**Original Article**

**Characterisation of key proteoglycans in the canine cranial cruciate ligament (CCL) from two dog breeds with a different predisposition to CCL disease and rupture**

S. Allaith a,c, S. R. Tew a,c, C. E Hughes d, P. D. Clegg a,c,E. G. Canty-Laird a,c, and E. J. Comerford a,b,c \*.

a *Department of Musculoskeletal and Ageing Science, Institute of Life Course and Medical Sciences, University of Liverpool, William Duncan Building, 6 West Derby Street, Liverpool L7 8TX, UK*

b *Small Animal Teaching Hospital, Leahurst Campus, Institute of Veterinary Science, University of Liverpool, Chester High Rd, Neston, CH64 7TE, UK*

c *The Medical Research Council Versus Arthritis Centre for Integrated Research into Musculoskeletal Ageing (CIMA)*

d *School of Biosciences, University of Cardiff, Sir Martin Evans Building, Museum Avenue, Cardiff, CF 10 3AX**, UK*

\* Corresponding author. Tel.: +44 (0)151 795 6100

*E-mail address:* [ejc@liverpool.ac.uk](mailto:s.allaith@liverpool.ac.uk) (E.J. Comerford).

**Abstract**

Cranial cruciate ligament disease and rupture (CCLD/R) is one of the most common orthopaedic conditions in dogs, eventually leading to osteoarthritis of the stifle joint. Certain dog breeds such as the Staffordshire bull terrier have an increased risk of developing CCLD/R. Previous studies into CCLD/R have found that glycosaminoglycan levels were elevated in cranial cruciate ligament (CCL) tissue from high-risk breeds when compared to the CCL from a low-risk breed to CCLD/R. Our objective was to determine specific proteoglycans/glycosaminoglycans in the CCL and to see whether their content is altered in dog breeds with a different predisposition to CCLD/R. Disease free CCLs from Staffordshire bull terriers (moderate/high-risk to CCLD/R) and Greyhounds (low-risk to CCLD/R) were collected and key proteoglycan/glycosaminoglycans were determined by semi-quantitative Western blotting, quantitative biochemistry, quantitative reverse transcription polymerase chain reaction, and immunohistochemistry.

Gene expression of fibromodulin (*P*=0.03), aggrecan (*P*=0.0003), and chondroitin-6-sulphate stubs (*P*=0.01) were significantly increased, and for fibromodulin this correlated with an increase in protein content in Staffordshire bull terriers compared to Greyhound CCLs (*P*=0.02). Decorin (*P*=0.03) and ADAMTS-4 (*P*=0.04) gene expression were significantly increased in Greyhounds compared to Staffordshire bull terrier CCLs. The increase of specific proteoglycans and glycosaminoglycans within the Staffordshire bull terrier CCLs may indicate a response to higher compressive loads, potentially altering their risk to traumatic injury. The higher decorin content in the Greyhound CCLs is essential for maintaining collagen fibril strength, whilst the increase of ADAMTS-4 indicates a higher rate of turnover helping to regulate normal CCL homeostasis in Greyhounds.

*Keywords:* Canine Cruciate Ligament; Gene expression; Protein expression; Proteoglycans

**Introduction**

Cranial cruciate ligament disease (CCLD) is a spontaneous non-contact degeneration of the cranial cruciate ligament (CCL) leading to ligament rupture (Vasseur et al., 1985; Narama et al., 1996), and different dog breeds have variable susceptibility to this condition (Whitehair et al., 1993; Duval et al., 1999). The extracellular matrix (ECM) of the CCL dry weight is primarily composed of collagen, elastin, and sulphated glycosaminoglycans (sGAG) (Smith et al., 2014) in the form of a variety of different proteoglycans having both structural and regulatory functions(Iozzo and Muroch, 1996; Iozzo and Schaefer, 2015). Small leucine rich proteoglycans (SLRPs), such as decorin, biglycan, lumican and fibromodulin bind to collagen fibrils and organise collagen fibrillogenesis (Scott and Haigh, 1988; Scott, 1996; Iozzo, 1999; Douglas et al., 2006). Knockout mice studies show that altered expression of SLRPs disrupts ligament ECM integrity(Svensson et al., 1999; Ezura et al., 2000; Chakravarti et al., 2000; Chakravarti et al., 2003; Liu et al., 2003; Zhang et al., 2009).

Aggrecan and versican are large aggregating proteoglycans(Vogel and Koob, 1989). Aggrecan is predominantly found in cartilage and its known to regulate its normal tissue homeostasis (Hardingham and Bayliss, 1990), whilst versican interacts with elastin and elastic fibers (Isogai et al., 2002),as well as with a number of growth factors such as transforming growth factor β and platelet derived growth factor (Schönherr et al., 1991). Increased proteoglycan content has been previously found in bovine deep digital flexor tendon that is subjected to compression (Vogel and Peters, 2005). The content of proteoglycans have also been shown to increase in pathological human tendons and anterior cruciate ligament (Riley et al., 1994; Lo et al., 1998; Corps et al., 2004; Fu et a., 2007; Samiric et al., 2009). Furthermore, proteoglycans and glycosaminoglycans have been found to be significantly increased in ruptured CCLs of the Labrador retriever compared to non-ruptured control CCLs (Comerford et al., 2004; Clements et al., 2008). The significant elevation of these molecules in injured/ruptured tendons and ligaments suggests that they could be involved in the pathology of CCLD/R. ADAMTS-4 and ADAMTS-5, on the other hand, are multi-domain proteases responsible for cleaving proteoglycans (Sandy et al., 2001; Kashiwagi et al, 2004; Porter et al., 2005; Melching et a., 2006), and are inhibited by tissue inhibitor metalloproteinase-3 (TIMP-3)(Lim et al., 2010). ADAMTS-5 has been reported to cleave aggrecan in cartilage, and to regulate collagen architecture in tendon (Wang et al., 2012).

