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Short Communication

Dietary Vitamin D Supplementation during Early Growth is not Protective for Medial Coronoid Disease Development in Labradors

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Keywords

- Medial coronoid disease
- Vitamin D supplementation
- Labrador retriever
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- chondrocyte

Abstract

Introduction: Medial coronoid disease (MCD) is a common heritable disease in young large-breed dogs and is characterized by fissures and/or fragmentation of the medial coronoid process (MCP), cartilage, and/or subchondral bone. MCD development has also been attributed to disturbed endochondral ossification. Vitamin D influences skeletal development and endochondral ossification by stimulating the terminal differentiation of chondrocytes and the mineralization of cartilage and the newly formed osteoid. The aim of this study was to investigate whether vitamin D supplementation of the standard puppy diet given to puppies after weaning can prevent the development of medial coronoid disease (MCD) by stimulating endochondral ossification, including terminal differentiation of chondrocytes, and mineralization of the cartilaginous template of the developing medial cornoid process (MCP).

Animals, material and methods: A litter of Labrador puppies was on purpose bred by mating a dam and a sire with MCD; these dogs are known to produce offspring with MCD. The puppies received a diet supplemented with 50,000 IU vitamin D per kg as fed. Development of MCD was monitored by computed tomography (CT) and plain radiographs every two weeks, and post-mortem by microCT, necropsy, histology, and immunohistochemistry and compared with data from a previous study.

Results and discussion: Vitamin D supplementation did result in increased plasma levels of 25- vitamin D and 1.25-vitamin D, but did not prevent development of MCD in growing Labradors. Instead, vitamin D supplementation resulted in increased collagen X staining ofthe MCP and irregular costal growth plates, demonstrating disturbed endochondral ossification.

Conclusion: Vitamin D supplementation does not prevent MCD development. Other causes for MCD rather than relative vitamin D deficiency causing disturbed endochondral ossification are more likely.

INTRODUCTION

Medial coronoid disease (MCD) is a common heritable disease in young large breed dogs, characterized by fissures and/or fragmentation of the medial coronoid process (MCP), cartilage and/or similar lesions in the subchondral bone (Fitzpatrick et al. 2009) [1]. It is regarded as one of the developmental abnormalities responsible for elbow dysplasia, with a high incidence in Labrador retrievers (Van Ryssen and van Bree 1997, Lavrijsen et al. 2012) [2,3] suggesting a genetic predisposition. Ad libitum feeding, overweight, heavy exercise, and heavy playing are considered risk factors for MCD development (Sallander et al. 2006) [4]. The exact cause of MCD is still unknown and is likely to be multifactorial (Danielson et al. 2006) [5]. As overweight and exercise are risk factors for MCD, uneven stress on the elbow is considered to be an important cause of MCD (Davidson

et al.2008) [6]. Uneven stress on the elbow may be caused by incongruity between the radius and ulna due to differences in growth in length of the radius and ulna or by physiological incongruity during loading (Davidson et al. 2008, House et al. 2009) [6,7]. MCD development has also been related to abnormalities of the subchondral bone; micro-cracks have been demonstrated histologically in the axial border of the MCP, which were more severe at the fragmented site of the MCP compared to the rest of the MCP (Danielson et al. 2006) [5]. Furthermore, MCD development has been attributed to disturbed endochondral ossification (Olsson and Reiland1978) [8]. Recently, an enlarged calcifying zone of the developing MCP and retained hyaline cartilage in the subchondral cancellous bone in the MCP region, were demonstrated in MCD-positive compared to MCD-negative Labrador retriever siblings of the same age (Lau et al. 2013b) [9]. In addition to fissures and fragmentation of the MCP, the initial lesions of MCD are characterized by a delayed ossification of the cartilaginous template of the MCP (Lau et al. 2013b) [9]. The enlarged calcifying zone indicates a disturbance of endochondral ossification due to delayed or abnormal terminal differentiation of the chondrocytes, and/or delayed calcification of the matrix, or disruption of the capillary blood flow through the subchondral bone, thus interfering with chondrocyte and osteocyte nutrition, causing cell death (Danielson et al. 2006) [5].

Vitamin D influences skeletal development and the process of endochondral ossification by stimulating terminal differentiation of chondrocytes, and mineralization of cartilage and the newly formed osteoid (Hazewinkel and Tryfonidou 2002) [10]. Dogs are unable to synthesize sufficient amounts of vitamin D in the skin under influence of UVB-light, and hence are solely dependent on dietary vitamin D intake (How et al. 1994) [11]. At birth, dogs have limited body reserves of vitamin D in the liver and thereafter depend solely on the bitch' milk or milk replacer as a source of vitamin D (Corbee et al. 2012) [12]. After weaning puppies depend solely on food and/or supplements for their vitamin D intake. The aim of this study was to investigate if supplementation of the standard puppy diet with vitamin D given to puppies after weaning can prevent the development of MCD. We hypothesized that supplementation of vitamin D would have a protective effect by stimulating endochondral ossification including terminal differentiation of chondrocytes, and mineralization of the cartilaginous template of the developing MCP.

