**Summary**

**Objectives:** This study aims to quantify numbers of elastic fibers in cranial cruciate ligaments from a dog breed at high risk of cranial cruciate ligament disease.

**Methods:** Macroscopically normal cranial cruciate ligaments were harvested from six Labrador retrievers. Sequential histological sections were assessed for extracellular matrix degeneration (haematoxylin and eosin stain) and elastic fiber staining (Miller’s stain). Elastic fibers were semi-quantified using previously published scoring systems. Each section was scored twice by two observers.

**Results:** Increased numbers of elastic fibres were seen with increasing cranial cruciate ligament degeneration (*p* = 0.001). Labrador retriever cranial cruciate ligaments had lower elastic fiber staining when compared to previous published findings in the racing greyhound.

**Clinical significance:** The cranial cruciate ligaments from a dog breed at high risk of cranial cruciate ligament disease vary in the quantity of elastic fibers in association with ligament degeneration. Breed variation in the quantity of elastic fibers may reflect differing risk of cranial cruciate ligament disease.

**Key Words: Dogs, Cruciate Ligament Disease, Elastic Fibres**

**Running Head: Variation in cranial cruciate ligament elastic fibers**

**Introduction**

Cranial cruciate ligament disease is a major source of morbidity in the dog, leading to severe osteoarthritis of the stifle joint.([1](#_ENREF_1)) To date, the pathogenesis of this debilitating condition is poorly understood.([2](#_ENREF_2), [3](#_ENREF_3)) In previous studies, we have suggested that cranial cruciate ligaments from dog breeds at a high risk of ligament disease ([4](#_ENREF_4)) such as the Labrador retriever, have altered extracellular matrix metabolism compared to low risk breeds.([5](#_ENREF_5)) Histological changes within the cranial cruciate ligament such as loss of collagen architecture, chondrogenic change and loss of ligament cells are considered degenerative in the dog and in other comparative species.([6-8](#_ENREF_6)) However such changes are seen in both the Labrador retriever and in the greyhound, a breed with a low risk of cranial cruciate ligament disease ([5](#_ENREF_5)) and these changes may reflect an healing response to microinjury in an exercising breed such as the racing greyhound. ([9](#_ENREF_9))

Microfibrils are polymers of fibrillins 1 and 2 and bundles of microfibrils are known as oxytalan fibers.([10](#_ENREF_10)) Elastin fibers comprise an outer scaffold of microfibrils with a central cross-linked core of elastin with many other associated molecules.([11](#_ENREF_11)) Collectively, oxytalan and elastin fibers are referred to as elastic fibers. Elastin has traditionally been considered a minor component of ligament tissue ([12](#_ENREF_12)) but it has been shown recently to form up to 20% of the canine cranial cruciate ligament.([9](#_ENREF_9)) A wide distribution of elastic fibers in the canine cranial cruciate ligament has been described, with abundant elastin fibers and oxytalan fibers running with collagen bundles ([13](#_ENREF_13)). Elastic fibers have important mechanical, biochemical and cell-regulatory functions in tissue ([14](#_ENREF_14)) thereby playing an important role in tissue morphogenesis and cellular responses to injury.([15](#_ENREF_15))

Compared to other ligaments, the canine cranial cruciate ligament has poor intrinsic healing capacity and it has been suggested ligament healing in breeds at low risk of cranial cruciate ligament disease may be superior to those at higher risk.([16](#_ENREF_16)) We have demonstrated a proportional increase in elastic fibers with advancing degenerative change in the greyhound cranial cruciate ligament which may be indicative of a healing response to microinjury as cranial cruciate ligament disease is extremely rare in this breed.([9](#_ENREF_9))

We have previously suggested that oxytalan fibers, may play an important role in pathogenesis of cranial cruciate ligament disease.([9](#_ENREF_9)) We hypothesize that numbers of oxytalan fibers will be increased in association with increasing cranial cruciate ligament degeneration in the Labrador retriever. Our aim is to quantify elastic fibers and degeneration in cranial cruciate ligaments from a breed of dog at high risk of cranial cruciate ligament disease, the Labrador retriever ([4](#_ENREF_4)).

**Materials and methods**

*Animals*

Nine cranial cruciate ligaments were harvested by sharp dissection from 6 skeletally mature Labrador retrievers with no gross evidence of stifle joint pathology. One sample was taken from the middle of each cranial cruciate ligament, taking both craniomedial and caudolateral bands, for analysis. An additional sample was taken from either proximal or distal ends of the ligament, again including both bands, in seven cranial cruciate ligaments. Animals were euthanatized for reasons other than musculoskeletal disease as part of another study (approved by University Ethics committee), and informed owner consent was obtained for tissue removal. All samples were obtained within 24 hours of death, fixed in formalin, embedded in paraffin blocks and processed as below in a single batch.