The presence of proteoglycans in the canine CCL have been demonstrated in a previous study (Yang et al., 2012).However, detailing the different types of proteoglycans and glycosaminoglycans between different predisposed dog breeds to CCLD/R remains unknown and this information may lead to a better understanding of their role in the breed-specific aetiology of this condition. Our aim was to determine if proteoglycan and/or sGAG content differed between dog breeds with different predisposition to CCLD/R. Therefore, the objectives of this study were to analyse sGAG content, water content, protein expression of proteoglycans, protein expression of sGAG stubs, gene expression of proteoglycans and of proteoglycan inhibitors (ADAMTS-4 and -5) in the CCLs from dog breeds with different predisposition to CCLD/R.

**Materials and Methods**

*Sample preparation*

Disease-free CCLs from Staffordshire bull terrier (*n*=20) cadavers were obtained with no macroscopic evidence of stifle joint pathology. Dogs were euthanised for purposes not related to this study and samples of joint tissues were clinical waste material donated to University of Liverpool. Ethical approval for use of this material was granted by the local ethics committee (VREC65). Archived disease-free CCLs from Greyhounds (*n*=20) were sourced from previous studies which had been collected as clinical waste material. The stifle joints were assessed both macroscopically and radiologically to confirm that there was no evidence of stifle joint osteoarthritis (Comerford, 2003). CCLs were stored at -20οC for biochemical analyses, snap frozen in liquid nitrogen and stored at -80οC for Western blotting, immersed in RNA later overnight at 4οC for reverse transcription quantitative real-time-PCR (RT-qPCR), or stored in 4% paraformaldehyde to be paraffin embedded for immunohistochemical analysis. The number of canine cadavers used for each experiment and their signalment which consisted of breed, age, bodyweight and gender were recorded (Table 1).

*Biochemical analysis*

CCL samples were digested overnight with 10U/ml papain in 100mM sodium-acetate, 2.4 mM EDTA and 5 mM cysteine HCL at 60οC. As previously described by Comerford and others (Comerford et al., 2004), sGAG content was determined using the 1,9-dimethlylmethylene blue (DMMB) dye binding assay(Farndale et al., 1986). For data analysis, sGAG content in CCL were normalised to ligament dry weight.

To determine the water content (%), snap frozen CCL wet weights were measured and then freeze dried for 24h and then reweighed to obtain their dry weight. The water content (%) was calculated using the following equation;

Water content (%) = ([wet weight–dry weight]/ wet weight) x 100

*Reverse transcription quantitative real-time-PCR*

Steps for RNA extraction and complementary DNA processing are detailed in Supplementary material 1. RT-qPCR was performed in a 25 μl volume containing; 50 ng cDNA, 6 pmol/μl forward and reverse primers designed for the particular gene of interest (Eurogentec), and GoTaq(R) qPCR Master Mix (Promega) diluted to 1x. Samples were run in duplicate on an Applied Biosystems 7300 Real-Time PCR machine. Primers were validated by performing a sequence (1 to 8) of 1:2 serial dilutions and were compared with those targeting two reference genes (GAPDH, B2M) (Supplementary material 2). Primers were validated by confirming that primers were found to lie within 10% of GAPDH and B2M percentage (Supplementary material 2). The selected primers (Table 2) were chosen to be normalised against GAPDH, and fold changes in gene expression level were calculated using the ΔCT method (Schmittgen and Zakrajsek, 2000).

