**Original Article**

**Investigation of fibrillin microfibrils in the canine cruciate ligament in dogs with different predispositions to ligament rupture.**

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**Abstract**

Cranial cruciate ligament disease (CCLD) is the most common cause of pelvic limb lameness in dogs but its precise aetiopathogenesis is uncertain. Fibrillin microfibrils (FM) are complex macro-molecular assemblies found in many tissues including ligaments, where they are thought to play an important mechanical role. We hypothesised that FM ultrastructural variation correlates with the differing predisposition of canine breeds to CCLD.

Non-diseased cranial and caudal cruciate ligaments (CCLs and CaCLs) were obtained from Greyhound (GH) and Staffordshire Bull Terrier (SBT) cadavers. Fibrillin microfibrils were extracted from the ligaments by bacterial collagenase digestion, purified by size-exclusion chromatography and subsequently visualized by atomic force microscopy (AFM). With AFM, FMs have a characteristic beads-on-a-string appearance. For each FM, periodicity (bead-bead distance) and length (number of beads/FM) was measured.

Fibrillin microfibril length was found to be similar for GH and SBT, with non-significant inter-breed and inter-ligament differences. Fibrillin microfibril periodicity varied when comparing GH and SBT for CCL (GH 60.2 ± 1.4nm; SBT 56.2 ± 0.8nm) and CaCL (GH 55.5 ± 1.6nm; SBT 61.2 ± 1.2nm). A significant difference was found in the periodicity distribution when comparing CCL for both breeds (*P*<0.00001), further, intra-breed differences in CCL vs CaCL were statistically significant within both breeds (*P*<0.00001). The breed at low risk of CCLD exhibited a periodicity profile which may be suggestive of a repair and remodelling within the CCL.

*Keywords:* fibrillin microfibrils; canine; cranial cruciate ligament; atomic force microscopy

**Introduction**

Cranial cruciate ligament disease (CCLD) is the most common cause of pelvic limb lameness in dogs (Lodato et al., 2013; Whitehair et al., 1993) with some breeds such as Rottweilers, Newfoundlands and Staffordshire Bull Terriers (SBT) having a greater predisposition for ligament rupture (Whitehair et al., 1993; Witsberger et al., 2008).

Despite the high level of incidence of CCLD within certain dog breeds (Duval et al., 1999; Whitehair et al., 1993) its precise aetiopathogenesis is complex, multifactorial and uncertain (Comerford et al., 2011; Cook, 2010; Griffin et al., 2000). Canine CCLD has been widely compared to non-contact anterior cruciate ligament (ACL) injury in man where similar degenerative changes are reported (Griffin et al., 2000; Hasegawa et al., 2013; Hasegawa et al., 2012). In the dog; breed, genetics, inflammation, changes to extracellular matrix (ECM) homeostasis, stifle joint laxity and instability, stifle conformation and abnormal gait have all been postulated in the aetiopathogenesis for CCLD previously (Comerford et al., 2011; Comerford et al., 2006; Duerr et al., 2007; Duval et al., 1999; Gyger et al., 2007; Hayashi et al., 2003; Inauen et al., 2009; Mostafa et al., 2010).

 Canine CCLD is recognised as ECM degradation eventually leading to ligament rupture (Comerford et al., 2011). Within the ECM of ligaments, collagen is the main structural component whilst elastin, proteoglycans and glycoproteins are considered minor components (Frank, 2004). Nevertheless, elastin is considered to play an increasingly important role in the mechanical behaviour of ligaments (Frank, 2004; Henninger et al., 2019). Elastin fibres are a central cross-linked core of highly extensible elastin surrounded by a sheath of fibrillin microfibrils (FM) (Kielty, 2006). Fibrillin microfibrils are complex macro-molecular assemblies composed of fibrillin-1 and numerous accessory proteins (Sherratt et al., 2003) which are thought to play important structural roles in ligaments (Jensen et al., 2012), ocular tissues (Ashworth et al., 2000) and arteries (Wagenseil and Mecham, 2009). Bundles of FMs are known as oxytalan fibres and collectively, oxytalan and elastin fibres are referred to as elastic fibres (Smith et al., 2014). An abundance of elastic fibres in the human ACL has been described (Strocchi et al., 1992) and more recently, elastin content was reported up to 19% dry weight of ligament tissue in canine cruciate ligaments (CL) (Smith et al., 2014). Within ligaments, elastic fibres help to mediate reversible elasticity (Eriksen et al., 2001) and are the dominant structural protein resisting transverse and shear deformation (Henninger et al., 2015).