*Histology*

Sequential longitudinal sections of 4 µm from paraffin-embedded samples were stained with 1) haematoxylin and eosin, and 2) Miller’s stain to show both elastin and oxytalan fibers.([17](#_ENREF_17)) This series allowed assessment of tissue architecture, and identification of elastic fibers. Images were recorded on a dedicated microscope (Eclipse 80i, Nikon, Tokyo, Japan). All sections were read by two observers blinded to sample identification (KDS and EJC) on two separate occasions at least 1 week apart. Scores for histological sections were averaged firstly for each observer then these scores were averaged between observers. To ensure the alteration in oxytalan fiber staining was not an artefact, staining as a positive control was confirmed as normal in areas unaffected by degenerate change such as blood vessels in synovium.

*Scoring Method for Extracellular Matrix Degeneration*

Sections were assessed for signs of extracellular matrix degeneration. All samples were graded 0-3 according to criteria previously described.([8](#_ENREF_8)) The broad grade 1 category was subdivided with a more detailed scoring system previously developed by the authors.([9](#_ENREF_9)) Briefly, a score from 0-4 was awarded based on the extent of the changes for each of eight factors resulting in a range of possible scores from 0-24 being awarded (Supplementary Table 1). These results are referred to as a modified Vasseur Score.

*Scoring method for Elastic Fibre Content*

A scoring system (Miller’s score) developed previously by the authors was used to quantify changes in elastic fibre staining.([9](#_ENREF_9)) Increased staining in interfascicular and interbundle regions, ligament substance (intrabundle), as well as the extent and degree of pericellular staining, could be awarded up to 2 points giving a score range of 0-10 (Supplementary Table 2).

*Statistical analysis*

Data were tested for normality using Anderson-Darling tests. Animal data was not normally distributed and are presented as median value, range and interquartile range. Vasseur score, modified Vasseur score and Miller’s score were not normally distributed and are presented as median value, range and interquartile range. The relationship between Vasseur score and Miller’s score was examined using Spearman’s correlation. Kendall’s coefficient of concordance was calculated for intra- and inter-observer concordance of Vasseur score, modified Vasseur score and Miller’s score. Kendall’s coefficient of concordance ranges from 0 (no agreement) to 1 (complete agreement). The relationship between age and modified Vasseur score and Miller’s score was tested using Spearman’s correlation. Data were analysed using Minitab 17 Statistical Software (Minitab, Coventry, UK). Significance was set at p < 0.05.

**Results**

*Animals*

Age range for Labrador retrievers was 13 to 144 months (median 60, interquartile range 98.7). Three were three female and three male dogs.

*Extracellular Matrix Degeneration*

Degenerative change was seen in all cranial cruciate ligaments. Eleven of 16 cranial cruciate ligament samples stained with haematoxylin and eosin were graded as grade 1 Vasseur score according to the published system.([8](#_ENREF_8)) Of the remaining cranial cruciate ligaments, 3 were grade 2 and 2 were grade 3 (median 1, range 1-3, interquartile range 1). Grade 1, as according to the original Vasseur score ([8](#_ENREF_8)), were also graded on the modified scoring system, with a median score of 16.3 ± 4.4 (range 8-22.5, interquartile range 3.3). No correlation was noted between age and Vasseur score (p = 0.30).

*Elastic Fibre Content*

For all cranial cruciate ligaments, Miller’s score median was 0 (range 0-2.5, interquartile range 0.45). For cranial cruciate ligaments of lower grade degeneration (grade 1 Vasseur scores), all Miller’s scores were 0 bar one sample of 0.25. A statistically significant positive Spearman’s correlation was seen between Vasseur score and Miller’s score (rs = 0.84, p < 0.001), suggesting an increased quantity of elastic fibres are seen with increased ligament degeneration. In cranial cruciate ligaments with minimal degeneration (modified Vasseur score of <10),sparse elastic fibres were found throughout the cranial cruciate ligament (Fig 1). No correlation between age and Miller’s score was noted (p= 0.75). Intra- and interobserver data is summarised in Table 1.

**Discussion**

Extracellular matrix degeneration was found in every sample examined, consistent with previous studies on canine cruciate ligaments. Advancing degeneration has been associated with mechanical weakness of the canine cranial cruciate ligament.([8](#_ENREF_8)) Generalised Vasseur grade 1 changes were described in both the cranial cruciate ligament and the caudal cruciate ligament in the greyhound, a breed at very low risk of canine cruciate ligament rupture, leading to the suggestion that these low-grade changes are not in fact degenerative but adaptive.([5](#_ENREF_5)) As grade 2 and 3 changes have not been described in the greyhound, ([5](#_ENREF_5)) we suggest that in grade 2 and 3 Labrador retrievers (high grade degeneration), the change may be truly degenerative and indicative of disease and may be a precursor to rupture.