*Western blot analysis and Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Canine CCLs were snap frozen in liquid nitrogen and pulverised with a dismembrator before being extracted with 4 M guanidinium chloride. Ligament extracts were dialysed using a 20,000kD cut-off membrane (Spectrapor, Breda) at 4oC overnight against 0.1 M sodium acetate buffer, pH 6.8 (containing proteinase inhibitors) (Rees et al., 2000). Samples were then digested with the appropriate glycosaminoglycan enzyme for 24h at 37oC with either chondroitinase ABC (Yamagata et al., 1968) (Sigma), keratanase I (Fukua and tsuboi, 1999) (AMS), and or keratanase II (Yamagishi et al., 2003) (AMS). CCL samples were further dialysed against deionised water, freeze dried and reconstituted in 1x Laemmli sample buffer (0.125 M Tris-HCl pH 6.8 containing; 4% (w/v) SDS; 20% (v/v) glycerol; 0.01% (w/v) Bromophenol Blue) with or without 5% (v/v) ß-mercaptoethanol (all from Sigma). Samples were heated (90˚C for 10m) and loaded onto NUPAGE 4-12% Bis-Tris Gels (Life Technologies) as equivalent mg per wet weight of tissue (Melrose et al., 2008; Plaas et al., 2011). Gels were transferred to nitrocellulose membranes (Whatman) using an X-Cell blot module (Invitrogen) for 90min at 35 volts in NUPAGE transfer buffer (Life technologies). Membranes were blocked in phosphate-buffered saline containing 5% (w/v) skimmed milk powder at 25oC for 1h. The membranes were then incubated in blocking buffer containing primary antibody (Table 3) and 0.05% Tween20 at 4oC overnight. The primary antibody was removed and membranes washed 3x 5min with phosphate buffer saline + 0.05% Tween20 (PBS-T) before adding the appropriate secondary antibody (Supplementary material 3). All secondary antibodies were diluted in blocking buffer and incubated with the membrane at 25oC for 1h. The membrane was washed 3x 5min in PBS-T.

Membranes were developed using an enhanced chemiluminescent detection image kit (ECL Plus Western Blotting Detection Reagents) according to the manufacturer’s protocol. Positive bands were visualised using UVP ChemiDoc-it imaging system. Images were captured and protein band density was quantified using VisionWorksLS acquisition and analysis software package. Area density was measured for each band to detect differences in GAGs and proteoglycans between Staffordshire bull terrier and Greyhound CCLs. Negative controls were performed by omitting the secondary antibody (Supplementary material 4).

*Immunohistochemistry*

Four micromillimetre sections of CCLs were rehydrated and endogenous peroxidase activity was blocked by incubating tissue sections with 3%(v/v) H2O2 (Sigma) at 25oC for 10min. Tissue sections were washed in water and pre-digested with chondroitinase ABC (0.5 U/ml) in 100 mM Tris-HCL pH 7.2-7.4 at 25oC for 30min. Non-specific binding was blocked using 20% goat serum in Tris buffer saline-Tween (TBS-T) at 25oC for 10min. The serum was removed and sections washed in TBS-T and incubated overnight at 4oC with primary antibody in TBS-T. Following incubation, sections were washed in TBS-T and incubated with secondary antibody (Supplementary material 3) diluted in 20% goat serum in TBS-T at 25oC for 30min before being finally washed in TBS-T. 3,3′-Diaminobenzidine (Sigma) was added to the sections to develop positive staining visible as a brown colour. Sections were rinsed in distilled water and counterstained with haematoxylin, dehydrated and cover slipped with dibutylphthalate polystyrene xylene (DPX) mountant for histology (Sigma). Negative controls were preformed using rabbit immunoglobulin (IgG), mouse IgG, mouse IgM or in the absence of primary antibody staining (Supplementary material 5 and 6).

**Data and statistical analysis**

Data from the DMMB, Western blot, and RT-qPCR assays were tested for normality using Kolmogorov-Smirnov test. Breed was the variable examined for each data set. If data had a normal distribution, the two groups were analysed by selecting a parametric test (Student t-test). Where data was not normally distributed, the two groups were analysed by selecting a non-parametric test (Mann-Whitney U test). Results are presented as mean values ± standard deviation (SD). Exact *P* values are presented and significance level of *P* <0.05 was used. Graphpad Prism (Version 7) was used for all graph generation and statistical analysis.

**Results**

*Biochemical analysis*

Mean water content (%) and sGAG (% per ligament dry weight) were 70±1.1% and 1.9±0.3% in Staffordshire bull terrier CCLs. For Greyhound CCL, values were 64±2.6% and 1.7±0.5% (*P*=0.55) (Fig. 1A, B). Staffordshire bull terrier CCLs had statistically significant higher percentage of water content (*P*=0.002) than Greyhound CCLs.

*Reverse transcription quantitative real-time-PCR*

Values were expressed in relation to GAPDH. Aggrecan gene expression demonstrated a significantly greater fold change (*P*=0.0003) in Staffordshire bull terrier CCLs (0.6±0.02) compared to that of Greyhounds (0.14±0.2) (Fig. 2A). There were not any statisically signficant differences in the gene expression of versican between Staffordshire bull terrier (1.4±2.8) and Greyhound (3.4±1.5 relative to GAPDH) (*P*=0.005) (Fig. 2B) CCLs. There was significantly higher fold change in decorin gene expression (*P*=0.03) in the Greyhound CCLs (260±218.3) when compared to the Staffordshire bull terrier CCLs (0.4±0.01) (Fig. 2C). Gene expression values for biglycan and lumican in Staffordshire bull terrier CCLs were 0.5±0.02 and 0.5±0.03 each relative to GAPDH. For Greyhounds, gene expression values were 1.1±1.0 and 4.5±4.4 (Fig. 2D and 2E, respectively). Biglycan (*P*=0.22) and lumican (*P=*0.08) showed no statistical significant differences in the gene expression between the two dog breeds. Fibromodulin gene expression had a significantly higher fold change (*P*=0.03) in Staffordshire bull terrier CCLs (9.7±4.7) in comparison to that of Greyhounds (3.5±2.3 relative to GAPDH) (Fig. 2F). Greyhound CCLs (0.7±0.6) had significantly higher fold change in its gene expression of ADAMTS-4 (*P*=0.04) as opposed to Staffordshire bull terrier (0.01±0.01) (Fig. 2G). ADAMTS-5 did not show any statistical significant differences in CCLs of Staffordshire bull terrier (0.2±0.2) or Greyhound (0.7±1.6) (*P*=0.15) (Fig. 2H).