 Our group has previously used histological staining to examine the organisation of elastin and FMs in the canine CL complex and observed that FMs were abundant throughout (Smith et al., 2011). Furthermore we found a positive correlation between increasing CL degradation and increased oxytalan fibre staining (Smith et al., 2014) suggesting that oxytalan fibres have an important reparative role in response to ligament loading (Smith et al., 2014). Oxytalan fibres are bundles of FMs, and FM ultrastructure can be examined using atomic force microscopy (AFM). When viewed using AFM, FMs take a bead-on-a-string appearance (Baldock et al., 2001), with length defined as the number of beads and periodicity as the bead-to-bead distance (Sherratt et al., 2005).

Studies examining FM ultrastructure have reported periodicity and length in numerous human and animal tissues, and *in vitro* (Akhtar et al., 2014; Baldock et al., 2001; Holmes et al., 2001; Kuo et al., 2007; Sherratt et al., 1997). Furthermore, studies have highlighted FM length and periodicity changes associated with disease, damage accumulation and remodelling (Akhtar et al., 2014; Eckersley et al., 2018). There is increasing evidence and interest in tissue specific FM ultrastructural diversity and the effect of damage accumulation (Eckersley et al., 2018; Hibbert et al., 2015). Although elastic fibres have been shown to be increased with ECM degeneration (Smith et al., 2014; Smith et al., 2011), to our knowledge, the ultrastructure of the FMs within the canine CL complex have never been reported. Additionally, ultrastructural differences between CLs in canine breeds at different risks of CCLD have not been investigated.

In this study, we hypothesised that variations in the ultrastructure of FMs would correlate with the differing predisposition of canine breeds to CCLD. To test this hypothesis the following questions were considered: (1) Does the ultrastructure of isolated FMs extracted from both cranial cruciate ligament (CCL) and caudal cruciate ligament (CaCL) differ for two breeds at differing risk of CCLD; Greyhound (GH) (low risk (Whitehair et al., 1993)) and SBT (increased risk (Duval et al., 1999; Whitehair et al., 1993))? (2) Is there a significant difference in the ultrastructure of FMs found in the CCL when compared with the CaCL of each breed?

**Materials and Methods**

*Tissue Samples*

Non-diseased CLs were obtained and pooled from skeletally mature (2 – 5 years old) SBTs (n=5) and ex-racing GHs (n=4) with no macroscopic evidence of stifle pathology. The CLs were from cadaveric material donated to the University of Liverpool for teaching (SBTs) or with full owner consent (GHs) under local institutional ethical guidelines (VREC65). The dogs were euthanised for reasons other than musculoskeletal disease and not for the purposes of this study.

The ligaments were dissected within 24 hours and either snap-frozen in liquid nitrogen, or frozen in Optimal Cutting Temperature (OCT) resin (Sakura Fintek Europe B.V, Alphen aan den Rijn, The Netherlands) in pre-cooled isopentane and stored at -80oC until required (as described previously (Akhtar et al., 2014)).