MATERIALS AND METHODS

This study was approved by the Utrecht University Institutional Committee on the Care and Use of Experimental Animals and was in compliance with national legislation on laboratory animal use (DEC 2012.III.07.068).

A litter of Labrador puppies was purposely bred by mating a dam and sire, both affected with MCD and known to produce offspring with MCD. Body weight of the puppies was recorded daily during the first 3 weeks of age, and every other day thereafter. The puppies were partially weaned at 3 weeks of age with a normal weaning diet (Royal Canin Large dog starter; Table 1) supplemented with 50,000IU of cholecalciferol per kg of food on an as fed basis. Total weaning was complete when the puppies were 7 weeks of age.

Blood samples were drawn at 5 weeks of age, and then every 2 weeks until euthanasia at 18 weeks of age, or earlier when MCD was diagnosed with computed tomography (CT). Blood samples were collected by jugular venipuncture in ethylenediaminetetra acetic acid (EDTA)-coated tubes on melting ice, centrifuged by 4^0C at 3000rpm; plasma was stored at -20^0C until further analysis. Plasma concentrations of total calcium (Ca), phosphate (P), total protein, and albumin were determined by the use of a Beckman

Table 1: Chemical analysis of the food. Chemical analysis of the food for the control group (Control) and the vitamin D supplemented group (vitamin D+).

	per 100g as fed	per 100g dry matter	per 100kcal ME
Crude protein (g)	30	32,6	6,8
Crude fat (g)	22	23,9	5
NFE (g)	31,3	34	7,1
Crude fibre (g)	6,4	7	1,4
Moisture (g)	8		
Calcium (g)	1,2	1,3	0,27
Phosphorus (g)	0,95	1,03	0,21
Sodium (g)	0,4	0,43	0,09
Chloride (g)	0,58	0,63	0,1
Potassium (g)	0,7	0,76	0,16
Magnesium (g)	0,07	0,08	0,016
EPA+DHA (g)	0,3	0,33	0,07
Vitamin A (IU)	2100	2283	474
Vitamin D (IU)	120	130	27,1
Vitamin E (mg)	60	65	13,5
Metabolizable Energy			
kcal/100g	443	482	
kJ/100g	1854	2016	
The vitamin D supplemented group received a supplement containing 5000 IU of vitamin D per mL.			
Per 100g of food, 1 mL of the supplement was added.			

system. The plasma concentrations of 25-hydroxyvitamin D (25-vitamin D) were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) after hexane extraction of 0.20 ml plasma by subsequent additions of 0.25 ml demineralized water, 0.50ml ethanol, 150 pmol of D6-25-vitamin D as internal standard, and 4 ml hexane containing 0.002% butylated hydroxytoluene as an anti-oxidant. Thereafter, samples were vortexed vigorously 3 times for 5 seconds. After centrifugation (5 min, 1000g) the upper phase was transferred to a new glass tube, hexane was evaporated under nitrogen gas and stored at -20°C. HPLC separation was performed as described by Testerink et al [13]. In short, the lipid fraction was reconstituted in 0.1 ml methanol/chloroform (1/1, v/v) and 10 µl was injected on a Lichrospher RP18-e column (5 µm, 250 x 4.6 mm; Merck, Darmstadt, Germany). A gradient was generated from acetonitrile : water 95/5 to acetone/chloroform 85/15, v/v, at a constant flow rate of 1 ml/min. Total run time per sample was 13 min. Mass spectrometry of lipids was performed using Atmospheric Pressure Chemical Ionization (APCI) on a Biosystems API-4000 Q-trap (MDS Sciex, Concord, ON, Canada). The system was controlled by Analyst version 1.4.2 software (MDS Sciex, Concord, ON, Canada) and operated in positive ion mode and in the multiple reaction monitoring (MRM) mode using the following settings: source temperature 420°C, nebulizer gas (GS1) 5, nebulizer current 3 µA, curtain gas 10, collision gas high and declustering potential and collision energy were empirically optimized for each compound. The MRM transitions (m/z) used was: 383.3 → 365.3 for 25-vitamin D, and 389.3 → 371.3 for D6-25-vitamin D. Quantification of 25-vitamin D was done relative to the internal standard. The exact concentration of the internal standard was determined before each experiment by spectrophotometry (D6-25-vitamin D at 264 nm using a molar absorption coefficient (M-1.cm-1) of 19400. The recovery was approximately 80% for the internal standard, and the intra-assay variation was 8.5% for 25-vitamin D. Plasma concentrations of 1,25-dihydroxyvitamin D (1,25-vitamin D) were determined after immuno-extraction in a competitive radioimmunoassay (RIA) (IDS AA-54F1, EliTech Systems Pvt Ltd., Gujarad, India) according to the manufacturer's instructions. Samples were diluted 6x with assay buffer. Limit of detection was 20pmol/L, and the inter-assay variation was 10% at 95pmol/L.