*Western blot analysis*

Aggrecan core protein band was 55kDaand the band density appeared to be more intense in Staffordshire bull terrier compared to Greyhound CCLs (Fig. 3A), whilst versican core protein showed molecular weights ranging from 40-260kDa (Samiric et al., 2004; ilic et al., 2005)(Fig. 4A). Decorin core protein revealed molecular weights ranging from 40-50kDa (Rees et al., 2000; Samiric et al., 2004; Ilic et al., 2005; Melrose et al., 2008; Yang et al., 2012) (Fig. 5A). Other molecular weight bands of interest for decorin were at 110kDa (Fig. 5A purple label) and at 30kDa (Fig. 5A black arrow). There were no statisticaly significant differences between the CCL in the two dog breeds in terms of monomeric decorin core protein (Fig. 5A green label). However, decorin dimeric core protein was significantly higher in Greyhound compared to Staffordshire bull terrier CCLs (*P*=0.008) (Fig. 5A). Biglycan core protein ranged from 40-50kDa (Rees et al., 2000; Samiric et al., 2004; Ilic et al., 2005; Melrose et al., 2008; Yang et al., 2012) (Fig. 6A). However, biglycan core protein band intensity was higher in the first and second donor compared to the other donors (Fig. 7A). Lumican and fibromodulin core protein were visible at 50kDa (Melrose et al., 2008; Yang et al., 2012)(Fig. 7A and 8A) and keratocan was present at 30kDa (Rees et al., 2009)(Fig. 8A). Fibromodulin core protein was found to be significantly higher in Staffordshire bull terrier CCLs (*P*=0.02) compared to Greyhounds CCLs (Fig. 9A). Chondroitin-6 sulphate stub (Fig. 10A) ranged from 80-260kDa (Rees et al., 2000; Samiric et al., 2004; Ilic et al., 2005). Chondroitin-4-sulphate stub ranged from 80-260kDa (Fig. 10B, green label) and between 40-50kDa (Rees et al., 2000; Samiric et al., 2004; Ilic et al., 2005) (Fig. 10B, purple label). Chondroitin-0-sulphate stub revealed a band at 260kDa (Rees et al., 2000; Samiric et al., 2004; Ilic et al., 2005) (Fig. 10C). Keratan sulphate stub (Fig. 10D) ranged from 80-260kDa. Chondroitin-6-sulphate stub was significantly higher in Staffordshire bull terrier (*P*=0.01) in relation to the Greyhound CCLs (Fig. 10A). Table 4 represents mean and SD of the arbitrary values after measuring band density.

*Immunohistochemistry*

Immunostaining for aggrecan was found in interfascicular regions and collagen bundles in the CCLs. Staffordshire bull terrier CCLs had increased aggrecan staining around the fibrocartilaginous type cells and was shown to localise intracellularly (within the cell) (Fig. 3B blue arrow). Aggrecan staining in Greyhound CCLs was rarely seen localised intracellularly (Fig. 3B blue arrow). Immunostaining of versican was observed in fascicular regions rather than collagen bundles in the CCLs (Fig. 4B black arrow). The immunostaining of decorin was also evident in fascicular regions and collagen bundles CCLs (Fig. 5B), whilst biglycan had less staining for collagen bundles and showed increased staining in fibrocartilaginous cells in the CCLs (Fig. 6B). Overall, biglycan had more intracellular staining in Staffordshire bull terrier CCLs compared to Greyhound CCLs (Fig. 6B blue arrow). Immunostaining of lumican, fibromodulin and keratocan was mainly present in the fascicular regions of the CCLs (Fig. 7B, 8B, and 9B black arrows). In addition, fibromodulin was found to surround fibrocartilaginous cells and stained some cells both pericellularly (around the cell) and intracellularly. This observation was prominent in Staffordshire bull terrier CCLs compared to Greyhounds (Fig. 8B blue arrow). Upon examination of the cellular morphology of the immunohistochemical sections, Staffordshire bull terrier CCLs mostly exhibited round shaped cells, whereas Greyhound CCLs consisted mainly of spindle shaped cells (Fig. 3B to 8B).

**Discussion**

This is the first study to demonstrate the presence of specific proteoglycans and their catabolic enzyme (ADAMTS-4) in CCLs from Staffordshire bull terriers (moderate-high risk to CCLD/R) and Greyhounds (low risk to CCLD/R).