*Isolation and Imaging of Fibrillin microfibrils*

Fibrillin microfibrils were extracted from the CLs by bacterial collagenase digestion, purified by size-exclusion chromatography at physiological pH and visualized by atomic force microscopy (AFM) (Kielty et al., 1991; Sherratt et al., 2003). The tissue was incubated with 1mg/ml type 1A bacterial collagenase in a high salt buffer (400mM NaCl, 50mM Tris–HCl, pH 7.4) supplemented with 10mM CaCl2 and protease inhibitors (2mM PMSF, 5mM NEM) for four hours at room temperature. All of these reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK). Digested samples were centrifuged at 5000g for five minutes and the supernatant was size fractionated using an AKTA primeplus system (GE Healthcare, Buckinghamshire, UK) on a Sepharose CL-2B column in the high salt buffer. The microfibril-rich excluded volume was used for subsequent investigations.

*Atomic force microscopy image analysis*

Atomic force microscopy images were analysed to characterise FM morphology in both groups. The images were analysed to determine FM length (number of beads per microfibril) and periodicity (bead-to-bead distance), as shown in the representative Figure 1.

 A computer code was written in Image SXM (Barrett, 2008) to allow semi-automated analysis of the AFM images to determine FM length and periodicity. The AFM images were loaded with 'line-by-line compensation' to reduce the scan line noise. The image was then inverted (to display dark beads on a light background) to enhance contrast. For each FM identified in the image, the x-y position of each bead was recorded to subsequently calculate FM periodicity and length. Table 1 provides a summary of each of the ligaments that were imaged and subsequently analysed.

*Statistical methods*

Data are expressed as means ± SEM. Standard deviation (SD) for periodicity are also reported. The Mann-Whitney U test was used for statistical analysis to compare FM periodicity and length measurements in the two breeds (where *P*<0.05 was considered statistically significant). The Kolmogorov–Smirnov test was used for statistical analysis of the periodicity distributions of the two groups, and for comparison of the two types of ligaments (CCL and CaCL). The kurtosis parameter was also used to compare periodicity distributions. Outliers were assessed by examining the percentage of values that were greater or less than two SD of the control mean (outliers) as described previously (Hibbert et al., 2019). All the statistical analysis was conducted with OriginPro 8.6 (OriginLab Corporation, Northampton, MA, USA).

**Results**

*Fibrillin microfibril length*

Abundant FMs were isolated and extracted from both SBT and GH ligaments (Figure 2). The length of FMs for the GH and SBT are shown in Figure 3. For the CCLs, the mean length for the GH and SBT groups was 31.4 ± 4.4 and 26.6 ± 3.4 respectively. There was no significant differences between the lengths of the two groups (Mann-Whitney U Test, *P*= 0.15). For the CaCLs, the mean length for the GH and SBT groups was 21.5 ± 2.8 and 21.5 ± 2.1. There was no significant difference between the lengths of the two groups (Mann-Whitney U Test, *P*=0.46).

*Fibrillin microfibril periodicity breed differences in CCL and CaCLs*

The mean FM periodicity for the groups is presented in Figure 4A ad Table 1 for each animal for both CCL and CaCL. The difference amongst the two breeds for both the CCLs and CaCLs were analysed in two ways. Firstly, box and whisker plots were used to compare the two ligaments for GH vs SBT, as shown in Figure 4B. The mean FM periodicity for both ligaments were more variable (large standard deviation) in the GH group (CCL SD=8.4nm; CaCL SD=11.3nm) as compared to the SBT group (CCL SD=6.8nm; CaCL SD=8.4nm). We found that the CaCLs were significantly different when comparing FM periodicity of pooled GH vs SBT samples (*P*<0.00001) whereas the difference in the CCL FM periodicity between pooled samples of both breeds was not statistically significant (*P*=0.0599) (Figure 4B). The median FM periodicity values for the GH and SBT CCLs were 57.8nm and 55.8nm respectively and for the CaCLs, median values were 53.3nm and 58.5nm respectively. The mean FM periodicity values for the GH and SBT CCL were 60.2 ± 1.4nm and 56.2 ± 0.8nm respectively and for the CaCL, mean values were 55.5 ± 1.6nm and 61.1 ± 1.2nm respectively (Figure 4B).