CT and plain radiographs of the elbow joints were performed under general anesthesia every 2 weeks from the age of 5 weeks (n=3) or 6 weeks (n=2) onward to monitor the development of the bony structures of the secondary ossification centers (Appendix A, Voorhout and Hazewinkel 1987) [14] and of the MCP of the elbow joints. CT was performed by the technique described previously (Lau et al. 2013a) [15] with a third-generation single-slice helical CT scanner (Philips Secura, Philips, Eindhoven,the Netherlands) using 120 kV and 120 mA with an exposure time of 1000ms. Radiographs of the elbow joints were made with a digital radiography system (Philips digital Rad TH, Philips, Eindhoven, the Netherlands) using 50 kV and 8 mA. Puppies that developed MCD as revealed on CT images of one or both elbow joints were euthanized with an IV overdose of barbiturate immediately after diagnosis. The remaining puppies were euthanized at 18 weeks of age, which was defined as the study endpoint, as most MCD-positive puppies in similar studies developed MCD at <18 weeks of age (Lau et al. 2013a, b) [9,15]. Immediately after euthanasia, the elbow joints and the growth plates of both 9th ribs were collected, examined macroscopically, and fixed in 4% neutral buffered formaldehyde.

Post mortal micro-CT was performed to visualize the MCP, and to identify the exact location of fissures or fragments of the MCP when present, by the technique described previously (Lau et al. 2013) [9]. In brief:the ulnas were scanned in a micro CT system (Scanco 80, Scanco Medical, Switzerland) from 2 cm proximal to the MCP to 2 cm distal to the MCP, using 55 kV, 145 mA, an exposure time of 1 s, and a pixel size of 36.9 µm.

After micro-CT, the MCP and costal growth plates were decalcified in 10% EDTA for 6 weeks, before being dissected and embedded in paraffin. Histological evaluation was performed on Haematoxylin and Eosin (HE), and Safranin O stained tissue sections. Immunohistochemistry was performed for collagen type Xas described previously (Lau et al. 2013b) [9].Collagen type X is a short chain collagen produced specifically by hypertrophic chondrocytes during endochondral ossification. Histomorphometry was performed with the aid of the Image J software (v1.47b Colour Convolution, NIH); the surface area of the four zones of the developing joint cartilage, i.e. tangential, transitional, radial, and calcifying zone, and the relative surface area positive for type X collagen were determined as described previously (Lau et al. 2013b) [9]. The mean width and standard deviation of each costal growth plate were determined by measurement at 12 different pre-set locations. The percentage of the standard deviation of the mean of the growth plate width (% sd) illustrated the irregularity of the width of the growth plate (Tryfonidou et al. 2002) [16]. The ratio between the width of the hypertrophic zone and proliferation zone of the costal growth plate was determined to demonstrate differences in stage of endochondral ossification.

The effects of vitamin D supplementation on skeletal development were compared to controls. As controls served a subset of data reported before (Lau et al. 2013b) [9] originating from MCD-positive and MCD-negative puppies of two different litters of the same parent animals that were raised on the same housing conditions and on the same standardized diet (containing 271 IU vitamin D per 1000kcal metabolizable energy (ME)) as the research animals described in this study. Statistical analyses were performed with the use of R-statistics (R i386 3.0.1). All data were tested for normal distribution by a Kolmogorov-Smirnov test. Data that were normally distributed are presented as mean±sd. Data that were not normally distributed are presented as median and range. A multivariate analysis was performed to correct for possible interactions between time points, sex, and diet group. A Fisher test was performed to correct for multiple comparisons; a p-value of <0.05 was set as the level of significance.

RESULTS

The control group consisted of 14 puppies, 9 males and 5 females, which were born from two litters, 7/14 dogs developed MCD, 5 males and 2 females. The youngest MCD-positive dog in the control group was 15 weeks old at the time of diagnosis based on CT. In a third litter, 5 puppies were born, 2 males and 3 females, which were raised on the vitamin D supplemented

food. The first appearance of MCD in the vitamin D supplemented group was detected by CT in a male puppy at 16 weeks of age, and the second puppy (female) of that group at 18 weeks of age being at corresponding ages as the controls (Table 2). The other 3 puppies (1 male, 2 females) of the vitamin D supplemented group did not demonstrate MCD on CT in the first 18 weeks of life. The puppies in the control group and the vitamin D supplemented group did not differ in their growth curves and the puppies were not lame during the study period. Vitamin D intake was 57 IU per $kg^{0.75}$ in the control group and 2427IU per $kg^{0.75}$ in the vitamin D supplemented group, respectively (Table 1).