Aggrecan gene expression had a significantly greater in Staffordshire bull terrier compared to Greyhound CCLs (Fig. 2A). 55kDa fragments of aggrecan (Fig. 3A) appeared to be enriched in Staffordshire bull terriers compared to Greyhound CCL. It is possible that our study was under powered, and larger numbers could have demonstrated significant differences in the 55kDa fragment between breeds. Immunostaining of CCLs for aggrecan showed increased staining for the fibrocartilaginous type cells in Staffordshire bull terriers CCLs compared to those of the Greyhounds (Fig. 3B). The greater presence of fibrocartilaginous type cells in Staffordshire bull terrier CCLs could be secondary to compression. Compressive loading in the CCL has been previously demonstrated in Labrador retrievers (high risk to CCLD/R) (Comerford et al., 2006a), where their stifle joints have a lower intercondylar notch index and femoral condyle height, resulting in a narrow intercondylar notch. Therefore, CCL function is impeded when passing through a narrow intercondylar notch, subjecting the CCL to compressive forces (Smith et. al., 2011). The CCL water content was significantly increased in Staffordshire bull terrier compared to Greyhound CCLs. Increased water content in the CCL of Staffordshire bull terrier may be indicative of subclinical tissue damage and therefore contribute to CCLD/ R, as Comerford and others have shown that ruptured CCLs had significantly increased water content compared to intact CCLs of the high-risk Labrador Retriever(Comerford et al., 2004). Pathological tendons have also been reported to demonstrate increased water content (Riley et al., 1994; Samiric et al., 2009). Decorin gene expression (Fig. 2C) and dimeric protein at 110kDa (Fig. 5A) were both significantly higher in Greyhounds compared to Staffordshire bull terrier CCLs, which may suggest differences in collagen fibrillogenesis regulated by decorin. The important role of decorin in collagen fibrillogenesis can be supported by the decorin knockout mice phenotype in tendon, where collagen fibrils had irregular outlines and were alterations in collagen fibril diameter distribution in cross sections (Danielson et al., 1997). Variable collagen fibril size can also be secondary to CCLD/R, where irregular shape of cross-sections of collagen fibrils has been detected in the Labrador CCL (high risk breed to CCLD/R) (Comerford et al., 2006b) and could also be explained by low concentrations of decorin in the CCLs for these high-risk breeds. ADAMTS-4 gene expression was significantly increased in Greyhound compared to Staffordshire bull terrier CCLs (Fig. 2G). ADAMTS4 has been shown to degrade decorin and other several proteoglycans as part of normal connective tissue homeostasis (Sandy et al., 2001; Kashiwagi et al, 2004; Porter et al., 2005; Melching et a., 2006), which may play a role in maintaining normal tissue homeostasis preventing rupture in this exercising breed. Evidence of increased catabolism in Greyhounds CCL associated with high expression of ADAMTS-4 could be linked to increased expression and catabolism of decorin substrate, since we have detected a catabolic fragment below the decorin core protein at 30kDa (Fig. 5A, black arrow), and we suggest that this fragment could be generated from ADAMTS-4 activity. Fibromodulin gene expression (Fig. 2F) and core protein at 50kDa (Fig. 8A) were shown to be significantly higher in Staffordshire bull terrier CCLs. Immunolocalisation of fibromodulin highlighted fibrocartilaginous type cells intracellularly and pericellularly (Fig. 8B) in Staffordshire bull terriers CCLs. The significant presence of fibromodulin could be associated with the development of more fibrocartilaginous type cells secondary to compression, indicative of more fibrocartilaginous matrices in Staffordshire bull terrier CCLs. Immunohistochemistry staining of fibromodulin was similarly found in fibrocartilaginous type cells at the enthesis site of human flexor carpi ulnaris tendon, pisometacarpal ligament, and pisohamate ligament(Adamczyk et al., 2008).

Densitometry of Western blot analysis showed Chondroitin-6-sulphate stubs were significantly higher in Staffordshire bull terrier compared to Greyhound CCLs (Fig. 10A). It is known that native chondroitin-6-sulphate increases in compressive areas in tendon (Huisman et al., 2014). This finding is consistent with fibromodulin previous findings in that Staffordshire bull terrier CCLs may have more fibrocartilaginous ECM. Interestingly, Table 5 highlights that most proteoglycans examined were located within the interfascicular regions in both breeds. However the “compressive’ proteoglycans tended to be located intracellularly in the Staffordshire bull terrier CCLs with little or no evidence of these proteoglycans being located intracellularly in the Greyhound CCL samples. The small sample size and lack of comprehensive history for all collected canine CCL samples was one of the limitations of this study, however, we believe that the significant changes found, particularly in decorin gene expression, between breeds are likely to be substantial findings not confounded by factors present in the signalment of the dog groups examined which were age-matched as much as possible.

## **Conclusions**

## Gene expression and protein content of proteoglycans vary in CCLs from dog breeds with a differing predisposition to CCLD/R. Increased decorin and ADAMTS-4 gene expression in Greyhound CCLs may help to maintain CCL strength and homeostasis. The presence of fibromodulin, and chondroitin-6-sulphate stubs in Staffordshire bull terrier CCLs could be due to an increase in fibrocartilaginous ECM as a response to higher compressive loads. The compressive loading in the CCL of Staffordshire bull terrier might lead to higher levels of stress, therefore predisposing the CCL to disease and rupture.

