Interact 1:363–376.
3. Cook JL, Kuroki K, Visco D, et al. 2010. The OARSI histopathology initiative—recommendations for histological assessments of osteoarthritis in the dog. Osteoarthritis Cartilage 18(Suppl 3):S66–S79.
4. Johnston SA. 1997. Osteoarthritis. joint anatomy, physiology, and pathobiology. Vet Clin North Am Small Anim Pract 27:699–723.
5. King MD. 2017. Etiopathogenesis of canine hip dysplasia, prevalence, and genetics. Vet Clin North Am Small Anim Pract 47:753–767.
6. Martinez S, Fajardo R, Valdes J, et al. 2007. Histopathologic study of long-bone growth plates confirms the basset hound as an osteochondrodysplastic breed. Can J Vet Res 71:66–69.
7. Brown EA, Dickinson PJ, Mansour T, et al. 2017. FGF4 retrogene on CFA12 is responsible for chondrodystrophy and intervertebral disc disease in dogs. PNAS 114:11476–11481.

8. Parker HG, VonHoldt BM, Quignon P, et al. 2009. An expressed fgf4 retrogene is associated with breed-defining chondrodysplasia in domestic dogs. Science 325:995–998.
9. Ornitz DM, Marie PJ. 2015. Fibroblast growth factor signaling in skeletal development and disease. Genes Dev 29: 1463–1486.
10. Teven CM, Farina EM, Rivas J, et al. 2014. Fibroblast growth factor (FGF) signaling in development and skeletal diseases. Genes Dis 1:199–213.
11. Eswarakumar VP, Schlessinger J. 2007. Skeletal overgrowth is mediated by deficiency in a specific isoform of fibroblast growth factor receptor 3. Proc Natl Acad Sci U S A 104: 3937–3942.
12. Tang J, Su N, Zhou S, et al. 2016. Fibroblast growth factor receptor 3 inhibits osteoarthritis progression in the knee joints of adult mice. Arthritis Rheumatol 68:2432–2443.
13. Vincent TL. 2012. Explaining the fibroblast growth factor paradox in osteoarthritis: Lessons from conditional knockout mice. Arthritis Rheum 64:3835–3838.
14. Zhou S, Xie Y, Li W, et al. 2016. Conditional deletion of Fgfr3 in chondrocytes leads to osteoarthritis-like defects in temporomandibular joint of adult mice. Sci Rep 6:24039.
15. Buchtova M, Oralova V, Aklian A, et al. 2015. Fibroblast growth factor and canonical WNT/beta-catenin signaling cooperate in suppression of chondrocyte differentiation in experimental models of FGFR signaling in cartilage. Biochim Biophys Acta 1852:839–850.
16. Nakamura Y, Nawata M, Wakitani S. 2005. Expression profiles and functional analyses of wnt-related genes in human joint disorders. Am J Pathol 167:97–105.
17. Blom AB, Brockbank SM, van Lent PL, et al. 2009. Involvement of the wnt signaling pathway in experimental and human osteoarthritis: prominent role of wnt-induced signaling protein 1. Arthritis Rheum 60:501–512.
18. Usami Y, Gunawardena AT, Iwamoto M, et al. 2016. Wnt signaling in cartilage development and diseases: Lessons from animal studies. Lab Invest 96:186–196.
19. Zhu M, Tang D, Wu Q, et al. 2009. Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. J Bone Miner Res 24:12–21.
20. Yasuhara R, Ohta Y, Yuasa T, et al. 2011. Roles of beta-catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells. Lab Invest 91:1739–1752.
21. Loughlin J, Dowling B, Chapman K, et al. 2004. Functional variants within the secreted frizzled-related protein 3 gene are associated with hip osteoarthritis in females. Proc Natl Acad Sci U S A 101:9757–9762.
22. Castano Betancourt MC, Cailotto F, Kerkhof HJ, et al. 2012. Genome-wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. Proc Natl Acad Sci U S A 109:8218–8223.
23. Smolders LA, Bergknut N, Grinwis GC, et al. 2013. Intervertebral disc degeneration in the dog. part 2: Chondrodystrophic and non-chondrodystrophic breeds. Vet J 195:292–299.
24. Mastbergen SC, Marijnissen AC, Vianen ME, et al. 2006. The canine "groove" model of osteoarthritis is more than simply the expression of surgically applied damage. Osteoarthritis Cartilage 14:39–46.
25. Farndale RW, Sayers CA, Barrett AJ. 1982. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 9:247–248.
26. Krenn V, Morawietz L, Burmester GR, et al. 2005. Synovialitis score: histopathological grading system for chronic rheumatic and non-rheumatic synovialitis. Z Rheumatol 64: 334–342.
27. Smolders LA, Meij BP, Riemers FM, et al. 2012. Canonical wnt signaling in the notochordal cell is upregulated in early intervertebral disk degeneration. J Orthop Res 30:950–957.