Plasma Ca, P, total protein, and albumin levels did not differ in the vitamin D supplemented group during the study period from the control group (i.e. total Ca 2.85±0.06 mmol/L, P 3.15±0.18 mmol/L, total protein 60±4 g/L, albumin 31±2 g/L). The plasma levels of25-vitamin D and 1,25-vitamin D were comparable at the start of the study between both groups. In the vitamin D supplemented group, 25-vitamin D and 1,25-vitamin D plasma levels were approximately twice as high compared to the control group (Figure 1).

The development of the secondary ossification centers was radiographically evaluated till 18 weeks of age; it was similar between the control and vitamin D supplemented group, and did not differ between MCD-positive and MCD-negative puppies (Appendix A).

At necropsy, both MCD-positive dogs at 16 weeks of age of the vitamin D supplemented group demonstrated an incomplete fissure in the articular cartilage of the right elbow, while no fissure was visible in the articular cartilage of the left elbow. There was also mild incongruence in the right elbow, caused by a shorter radius. The MCD-positive dog at 18 weeks of age of the vitamin D supplemented group demonstrated an incomplete fissure in the articular cartilage of the left elbow, while no fissure was visible on the articular cartilage of the right elbow. These findings were in correspondence with the MCD-positive dogs of the control group. All MCD-negative dogs in both groups demonstrated normal elbow joints at necropsy (Table 2).

Within the vitamin D supplemented group, micro-CT revealed a bilateral fragmentation of the MCP of the16-weeks old dog, and a bilateral fissure of the MCP of the18-weeks old dog (Figure 2). The remaining dogs in the vitamin D supplemented group did not reveal any abnormalities at the study endpoint (i.e. 18 weeks of age). Representative examples of micro-CT imaging, histology, and immunohistochemistry of the MCD-positive and MCD-negative dogs of the vitamin D supplemented and the control group are demonstrated in Figure 2. The calcifying zone of the joint cartilage at the side of the MCP in MCD-positive dogs was significantly thicker compared to MCD-negative dogs in both the vitamin D supplemented and the control group (Figure 2, Table 3). In both MCD-positive dogs of the vitamin D supplemented group, the fissure was located at the junction of the cartilage and the subchondral bone (Figure 2), with cell death of chondrocytes in the calcifying zone of the cartilage (Figure 3), which is in correspondence with the MCD-positive dogs of the control group. The subchondral bone of the MCP and the primary spongiosa contained retained hyaline cartilage, which appeared to be more in the MCD-positive dogs compared to the MCD-negative dogs of both groups, and even more in the vitamin D supplemented group compared to the control group (Figure 3, 4). The vitamin D supplemented group had more irregular costal growth plates compared to the normal fed controls, as depicted

Table 2: Development of the secondary ossification centers of the elbow joint and of the distal ulna. Development of the secondary ossification centers (SOCs) of the elbow joint and of the distal ulna in the control group (c) and the vitamin D supplemented group (vitD) in dogs with (MCD+) or without medial coronoid disease (MCD-). Explanation of the SOCs can be found in Appendix 1.

	cMCD-	cMCD+	vitDMCD-	vitDMCD+	cMCD-	cMCD+	vitDMCD-	vitDMCD+	cMCD-	cMCD+	vitDMCD-	vitDMCD+
	9 weeks of age				**12 weeks of age**				**16 weeks of age**			
Olecranal apophysis												
Irregular shape												
Round	4	5										
Rounded edges	3	2	3	2	1	1		1				
Complete ossification					6	6	3	1	7	7	3	2
Distal Ulnar Metaphysis												
Flattened or rounded												
Cartilage cone	6	7	3	2								
Irregular structure	1				7	7	3	2				
Normal shape									7	7	3	2
Medial Humeral epicondyl												
Round	4	7	1	2								
Round edges	3		2									
Complete ossification					7	7	3	2	7	7	3	2
Anconeus												
No ossification	7	7	3	2								
Some ossification					5	4	1	2				
Complete ossification					1	3	1					
Fusion					1		1		7	7	3	2