*Fibrillin microfibril periodicity distributions*

To determine overall trends, FM periodicity distribution was analysed in-depth by pooling together all replicates from each ligament, as shown in Figure 5. These plots are composed of each measured periodicity with a minimum of 1000 measurements in each group.

Histogram distributions demonstrated interesting trends when comparing inter-breed and intra-breed variations in the two ligaments. When examining the CCL in the GH vs SBT there was a significant difference in the periodicity distribution (Kolmogorov-Smirnov Test, *P*<0.00001) with the difference largely being in the periodicities above 65nm (Figure 5A). When examining CaCL in GH vs SBT, it was found that the two distributions were approximately centered around the same value (60nm), but the GH distribution was sharper with less values above 70nm (Kolmogorov-Smirnov Test, *P*<0.00001) (Figure 5B). We also examined intra-breed differences in CCL vs CaCL (Figures 5C and 5D). In each case, the differences in CCL and CaCL were statistically significant within the SBT and GH (Kolmogorov-Smirnov Test, *P*<0.00001). However, for SBT it was noted that the shape and the peak center of the distribution for the two ligaments differed more than that for GH. The SBT CCL (Figure 5C) followed a sharper peak centred at 58.7nm as compared to a broader curve for the SBT CaCL which had peaks at 58.8nm and 63.8nm. In contrast, GH CCLs and CaCLs (Figure 5D) both exhibited sharp distributions which were approximately similarly centred (63.75nm and 61.25nm respectively).

The kurtosis parameter and outlier assessment showed consistency with the periodicity distributions shown in Figure 5 (Table 2). Specifically, the GH CCL had a higher percentage of periodicities within the tails of the distribution (kurtosis 8.79), in comparison to the SBT CCL (kurtosis 4.31). The outlier calculation showed that for GH CCL, a greater percentage of outliers were located in the tail associated with the higher periodicities (3%) as compared to the tail associated with lower periodicities (1.1%). In comparison, for SBT CCL, the outliers were split approximately equally amongst the two tails (Table 2).

**Discussion**

We hypothesised that differences in FM ultrastructure may explain the relative prevalence of CCLD in dog breeds with a differing predisposition to ligament rupture. This study compared the morphology of FMs from two breeds; GH (low risk) and SBT (increased risk) and to the best of our knowledge is the first to report FM ultrastructure in canine ligaments.

We have found that the mean FM length for GH and SBT CCLs was 31.4 and 26.6 respectively. For the CaCLs the mean FM length was 21.5 for both the GH and SBT. Previous studies have noted normal FM length in the aorta as 21 and skin as 27 (Akhtar et al., 2014; Sherratt et al., 2010). The lack of very short FM lengths and, of inter- and intra-ligament differences would suggest fragmentation does not lead to a predisposition for CCLD.

For the CCL, the mean FM periodicity in the GH and SBT was 60.2nm and 56.2nm, whereas the mean periodicity for CaCL in GH and SBT was 55.5nm and 61.2nm (Figure 4). Studies of un-tensioned FMs have reported mean periodicities of between 55nm – 57.2nm (Akhtar et al., 2014; Baldock et al., 2001; Kielty et al., 1998; Lu et al., 2005), including 56.1nm in bovine nuchal ligaments (Sherratt et al., 1997). To date, we do not know of any literature reporting periodicity measurements for the human or canine CLs. Our findings suggest the FM periodicity within the canine CL is greater than what is currently reported in other tissues and species. Recent literature on FM ultrastructure showed that morphology and periodicity was tissue source-dependent (Eckersley et al., 2018). Therefore, apparent periodicity differences could be attributable to species variation or due to functional differences associated with CL tissue.