28. Tellegen AR, Jansen I, Thomas R, et al. 2018. Controlled release of celecoxib inhibits inflammation, bone cysts and osteophyte formation in a preclinical model of osteoarthritis. Drug Delivery 25:1438–1447.
29. Frost-Christensen LN, Mastbergen SC, Vianen ME, et al. 2008. Degeneration, inflammation, regeneration, and pain/disability in dogs following destabilization or articular cartilage grooving of the stifle joint. Osteoarthritis Cartilage 16: 1327–1335.
30. Sniekers YH, Intema F, Lafeber FP, et al. 2008. A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models. BMC Musculoskelet Disord 9:20.
31. Mastbergen SC, Marijnissen AC, Vianen ME, et al. 2006. Inhibition of COX-2 by celecoxib in the canine groove model of osteoarthritis. Rheumatology (Oxford) 45:405–413.
32. Smith GK, Mayhew PD, Kapatkin AS, et al. 2001. Evaluation of risk factors for degenerative joint disease associated with hip dysplasia in German shepherd dogs, golden retrievers, labrador retrievers, and rottweilers. J Am Vet Med Assoc 219: 1719–1724.
33. Marijnissen AC, van Roermund PM, TeKoppele JM, et al. 2002. The canine 'groove' model, compared with the ACLT model of osteoarthritis. Osteoarthritis Cartilage 10:145–155.
34. Lafeber FP, Vander Kraan PM, Huber-Bruning O, et al. 1993. Osteoarthritic human cartilage is more sensitive to transforming growth factor beta than is normal cartilage. Br J Rheumatol 32:281–286.
35. van Valburg AA, van Roermund PM, Marijnissen AC, et al. 2000. Joint distraction in treatment of osteoarthritis (II): Effects on cartilage in a canine model. Osteoarthritis Cartilage 8:1–8.
36. Sawilowsky SS. 2009. New effect size rules of thumb. J Mod Appl Stat Meth 8:597–599.
37. Vargha A, Delaney HD. 2000. A critique and improvement of the CL common language effect size statistics of McGraw and wong. J Educ Behav Stat 25:101–132.
38. Ireland PJ, Pacey V, Zankl A, et al. 2014. Optimal management of complications associated with achondroplasia. Appl Clin Genet 7:117–125.
39. Klag KA, Horton WA. 2016. Advances in treatment of achondroplasia and osteoarthritis. Hum Mol Genet 25:R2–R8.
40. Karbe GT, Biery DN, Gregor TP, et al. 2012. Radiographic hip joint phenotype of the Pembroke welsh corgi. Vet Surg 41:34–41.
41. Fujita Y, Hara Y, Nezu Y, et al. 2006. Proinflammatory cytokine activities, matrix metalloproteinase-3 activity, and sulfated glycosaminoglycan content in synovial fluid of dogs with naturally acquired cranial cruciate ligament rupture. Vet Surg 35:369–376.
42. Fenwick SA, Gregg PJ, Rooney P. 1999. Osteoarthritic cartilage loses its ability to remain avascular. Osteoarthritis Cartilage 7:441–452.
43. Attur M, Al-Mussawir HE, Patel J, et al. 2008. Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: Evidence for signaling via the EP4 receptor. J Immunol 181: 5082–5088.
44. Hugle T, Geurts J. 2017. What drives osteoarthritis?-synovial versus subchondral bone pathology. Rheumatology (Oxford) 56:1461–1471.
45. Beekhuizen M, Bastiaansen-Jenniskens YM, Koevoet W, et al. 2011. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage:

Establishment and characterization of a long-term cartilage-synovium coculture. Arthritis Rheum 63:1918–1927.
46. Funck-Brentano T, Bouaziz W, Marty C, et al. 2014. Dkk-1-mediated inhibition of wnt signaling in bone ameliorates osteoarthritis in mice. Arthritis Rheumatol 66:3028–3039.
47. Honsawek S, Tanavalee A, Yuktanandana P, et al. 2010. Dickkopf-1 (dkk-1) in plasma and synovial fluid is inversely correlated with radiographic severity of knee osteoarthritis patients. BMC Musculoskelet Disord 11.
48. Snelling SJ, Davidson RK, Swingler TE, et al. 2016. Dickkopf-3 is upregulated in osteoarthritis and has a chondroprotective role. Osteoarthritis Cartilage 24:883–891.
49. Calce SE, Kurki HK, Weston DA, et al. 2018. The relationship of age, activity, and body size on osteoarthritis in weight-bearing skeletal regions. Int J Paleopathol 22:45–53.
50. Smith GK, Paster ER, Powers MY, et al. 2006. Lifelong diet restriction and radiographic evidence of osteoarthritis of the hip joint in dogs. J Am Vet Med Assoc 229:690–693.

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