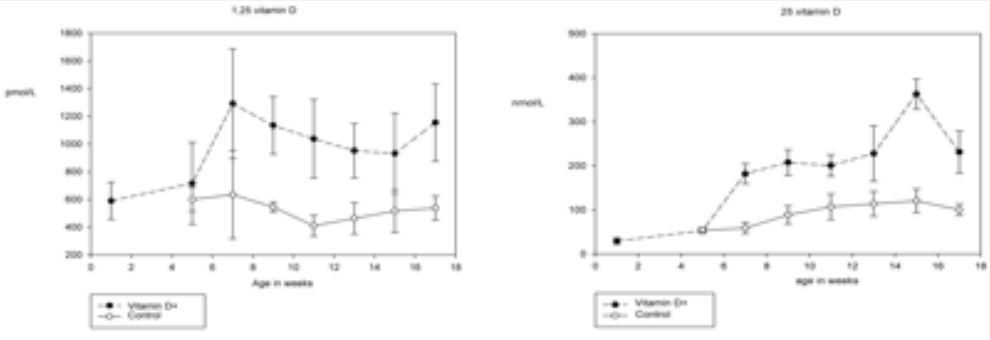

Figure 1 25-vitamin D and 1,25-vitamin D plasma concentrations of the puppies of the vitamin D supplemented group, and the control group (* p<0.05, ** p<0.01).

