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. 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. 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, accompanied by an increase in Wnt antagonists such as members of the Dickkopf family. Both gain- and loss-of-function of β-catenin in cartilage induced osteoarthritic changes in mice. Genetic studies in humans suggest that the canonical Wnt pathway participates in the pathogenesis of OA in at least a subset of patients. 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.

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. 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. 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.

**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]
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; PA Laboratory, Cologne, 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 (210-1A; 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-Aldrich, Saint Louis, MO), and 1 ng/mL bovine serum albumin (A9418; Sigma-Aldrich). To assess the response of CD and NCD tissue to an anti-inflammatory drug, celecoxib (10⁻⁶M) 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, LRPS5A, 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 H₂NaPO₄·2 H₂O (21254; Boom BV, Meppel, the Netherlands), pH 6.1) 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 dimethylmethylenblue (DMMB) assay was performed to measure glycosaminoglycan (GAG) levels.25 Prostaglandin E₂ (PGE₂) 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 PGE₂ release were calculated over the 21 day culture period.

Histology

Cartilage and synovial explants were fixed in neutral buffered formalin (NBF 4%, 4286, Klinpath, 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.). 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.).

Immunohistochemistry for collagen type I and II (cartilage quality), β-catenin (canonical Wnt pathway),
cyclooxygenase 2 (COX-2) (inflammation marker) 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 (n = 19 Beagles; n = 12 Labrador Retrievers). While Labrador Retrievers are predisposed to the development of secondary OA to elbow and hip dysplasia, 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. 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) and average values calculated for each joint. Furthermore, synovitis severity was scored in the synovial tissue collected from three locations in the joint (medial, middle, and lateral infrapatellar). For biochemical analysis, the cartilage samples were assayed, as described previously. 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.

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) and, for non-parametric data, Cliff’s delta was assessed (0.28 ≤ ES ≤ 0.43, medium; 0.43 ≤ ES ≤ 0.7, large; ES ≥ 0.7, very large). Differences were considered significant when p < 0.05, or when 0.05 ≤ p-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...
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 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 cartilage at day 0 (Table 3A). 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). PGE₂ 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 \textit{ACAN}, \textit{COL1A1}, \textit{COL2A1}, \textit{ADAMTS5}, and \textit{MMP13} were not significantly different between CD and NCD cartilage after 1 week of culture (Supplementary File 1). Gene expression of \textit{FZD1}, \textit{IL1β}, \textit{IL6}, \textit{LRP5}, \textit{PTGES1}, and \textit{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 \textit{AXIN2} and \textit{CCND1} messenger RNA (mRNA) expression (Fig. 4A and B; \(p = 0.17, p = 0.16\), respectively), although mRNA expression of the Wnt antagonist \textit{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.\(^{24,30,39}\) 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; \(p = 0.11, \text{large ES, respectively}\)).

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
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. These abnormalities predispose patients to degenerative joint diseases and spinal conditions, but they surprisingly exhibit a lower incidence of OA. In chondrodystrophic dogs, these bony deformities are seen as well, and are also accompanied by lower conventional OA rates. 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. 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, but could also reflect a lower total GAG production because of activated FGFR3 signaling. 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. 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
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 donors. 

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]
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 PGE2 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 PGE2 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 pro-inflammatory 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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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.