Periodicity distributions were significantly different both within breed and between breeds (Figure 5). When comparing FM periodicities between SBT and GH CCLs, the data showed that GHs tended to have a larger percentage of periodicity measurements above 65nm (36.3% for GH vs 24.8% for SBT), and SBTs had a larger percentage of periodicity measurements below 50nm (13.7% for GH and 16.9% for SBT). An opposite relationship was found when comparing the CaCL periodicity data between SBT and GH (<50nm: 22.7% for GH and 17.9% for SBT, >65nm: 25.6% for GH and 37% for SBT). For the SBT, the CCL distribution follows a very sharp distribution relative to the CaCL (Figure 5C). In contrast, for the GH the two ligaments have a similar peak sharpness, however for the CCL the higher number of periodicities >65nm is clearly visible (Figure 5D).

Newly synthesised cultured FM periodicities have been shown to be significantly higher than mature ex vivo-derived cells (Eckersley et al., 2018). It could be postulated that the significantly increased periodicity distribution in the GH may be associated with damage repair and new FM synthesis possibly acting as a protective remodelling within the breed at low risk of CCL rupture. Our previous work has demonstrated that CLs from ‘non-diseased’ ex-racing GHs exhibited histological changes consistent with mild degeneration and suggested that these changes might be a physiological response to normal CL loading and/or exercise in this breed (Smith et al., 2014). Our findings on isolated FMs mimic the trends observed in pathological tissue such as in the diabetic aorta where there is a profound change in FM periodicity (Akhtar et al., 2010). However, in the case of the GH CCL, these changes are not associated with any pathological condition. In contrast, damage accumulation to the SBT CCL could explain the lower periodicity shift and subsequently a lack of repair initiation potentially seen in the GH CCL.

Studies have reported stretching potential of FMs and suggest that individual FMs are elastic (Holmes et al., 2001; Keene et al., 1991). Therefore, given the reduced number of larger periodicities within the SBT CCL, it could be hypothesised that the SBT CCL is more vulnerable to insult from loading and, the increased periodicity of GH FMs may provide greater resistance to stretching resulting in protection from CCL rupture.

The SBT had larger periodicity distributions on analysis of the CaCL in comparison to the CCL whereas in the GH group the opposite was true (Figure 5C and 5D). The data showed that the CCL and CaCL periodicity measurements within the GH were much more similar with regards periodicity curves and peaks, in comparison to the SBT ligaments for which the curve shapes and peaks were less closely matched (Figure 5C and 5D). Caudal CL disease incidence is unknown within dogs but isolated rupture is accepted as a rare occurrence (Kowaleski et al., 2018), however similar to the CCL in GHs, the increased periodicity associated with the CaCL of the SBT may be a consequence of damage repair in a breed at high risk of stifle disease.

There were several limitations to this study, including the small number of dogs from which CLs were recruited. The small number of dogs may have led to statistical errors however to counter-act this limitation a substantial number of periodicity measurements (n>500/dog) were obtained to provide a good representation of FM morphology in each animal and across the two breeds. There is also the potential for individual FM lengths to skew the periodicity measurements, for example, a few very long FMs may influence the periodicity data and subsequent statistical analysis. The large number of periodicity measurements were performed to counter-act such a limitation similar to previous studies (Akhtar et al., 2014).

A further limitation was the lack in detail specifically associated with the gender and neuter status. This was a consequence of obtaining ethically sourced donated clinical research material. Although these details may have been useful, the association with gender or neutering status and CCLD is contested within veterinary literature (Adams et al., 2011; Duval et al., 1999; Ekenstedt et al., 2017; Guthrie et al., 2012; Witsberger et al., 2008).

Finally, there may be a possible limitation in the manner in which the FMs are extracted and isolated in this study potentially inducing structural and compositional differences. However, this was addressed by ensuring that all of the samples were treated with the same protease and subjected to the same buffer conditions.

**Conclusions**

In conclusion, our study has reported the FM length and periodicity of the CCL and CaCL in two different canine breeds with differing risks to CCLD and rupture. There was no significant difference between the FM length between the breeds suggesting the number of beads does not influence CCLD. However, there was a significant difference in FM periodicity distributions for the CCL and CaCL between the breeds. The breed at low risk of CCLD had a higher proportion of CCL periodicity measurements above 65nm suggestive of a repair and remodelling mechanism protecting against CCLD.