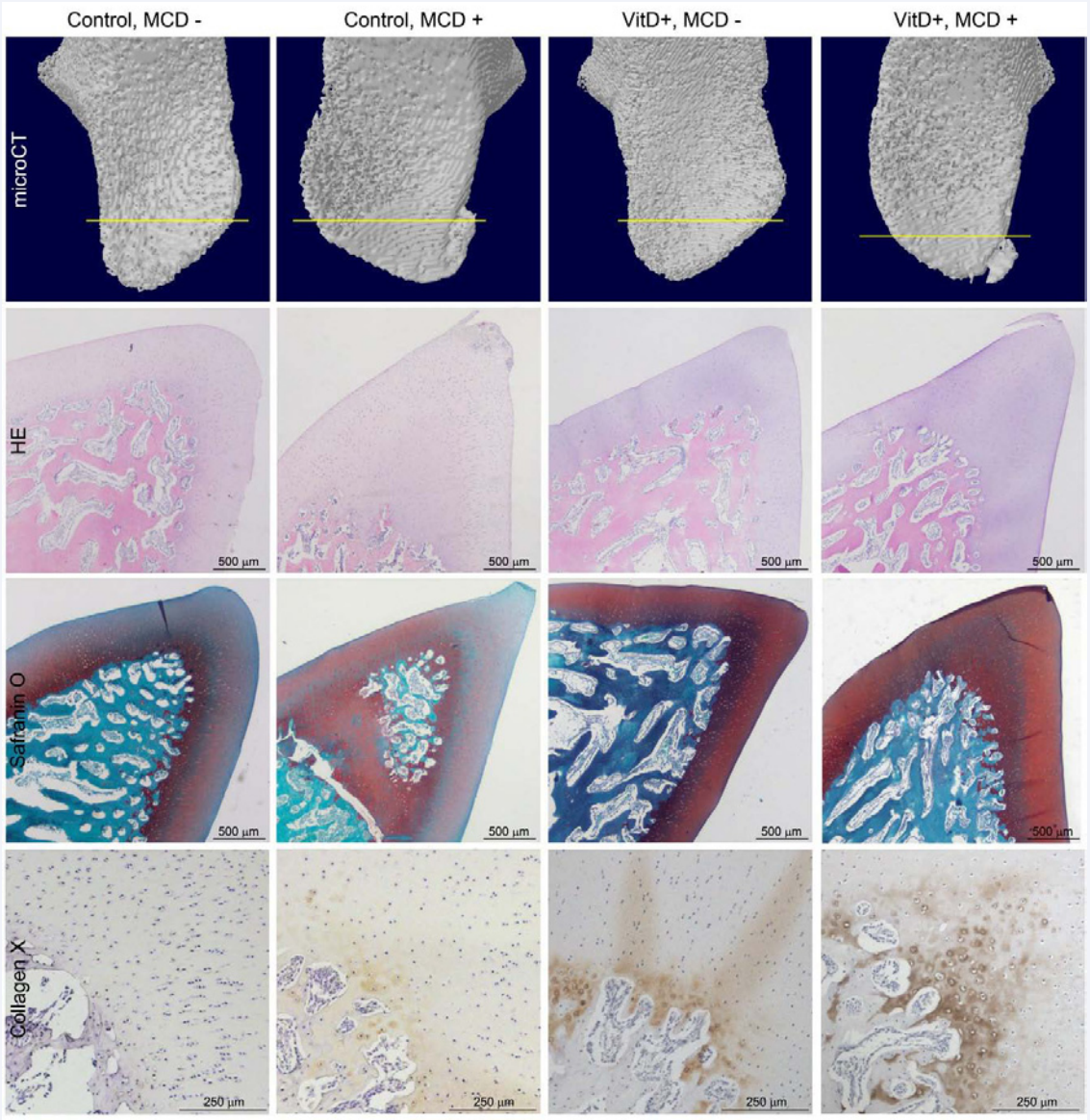

Figure 2 Comparison of the proximal view of the 3-D reconstructed micro-computed tomography (MicroCT) images of the medial coronoid process with corresponding (cut at yellow line), Haematoxylin and Eosin, Safranin O, and collagen X staining in medial coronoid disease (MCD)- negative and – positive joints of dogs. Representative examplesfrom the control group and from the vitamin D supplemented group of the same age (18 weeks old).

Table 3: Histology findings. Necropsy findingsin the control group and the vitamin D supplemented group. Sex is male (M) or female (F). Limb is left (L) or right (R). CT findings are negative, suspicion of medial coronoid disease (Suspect), or positive for medial coronoid disease. Macroscopic evaluation at necropsy of the cartilage demonstrated no fissure, incomplete fissure (Inc. Fissure), or complete fissure (Fissure). Micro-computed tomography (MicroCT) revealed a normal medial coronoid process (Normal), a non-mineralized line within the mineralized area (Non-mineralized zone), or a non-mineralized area between 2 mineralized areas (Non-mineralized area).

	Control		Vitamin D+		
	MCD-	MCD+	MCD-	MCD+	
Surface area cartilage MCP (mm^2)	2.0 ± 0.2^A	2.6 ± 0.4^B	2.0 ± 0.2^A	2.5 ± 0.4^B	p=0.019
Width of cartilage layer MCP (mm)	$0.79\ (0.75\text{-}0.81)^A$	$2.07\ (1.67\text{-}2.77)^B$	$1.00\ (0.82\text{-}1.02)^A$	$2.42\ (2.21\text{-}2.50)^B$	p=0.013
Collagen X ratio MCP in %	$0.02\ (0.00\text{-}2.55)^A$	$1.43\ (0.00\text{-}11.83)^B$	$0.83\ (0.21\text{-}3.60)^C$	$10.16\ (1.14\text{-}11.42)^D$	p<0.05
Growth plate (9th rib) width in mm	0.83 (0.78-1.02)	N/A	0.81 (0.69-1.02)	(0.81-1.17)	NS
Growth plate (9th rib) irregularity in %sd	10 ± 1^A	N/A	10 ± 1^A	17 ± 9^B	p=0.041
Ratio hypertrophy zone/proliferation zone (9th rib)	0.77 (0.63-0.84)	N/A	0.74 (0.65-0.87)	(0.72-0.81)	NS

Table 4: Histology findings in the control group (Control) and the vitamin D supplemented group (vitamin D+) in dogs with (MCD+) or without medial coronoid disease (MCD-).^{A} significantly different from ^{B,C,D} (p<0.05).

	Control		Vitamin D+		
	MCD-	MCD+	MCD-	MCD+	
Surface area cartilage MCP (mm^2)	2.0 ± 0.2^A	2.6 ± 0.4^B	2.0 ± 0.2^A	2.5 ± 0.4^B	p=0.019
Width of cartilage layer MCP (mm)	$0.79\ (0.75\text{-}0.81)^A$	$2.07\ (1.67\text{-}2.77)^B$	$1.00\ (0.82\text{-}1.02)^A$	$2.42\ (2.21\text{-}2.50)^B$	p=0.013
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Growth plate (9th rib) width in mm	0.83 (0.78-1.02)	N/A	0.81 (0.69-1.02)	(0.81-1.17)	NS
Growth plate (9th rib) irregularity in %sd	10 ± 1^A	N/A	10 ± 1^A	17 ± 9^B	p=0.041
Ratio hypertrophy zone/proliferation zone (9th rib)	0.77 (0.63-0.84)	N/A	0.74 (0.65-0.87)	(0.72-0.81)	NS