**Conflict of interest statement**

The author declares that there are no financial or personal relationships with other people or organisations that could inappropriately inﬂuence or bias the content of the paper.

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**Supplementary material**

Supplementary data associated with this article can be found, in the online version, at https:// doi.org/ XXXX

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**Table 1**

Summary of the age, bodyweight and gender of cadaveric canine cranial cruciate ligament (CCL) samples. RT-qPCR, reverse transcription quantitative real-time-PCR; S, Staffordshire bull terrier; G, Greyhound; N/ A, not available.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Method | Biochemical analysis | Western blot analysis | RT-qPCR | Immunohistochemistry |
| Number of S donors | *n* = 5 | *n* = 5 | *n* = 5 | *n* = 5 |
| S age (mean ± SD), *n* = 5 | Skeletally mature | Skeletally mature | Skeletally mature | Skeletally mature |
| S weight (mean ± SD), *n* = 5 | 20 ± 1.7 kg | 20.3 ± 2.8 kg | 19 ± 1.7 kg | 18.2 ± 2.3 kg |
| S gender, *n* = 5 | Female, *n* = 5 | Female, *n* = 5 | Male, *n* =4 Female, *n* = 1 | Male, *n* = 4  Female, *n* = 1 |
| Number of G donors | *n = 5* | *n = 5* | *n = 5* | *n = 5* |
| G age, *n* = 5 | Skeletally mature | Skeletally mature | Skeletally mature | Skeletally mature |
| G weight (mean ± SD), *n* = 5 | N/ A | N/ A | 25 ± 1.2 kg | N/ A |
| G gender, *n* = 5 | N/ A | N/ A | Male, *n* = 1  Female, *n* =2  N/ A, *n* = 2 | N/ A |

**Table 2**

Reference and target gene sequences for real-time qRT-PCR.RT-qPCR, reverse transcription quantitative real-time-PCR.

|  |  |
| --- | --- |
| Gene | Sequence |
| GAPDH a | Forward 5' CTGGGGCTCACTTGAAAGG 3'  Reverse 5' CAAACATGGGGGCATCAG 3' |
| Aggrecan a | Forward 5' GGGACCTGTGTGAGATCGAC 3'  Reverse 5' GTAACAGTGGCCCTGGAACT 3' |
| Versican a | Forward 5' GGGACCTGTGTGAGATCGAC 3'  Reverse 5' GTAACAGTGGCCCTGGAACT 3' |
| Decorin a | Forward 5' CGCTGTCAGTGCCATCTC 3'  Reverse 5' GGGGGAAGATCTTTTGGTACTT 3' |
| Biglycan a | Forward 5' CAGAACAACGACATCTCAGAGC 3'  Reverse 5' TCACCAGGACGAGAGCGTA 3' |
| Lumican a | Forward 5' ACCTGGAAATTCTTTTAATGTATCATC 3'  Reverse 5' CGGTATGTTTTTAAGCTTATTGTAGGA 3' |
| Fibromodulin b | Forward 5' CCTCCAAGGCAATAGGATCA 3'  Reverse 5' GAAGTTCATGACGTCCACCAC 3' |
| ADAMTS-4 a | Forward 5' GACCAGTGCAAACTCACCTG 3'  Reverse 5' CAGGGAGTCCCATCTACCAC 3' |
| ADAMTS-5 a | Forward 5' TGGGTTCCCAAATATGCAG 3'  Reverse 5' CTGTCCCATCCGTCACCT 3' |

a Gene sequence adapted from Clements and others (Clements et al., 2006; Clements et al., 2008).

b Gene sequence adapted from Yang and others (Yang et al., 2012).

**Table 3**

Primary antibodies used in Western blot analysis and immunohistochemical staining.

|  |  |
| --- | --- |
| Primary antibody (dilution for western blot) {dilution for immunohistchemistry} | Code |
| Anti-decorin mouse monoclonal antibody IgG (1:200) {1:50} a | DECN 70.6 |
| Anti-biglycan mouse monoclonal antibogy IgG (1:200) {1:50} a | PR8A4 |
| Anti-lumicanmouse monoclonal antibody IgM (1:200) {1:50} a | LUM1 |
| Anti-keratocan mouse monoclonal antibody IgG (1:200) {1:50} a | KER1 |
| Anti-fibromodulin rabbit polyclonal antibody IgG (1:500) {1:50} b | PR184 |
| Anti-aggrecan mouse monoclonal antibody IgG (1:500) {1:50} a, d | 7D1 |
| Anti-versican mouse monoclonal antibody IgG (1:200) {1:50} c | 12C5 |
| Anti-ChABC generated chondroitin-6-sulphate stub mouse monoclonal antibody IgG (1:200) a | 3B3 |
| Anti-ChABC generated chondroitin-4-sulphate stub mouse monoclonal antibody IgG (1:200) a | 2B6 |
| Anti-ChABC generated chondroitin-0-sulphate stub mouse monoclonal antibody IgG (1:200) a | 1B5 |
| Anti-KI generated keratan sulphate stub mouse monoclonal antibody IgG (1:200) a | BKS1 |

aDonated by Professors Bruce Caterson and Clare Hughes.

bDonated by Professor Peter Roughley.

cAntibody purchased from developmental ctudies of hybridoma bank, University of Iowa, department of biological sciences, USA.

d7D1 epitope detects the first globular domain fragment of the aggrecan core protein.