Elastic fibre quantity in the Labrador retriever showed a significant relationship with degeneration. As degeneration became more severe (Vasseur grade 2 and 3), there was an increase in oxytalan fibre quantity. The pattern of elastic fibre distribution was similar to our previous study on the greyhound and the use of control slides confirmed normal elastic fibre staining.([13](#_ENREF_13)) Furthermore, a similar relationship between degeneration and elastic fibres was described in the breed. ([9](#_ENREF_9)) Thus, we believe this relationship in the Labrador retriever is a genuine relationship reflecting ligament physiology. The role of elastic fibres in cranial cruciate ligament tissue is unknown and may involve provision or maintenance of elasticity, stabilization of blood vessels, anchoring tissue or guidance of cell migration.([18](#_ENREF_18), [19](#_ENREF_19)) Previously we proposed that increased quantity of elastic fibres observed in greyhound cranial cruciate ligaments may reflect a healing response ([9](#_ENREF_9)) as assembly of oxytalan fibres is commonly seen in healing responses in artery, ([20](#_ENREF_20)) myocardium, ([21](#_ENREF_21)) muscle ([22](#_ENREF_22)) and skin. ([23](#_ENREF_23)) Miller’s score ranges from 0 to 10 and in the Labrador retriever the highest recorded score was 2.5, observed in two samples where Vasseur score was 3. Using identical methodology, higher maximal values were seen in the greyhound ([9](#_ENREF_9)) and beagle (unpublished data). Additionally, none of these greyhounds had high-grade degeneration (Vasseur score 2 and 3), whereas 5/16 Labrador retrievers did. These findings are suggestive of a fundamental difference between the cruciate ligaments of Labrador retrievers and greyhounds and these findings may reflect reduced production or assembly, or increased destruction of fibrillin. Although the greyhounds in this previous study were not significantly older than the Labrador, this variation may have arisen from differences in husbandry such as exercise and it is not possible to conclude whether these differences are genotypic or phenotypic. Regardless of the cause of these differences, assembly of elastic fibres is commonly seen in healing responses and this relative lack of elastic fibres in degenerating Labrador retriever cruciate ligament tissue may contribute to the eventual rupture of the ligament through a failure of healing. Although poor healing has been described in the canine cranial cruciate ligament,([24-27](#_ENREF_24)) this does not mean that intrinsic healing capacity of cranial cruciate ligaments in all canine breeds is uniform.

Many of the limitations in this study have arisen through limitations in tissue availability as obtaining intact Labrador cruciate ligaments from healthy stifles is challenging. One or two samples were used from each cruciate ligament to improve sample size. Previous work by our group and others has shown regional variation in the degeneration within individual ligaments and this may reflect local variation in mechanical environment.([8](#_ENREF_8), [28](#_ENREF_28)) Furthermore, the correlation between degeneration and elastic fibre content was independent of location within the ligament. Thus, we believe using multiple samples is justified. We would have preferred to conduct a more thorough analysis of regional variations in elastic fibre staining and this will be part of future work. There was a wide variation in age in our samples, but no relationship was noted between degeneration, elastic fibre staining and age. Other authors have similarly found no clear relationship between cruciate ligament degeneration and age. Indeed neither of the Vasseur grade 3 ligaments was from the oldest dog in the study and we do not believe variation in age undermines the results of our study. However, in comparison with other breeds, while there was no statistical difference in age, other variants such as exercise and diet may have altered ligament physiology. As mentioned earlier, we believe the differences, whether genotypic or phenotypic, to be real and age- and husbandry-matched ligaments would be required to further assess this. The relative lack of elastic fibre staining in Labrador retrievers was checked by reference to known sites of normal elastic fibres, blood vessels and epiligament, where elastic fibres could be seen. Future work will include analysis of other breeds with differing risk of cranial cruciate ligament disease to validate our findings as a possible mechanism in the aetiopathogenesis of this condition. In summary, we have demonstrated that Labrador retriever cranial cruciate ligaments show increased numbers of elastic fibres with advancing degeneration. However, elastic fibres were seen in lower numbers in the Labrador retriever than in the greyhound, a breed at low risk of cranial cruciate ligament disease. These differences suggest that the cranial cruciate ligaments of differing breeds of dog vary in their response to cranial cruciate ligament degeneration, and the presence or absence of elastic fibres may affect cranial cruciate ligament healing, reflecting their differing risk of cranial cruciate ligament disease. Whether these differences arise through genotypic or phenotypic mechanisms remains to be elucidated. Tissue integrity is a balance of damage and repair and failure to generate or maintain sufficient elastic fibres in cranial cruciate ligament tissue. The findings presented here may be a key to understanding the aetiopathogenesis of this disease.

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