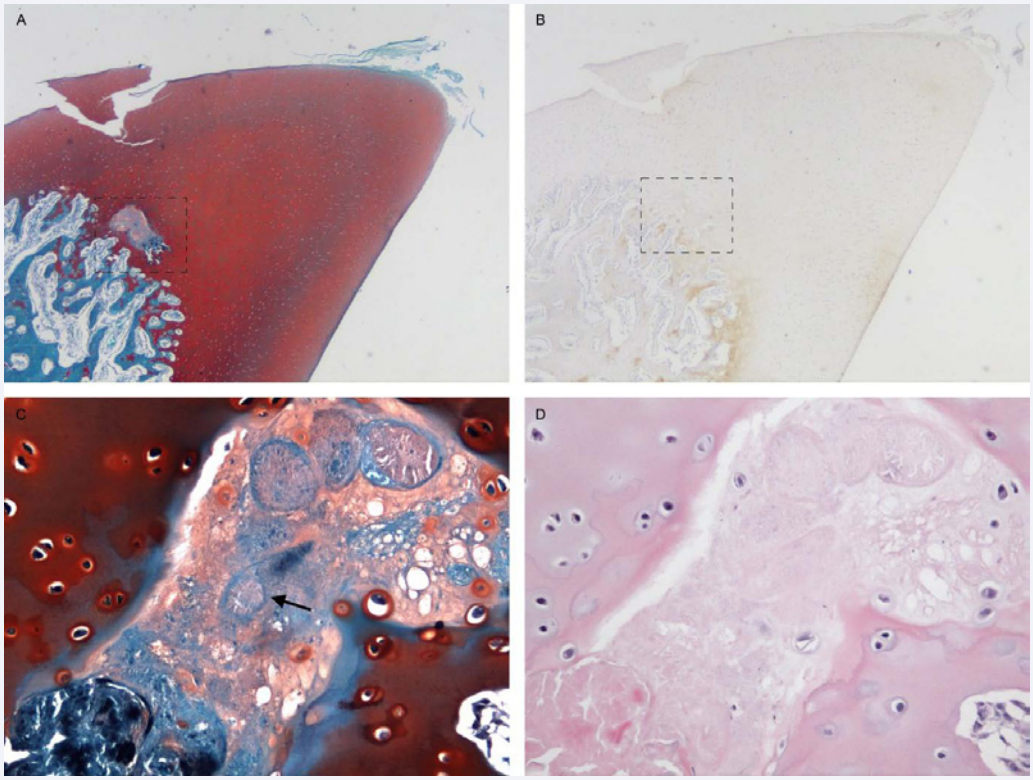

Figure 3 Lesion within the cartilage template of the developing medial coronoid process in a dog of 16 weeks old with medial coronoid disease. Haematoxylin and Eosin, Safranin O, and collagen X. Note that section is located at the beginning of the fissure. Thelesion is located in bone and cartilage with some pycnotic nuclei in the degenerative cartilage cells, vacuolization and debris of both cartilage and subchondral bone. Degeneration of chondrocytes surrounding the defect is also visible illustrated by the loss of GAG (red; Safranin O staining).

by a difference in %sd of the growth plate thickness, as well as by the less well organized columns of proliferating and hypertrophic chondrocytes (Table 4, Figure 4). The ratio between the width of the hypertrophic zone and proliferation zone of the costal growth plate did not differ significantly between the vitamin D supplemented group and the control group (Table 4).

In both the MCD-negative group and the MCD-positive group collagen X staining was present in the calcifying zone, as well as in the non-mineralized cartilage matrix of the MCP (Figure 2). In the vitamin D supplemented group, significantly more collagen X staining was demonstrated compared to the normal fed controls, both in MCD-positive (p=0.039) as in MCD-negative dogs (p=0.003) (Figure 5, Table 3).

DISCUSSION

We have demonstrated in a previous study that MCD-positive growing Labrador retriever sreveal a disturbed process of endochondral ossification of the developing MCP (Lau et al. 2013b) [9]. Within the process of endochondral ossification, mineralization of the cartilaginous matrix is an important step in the replacement of the cartilage template by bone. The delayed maturation of the cartilage of the developing MCP results in a thicker cartilage layer in dogs with MCD. Due to the presence of retained cartilage within the developing MCP, the MCP is more vulnerable to micro-trauma and / or susceptible to relative mechanical overloading. Delayed skeletal maturation in large breed dogs with rapidly increasing body weight and activity, may cause mechanical damage to the vulnerable cartilage (Olsson and Reiland 1978) [8]. Taking into consideration the role of vitamin D in skeletal development and mineralization (Hazewinkel and Tryfonidou 2002) [10], we hypothesized that vitamin D supplementation of Labradors at risk for MCD would augment mineralization of the developing MCP at young age and thus prevent the development of MCD.

This prospective study employed growing Labrador retrievers at risk to develop MCD; their parents were a MCD-affected dam and sire with known off spring that developed MCD. Successful vitamin D supplementation was reflected by the significantly increased plasma levels of both 25-vitamin D and 1,25-vitamin D compared to the control group. Although dietary vitamin D intake was 43-fold increased, Ca and P concentrations remained unchanged; hence indicated that there were no signs of vitamin D intoxication. This is in accordance with previous studies in young dogs raised on food with increased vitamin D content, demonstratingan up-regulated 24-hydroxylase activity, catabolizingexcess 25-vitamin D and 1,25-vitamin D to prevent intoxication (Tryfonidou et al. 2003b) [17].