**Table 4**

Densitometry results from Western blot analysis for proteoglycans and glycosaminosglycans (GAGs) found in the canine cranial cruciate ligaments (CCLs) from differentially predisposed dog breeds to cranial cruciate ligament disease/rupture (CCLD/ R). Values shown as mean ± SD of arbitrary units, *n* = 5 of each dog breed. S, Staffordshire bull terrier; G, Greyhound; HMW, high molecular weight; LMW, low molecular weight.

|  |  |  |
| --- | --- | --- |
| Proteoglycan/ glycosaminoglycan *P* value at *P* < 0.05 | S | G |
| Aggrecan core protein fragment at 50 kDa *P*=0.21 | 530.7±385.2 | 248.9±252.4 |
| Versican core protein fragments at 40-260 kDa *P*=0.55 | 4873.6±630.8 | 5335.8±1518.1 |
| Decorin core protein at 40-50 kDa *P*=0.48 | 8283.8±849.5 | 7902.5±765.1 |
| Decorin band at 110 kDa a *P*=0.008 | 3507.3±1079.7 | 5442.6±391.9 |
| Biglycan core protein at 40-50 kDa *P*=0.14 | 532.9±95.5 | 764.3±300.2 |
| Lumican core protein at 50 kDa *P*=0.78 | 3314.1±573.1 | 3145±1192.9 |
| Fibromodulin core protein at 50 kDa a *P*=0.02 | 5084.5±798 | 2554±1666.3 |
| Keratocan core protein at 30 kDa *P*=0.45 | 4648.4±1076.3 | 3813±1796.6 |
| Chondroitin-0-sulphate stub ≤ 260 kDa *P*=0.29 | 4848±6035.1 | 1749±983.6 |
| Chondroitin-4-sulphate stub (HMW at 80-260 kDa) *P*=0.19 | 8945.6±1042.4 | 7408.6±2192 |
| Chondroitin-4-sulphate stub (LMW at 40-50 kDa) *P*=0.29 | 7669.5±1395.6 | 6835.5±876.7 |
| Chondroitin-6-sulphate stub at 80-260 kDa a *P*=0.01 | 6357.5±1354 | 3603.5±1442.5 |
| Keratan sulphate stub at 80-260 kDa *P*=0.30 | 2842.5±1796.2 | 3596.9±1138.5 |

a indicates a significant difference at P < 0.05 was found.

**Table 5.**

Immunohistochemical staining summary of proteoglycans in the CCL of different dog breeds. S, Staffordshire bull terrier; G, Greyhound; CCL, cranial cruciate ligament; ++ , present; +, present in low amounts; -, absent; +/-, rarely observed.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Dog breed | CCL Region | Aggrecan | Versican | Decorin | Biglycan | Lumican | Fibromodulin | Keratocan |
| S | Interfascicular | ++ | ++ | ++ | +/- | ++ | ++ | ++ |
| Collagen bundles | ++ | +/- | ++ | +/- | +/- | +/- | +/- |
| Intracellular | ++ | - | - | ++ | - | ++ | - |
| Pericellular | - | - | - | - | - | ++ | - |
| G | Interfascicular | ++ | ++ | ++ | +/- | ++ | ++ | ++ |
| Collagen bundles | ++ | +/- | ++ | +/- | +/- | +/- | +/- |
| Intracellular | +/- | - | - | + | - | + | - |
| Pericellular | - | - | - | - | - | + | - |

**Figure legends**

Fig. 1. Water content and sGAG content in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Water content presented as % of wet weight/ mg of CCL dry weight. Staffordshire bull terrier CCLs contained significantly higher % water content when compared to Greyhound CCL (*P*=0.002). B) sGAGs presented as % of CCL dry weight. Values are shown as mean±SD. \* indicates a significant difference at *P* < 0.05, *n* = 5 of each dog breed. CCL, Cranial cruciate ligament; sGAG, Sulphated glycosaminoglycans; S, Staffordshire bull terrier; G, Greyhound.

