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Infrared spectroscopy of synovial fluid as a potential screening approach for the diagnosis of naturally occurring canine osteoarthritis associated with cranial cruciate ligament rupture☆

Sarah Malek [a,*], Federico Marini [b], Mark C. Rochat [a], Romain Béraud [c], Glenda M. Wright [d], Christopher B. Riley [e]

[a] Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, 625 Harrison St., West Lafayette, IN, USA
[b] Department of Chemistry, University of Rome La Sapienza, P.le Aldo Moro 5, I-00185, Rome, Italy
[c] Centre Vétérinaire Daubigny, Quebec, QC, Canada
[d] Atlantic Veterinary College, Department of Biomedical Sciences, University of Prince Edward Island, 550 University Ave., Charlottetown, PE, Canada
[e] School of Veterinary Science, Massey University, Palmerston North, New Zealand

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ABSTRACT

Objective: To evaluate infrared (IR) spectroscopy of synovial fluid (SF) as tool to differentiate between knees of dogs with naturally occurring OA associated with cranial cruciate ligament rupture (CrCLR) and controls.
Method: 104 adult dogs with CrCLR (affected group) and 50 adult control dogs were recruited in a prospective observational study. Synovial fluid (SF) samples were collected preoperatively from dogs with CrCLR and from a subset of these at 4-, and 12-week post-surgery. Knee samples were collected bilaterally once from control dogs. Dried synovial fluid films were made, and IR absorbance spectra acquired. After preprocessing, partial least squares discriminant analysis (PLS-DA) and ANOVA-simultaneous component analysis (ASCA) were used to evaluate group and temporal differences, and to develop predictive models.
Results: There were statistically significant spectral differences between the SF of OA affected and control dogs at all three time-points ($P < 0.001$). Pairwise comparison of spectral SF of knees with CrCLR over time showed statistically significant differences amongst all three time-points ($P < 0.001$). The predictive model for identifying the affected group from control had sensitivity, specificity and overall accuracy of 97.6%, 99.7% and 98.6%, respectively.
Conclusions: The findings demonstrate the ability of FTIR-spectroscopy of synovial fluid combined with chemometric methods to accurately differentiate dogs with OA secondary to CrCLR from controls. The role of this IR-based screening test as a diagnostic and monitoring biomarker for OA specific to the joint being sampled warrants further investigation.

1. Introduction

Canine knee OA, secondary to cranial cruciate ligament rupture (CrCLR), is a common musculoskeletal condition which similar to OA of the human knee, progresses, despite medical and surgical interventions [1–4]. Dogs have been a candidate translational research model for human knee OA due to the similarities in anatomy and the pathophysiology of the disease [5,6]. Naturally occurring OA secondary to CrCLR (a degenerative, non-traumatic condition) is of interest due to the high prevalence of the disease and incidence of the subsequent development of CrCLR in the contralateral knee [7,8]. The clinically stable contralateral knees in dogs with unilateral CrCLR provides an interesting preclinical OA model. Development of disease in the contralateral knee of dogs unilaterally affected with CrCLR has been the subject

☆ This study was conducted at the Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island. The statistical analyses of the spectral data were performed at the Department of Chemistry, University of Rome La Sapienza.

* Corresponding author. Lynn Hall, Dept. of Veterinary Clinical Sciences, Purdue University College of Veterinary Medicine, 625 Harrison St., West Lafayette, IN, 47907-2026, USA.
E-mail addresses: maleks@purdue.edu (S. Malek), federico.marini@uniroma1.it (F. Marini), mrochat@purdue.edu (M.C. Rochat), rberaud@daubigny.ca (R. Béraud), gwright@upei.ca (G.M. Wright), C.B.Riley@massey.ac.nz (C.B. Riley).

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of research directed at understanding the pathophysiology of the OA development and prognosis [9–12]. Ongoing research in human and animal models of knee OA have resulted in identification of various biochemical markers that reflect different aspects of this complex condition [13,14]. However, the complexity of disease phenotype and multifactorial nature of OA warrants further investigation of validity of novel or existing candidates to define their role as disease burden, investigative, prognostic, efficacy of intervention, diagnostic and safety biomarkers [15].