Vitamin D influences skeletal development by stimulating terminal differentiation of chondrocytes, matrix remodeling, and mineralization of bone (Hazewinkel and Tryfonidou 2002) [10]. Vitamin D supplementation, up to 43-fold, did not lead to normalization of the process of endochondral ossification in the MCP area nor to prevention of the development of MCD. On histological examination the costal growth platesin the vitamin D supplemented group were even more irregular and contained moderately disorganized chondrocyte columns compared to the control group. Even more so, in the vitamin D supplemented group there appeared to be moreretained cartilage in the primary spongiosa of the growth plate compared to the control group, altogether indicating disturbed endochondral ossification. In line with these observations, the area stained positive for collagen type X within the MCP region in both MCD-positive and MCD-negative dogs was increased in the vitamin D supplemented group compared to the control group. Based on these observationsa43-fold vitamin D supplementation predisposes the dogs in the vitamin D supplemented group to generalized disorders of endochondral ossification and hence to developmental orthopedic disease. In line with these findings, growing Great Danes on a 10-fold and 135-fold vitamin D supplemented diet have also been reported to develop more severe disorders of endochondral ossificationthan Great Danes raised on a control food (Tryfonidou et al. 2003a) [18].

This study confirms that MCD is initiated within the deeper layers of the developing MCP, rather than the superficial layers of the articular cartilage (Lau et al. 2013b) [9]. After physiological regression of the cartilage canals, persistence of hyaline cartilage

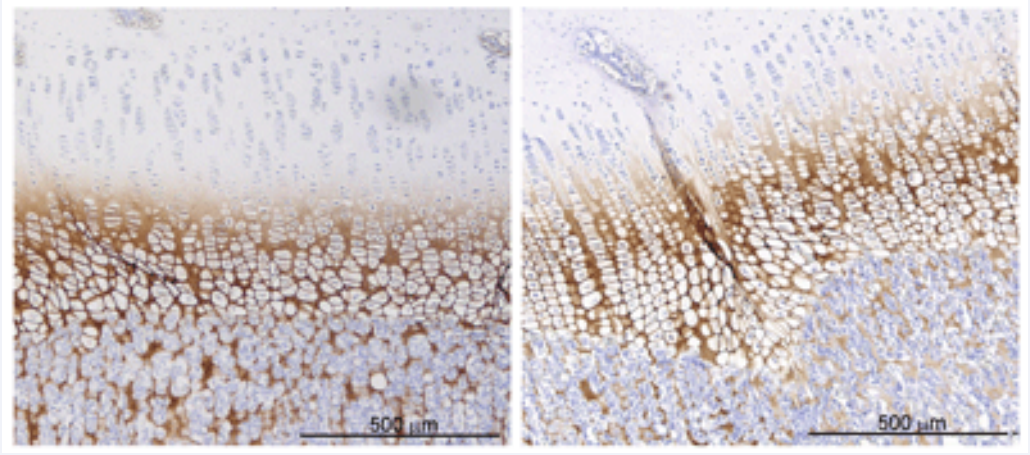

Figure 4 Representative examples of regular (MCD-negative dog) and irregular (MCD-positive dog) growth plate from the 9th ribs (of the vitamin D supplemented group) on collagen X. Note the retained cartilage in the subchondral bone in the MCD-positive dog.

within the developing MCP, probably ultimately hinders diffusion of nutrients originating from the synovial fluid and from the vessels in the epiphyseal intra-trabecular connective tissue into the deeper cartilage layers. In both experimental groups, the prolonged presence of the unmineralized cartilage template within the MCP may predispose to fissure formation of the loaded MCP, but we cannot exclude that the micro-trauma precedes chondrocyte cell death. Whether mechanical (ab) normal loading is responsible for disturbed vascularization and/or disturbed mineralization of the cartilage template within the MCP remains speculative. MCD can be considered a manifestation of osteochondrosis, often seen in rapid growing animals at different skeletal sites because of the shared the morphological similarities (Olsson and Reiland 1978, Lau et al. 2013b) [8,9].

CONCLUSIONS

MCD is initiated in the deeper layers of the developing MCP and is characterized by delayed endochondral ossification, and thus retention of cartilage within the subchondral bone.Vitamin D supplementation did not accelerate the process of endochondral ossification in Labrador retriever puppies at risk for MCD development. However, 43-fold vitamin D supplementation caused a generalized disturbed process of endochondral ossification, characterized by irregular growth plates and retained cartilage within the primary spongiosa of the costal growth plate. Optimal dietary vitamin D intake in dogs at risk for MCD is important in predisposed dogs to prevent aggravation of MCD and development of other orthopedic diseases.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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