Fig. 2. Aggrecan (A) Versican (B) Decorin (C) Biglycan (D) Lumican (E) Fibromodulin (F) ADAMTS4 (G) and ADAMTS5 (H) ΔCT values as determined by RT-qPCR in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Aggrecan gene expression was shown to be significantly higher in the Staffordshire bull terrier CCLs compared to those of the Greyhounds (*P*=0.0003). B) There were no statistically significant differences in versican gene expression between the Staffordshire bull terrier and Greyhound CCLs. C) Decorin gene expression was shown to be significantly lower in the Staffordshire bull terrier CCLs compared to those of the Greyhounds (*P*=0.03). D) There were not any statistically significant differences in biglycan gene expression between the Staffordshire bull terrier and Greyhound CCLs. E) There were no statistically significant differences in lumican gene between Staffordshire bull terrier and Greyhound CCLs. F) Fibromodulin gene was shown to be significantly higher in the Staffordshire bull terrier CCLs compared to the Greyhound CCLs (*P*=0.03). G) ADAMTS4 gene was shown to be significantly lower in the Staffordshire bull terrier CCLs compared to Greyhound CCLs (*P*=0.04). H) There were no statistically significant differences in ADAMTS5 gene between the Staffordshire bull terrier and Greyhound CCLs. Values shown as mean±SD, *n* = 5 of each dog breed \*indicates a significant difference at *P* < 0.05, *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 3. Aggrecan protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Aggrecan core protein determination using Western blot analysis: The core protein fragment(s) of aggrecan is shown at 50 Kda and is labelled in green. B) Aggrecan localisation as determined by immunohistochemistry: There is prominent staining for the aggrecan antiboody intracellularly (within the cells) of the Staffordshire bull terrier CCLs (blue arrow, S), whereas little staining of aggrecan antibody was evident intracellularly in the Greyhound CCL (blue arrow, G). There is also evidence of aggrecan staining in the collagen bundles (white arrow, S) (Bar 100μm), *n* = 5 of each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 4. Versican protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Versican core protein determination using Western blot analysis: The fragment(s) of verscian core protein is shown between 40-260kDa and is labelled in green. B) Versican localisation as determined by immunohistochemistry: Versican antibody staining tends to be more prominent in the interfascicular regions (black arrow, S and G) compared to the collagen bundles (white arrow, S and G) in both dog breeds (Bar 100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 5. Decorin protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Decorin core protein determination using Western blot analysis: The core protein of decorin is shown at 40-50kDa and is marked in green. Other bands of interest are the 110kDa band marked in purple, and the faded band at 30kDa (black arrow). Decorin 110kDa band was significantly higher in Greyhound (*P*=0.008) compared to Staffordshire bull terrier CCL. B) Decorin localisation as determined by immunohistochemistry: Decorin antibody was found to stain the interfascicular regions and collagen bundles (white arrow, S and G) in the CCLs from both dog breeds (Bar 100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 6. Biglycan protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Biglycan core protein determination using Western blot analysis: the core protein of biglycan is shown at 40-50kDa and is marked in green. B) Biglycan localisation as determined by immunohistochemistry: Biglycan antibody was localised in rounded cells (blue arrow, S) of the Staffordhsire bull terrier CCLs compared to those of the Greyhounds (blue arrow, G). Biglycan also show evidence to stain the collagen bundles (white arrow, S) in Stafforshire bull terrier CCLs. (Bar 100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 7. Lumican protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Lumican core protein determination using Western blot analysis: the core protein of lumican is shown at 50kDa and is marked in green. B) Lumican localisation as determined by immunohistochemistry: staining for the lumican antibody is evident in the collagen bundles (white arrow, S) and in the interfascicluar regions (black arrow, G) of both dog breeds (Bar 100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 8. Fibromodulin protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Fibromodulin core protein determination using Western blot analysis: The core protein of fibromodulin is shown at 50kDa and is marked in green. Fibromodulin was significantly higher in Staffordshire bull terrier (*P*=0.02) compared to Greyhound CCLs. B) Fibromodulin localisation as determined by immunohistochemistry: staining of the fibromodulin is evident around the collagen bundles (white arrow, S and G) in both dog breeds, and in the interfascicular region of the Staffordshire bull terrier CCL. There is also evidence of fibromodulin staining in intrafascicluar regions (black arrow, S) in Staffordshire bull terrier CCLs. A greater intensity of antibody staining was found around the rounded ligament cells in Staffordshire bull terrier CCLs (blue arrow, S) (Bar 100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 9. Keratocan protein expression determined by Western blot analysis and immunohistochemistry in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Keratocan core protein determination using Western blot analysis: The core protein of keratocan is shown at 30kDa and is marked in green. B) Keratocan localisation as determined by immunohistochemistry: there is evidence of keratocan antibody staining around the collagen bundles (white arrow, S and G) in both dog breeds. There is also evidence of keratocan staining in intrafascicluar regions in Staffordshire bull terrier CCLs (Black arrow, S). (Bar100μm), *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.

Fig. 10. Chondroitin sulphate and keratan sulphate stubs core protein determination by using Western blot analysis in two dog breeds with a different predisposition to cranial cruciate ligament disease and rupture. A) Chondroitin-6 sulphate stubs ranged between 80-260kDa. B) Chondroitin-4 sulphate stubs. C) Chondroitin-0 sulphate stubs. D) Keratan sulphate stubs. The high molecular weights of chondroitin -6, -4, -0, keratan sulphate stubs (80-260 kDa), and chondroitin sulphate stubs (260 kDa and above) are highlighted in green, and the low molecular weight of chondroitin -4 sulphate stubs (40 kDa) are highlighted in purple. Chondroitin-6-sulphate stubs core protein at >260kDa was found to be significantly higher in Staffordshire bull terrier (*P*=0.01) compared to Greyhound CCLs, *n* = 5 for each dog breed. CCL, Cranial cruciate ligament; S, Staffordshire bull terrier; G, Greyhound.