**Conflict of interest statement**

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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**Table 1**

Summary of canine cruciate ligaments (CLs) fibrillin microfibrils that were imaged with AFM and the resulting mean (SD) for each ligament.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample ID** | **Canine Breed** | **Ligament Type** | **Number of microfibrils imaged** | **Periodicity (nm)****Mean (SD)** |
| GH1 | GH | CCL | 20 | 57.0 (6.3) |
| GH2 | GH | CCL | 16 | 64.1 (9.2) |
| GH3 | GH | CaCL | 30 | 54.7 (12.3) |
| GH4 | GH | CaCL | 20 | 56.8 (9.8) |
| SBT1 | SBT | CCL | 14 | 55.3 (4.4) |
| SBT2 | SBT | CCL | 38 | 55.5 (5.8) |
| SBT3 | SBT | CCL | 18 | 58.6 (9.8) |
| SBT4 | SBT | CaCL | 26 | 63.3 (9.0) |
| SBT5 | SBT | CaCL | 27 | 59.1 (7.4) |

**Table 2**

Summary of data when all periodicity values were pooled together to examine the fibrillin microfibril periodicity distributions for each ligament comparing inter- and intra-breed differences. The kurtosis parameter and percentage outliers were determined.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | N | Mean (nm) | SD (nm) | Kurtosis | 2SD (nm) | Mean + 2SD(nm) | Mean – 2SD (nm) | Overall outliers (%) | Minimum valueoutlier (%) | Maximum value outlier (%) |
| GH CCL | 1000 | 63.03 | 14.5 | 8.79 | 29.0 | 92.1 | 33.1 | 4.1 | 1.1 | 3 |
| GH CaCL | 1000 | 60.0 | 15.8 | 2.22 | 31.7 | 91.7 | 28.3 | 6.4 | 1.4 | 5 |
| SBT CCL | 1500 | 59.1 | 12.0 | 4.31 | 24.1 | 83.2 | 35.1 | 5.3 | 2.5 | 2.7 |
| SBT CaCL | 1000 | 62.0 | 14.0 | 1.40 | 28.1 | 90.1 | 33.9 | 4.8 | 1.3 | 1.3 |

**Figure Legends**

**Figure 1** Representative microfibril atomic force microscopy (AFM) image from a Greyhound (GH) showing length (number of beads) and periodicity (bead-bead distance) (nm).

 **Figure 2** Representative atomic force microscopy (AFM) images showing isolated microfibrils (FMs) from cranial cruciate ligaments (CCLs) for (A) Greyhound (GH) (Sample ID GH2) and (B) Staffordshire Bull Terrier (SBT) (Sample ID SBT3).

 **Figure 3** Fibrillin microfibril lengths (A) cranial cruciate ligament (CCL) and caudal cruciate ligament (CaCL) shown for each animal within each breed (B) CCL and CaCL averaged for each breed (GH-greyhound and SBT-Staffordshire bull terrier)

 **Figure 4** Fibrillin microfibril periodicity (nm) (A) cranial cruciate ligament (CCL) and caudal cruciate ligament (CaCL) shown for each animal within each breed (B) CCL and CaCL averaged for each breed (GH-greyhound and SBT-Staffordshire bull terrier).

**Figure 5** Periodicity distribution of fibrillin microfibrils (nm) (A) Greyhound (GH) vs Staffordshire Bull Terrier (SBT) cranial cruciate ligament (CCL) (B) GH vs SBT caudal cruciate ligament (CaCL) (C) GH CCL vs CaCL (D) SBT CCL vs CaCL. In total, there were 1500 measurements for the SBT CCL and 1000 measurements for the GH CCL. There were 1000 measurements for the CaCL in both groups (GH-greyhound and SBT-Staffordshire bull terrier).