Fourier transform infrared (FTIR) spectroscopy is used in detection of biological patterns based on the infrared-active organic molecular bonds in biological fluids [16,17]. The spectral pattern from each sample is the result of frequency-dependent absorption of infrared (IR) radiation, reflecting the sum of all infrared active organic molecules in that sample [18]. Therefore, the spectrum represents a biochemical "fingerprint" of the tested sample, rather than a few select or known biomarkers [17,19]. Analysis of spectral features using chemometric methods detects differences that are characteristic of a disease or natural state of a biological sample [18]. This technology can be used for automated repetitive reagent-free analyses with small sample volume requirements [17,19]. This approach has shown promising results as a potential screening tool for the diagnosis of human arthritic disorders based on synovial fluid (SF), trauma-induced OA in rabbits (serum), naturally occurring OA associated with CrCLR in dogs (serum), as well as traumatic OA (SF) and osteochondrosis (SF) in horses [20–28]. Despite the overall successful performance of FTIR spectroscopy in distinguishing serum samples of OA dogs from controls, this approach does not have the ability to directly identify contributions to serum from individual joints [28]. To the authors' knowledge, the performance of IR spectroscopy of SF in detecting OA associated with naturally occurring CrCLR in dogs has not been investigated. The first null hypothesis for this study was that FTIR spectroscopy cannot distinguish between knee SF samples of dogs with OA associated with naturally occurring CrCLR, contralateral stable knees of dogs with unilateral CrCLR, and control dogs. The second was that FTIR spectroscopy cannot distinguish between spectra of SF samples from dogs with OA associated with naturally occurring CrCLR and their contralateral stable knees sampled at different intervals after surgical stabilization of the CrCL deficient knee.

2. Method

The Animal Care Committee of the University of Prince Edward Island (#11–062) approved this prospective observational clinical cohort research for which dogs were enrolled after informed written consent was obtained from their respective owners. Sample size calculation was based on a 95% confidence level and 95% power range (for an unmatched case-control study; single control per two OA cases). This resulted estimated group size of 27 dogs in the OA group (effect size of 1.29 was based on a hypothetical 80% detection rate of OA). Due to the lack of data on knee OA prevalence in dogs, the number of dogs was increased to 100 in the OA and 50 in the control groups. This was based on the previous comparable studies in horses [20,21], to accommodate independent validation and calibration data sets. In an effort to account for potential loss of cases during follow up rechecks that required a 45–50 subset of the OA group dogs, and subsequent exclusion or loss of samples, a 10% additional allowance for recruitment was made.

2.1. Animals

OA group

Inclusion criteria for the OA group included client-owned, adult, medium to large breed dogs (>15 kg body weight) presented for naturally occurring (non-acute, non-traumatic) cranial cruciate ligament rupture (CrCLR) of one or both knees. Cases were recruited at the Atlantic Veterinary College, PE, Centre Vétérinaire Laval, QC, and veterinary clinics in Newfoundland and Labrador, Canada. Eligible dogs had no history of previous surgery or treatment with systemic corticosteroids during the four weeks preceding enrolment. All dogs underwent complete physical examination including neurologic and orthopedic exams. Complete blood count and serum biochemistry profile tests were performed to exclude presence of concurrent systemic abnormalities. Clinical diagnosis of CrCLR in one or both knees was based on orthopedic examination [7]. Dogs that were diagnosed with OA or pain in joints other than the knees, and other causes of knee instability (e.g., luxating patella) based on orthopedic examination and confirmed on radiographs were excluded. Both knees in dogs in the OA group were assessed using orthogonal radiographic views to confirm and grade the radiographic signs of OA, and rule-out non-OA radiographic abnormalities. Grading of OA of knee joints was based on a previously described scheme (i.e., osteophytosis (0–3), joint effusion (0–2), intra-articular mineralization (0–2), global score (0–3)) [29]. A subset of OA group dogs (~45) were randomly assigned at their initial visit (T1) to return for two additional rechecks for evaluation and sampling at 4 (T2), and 12 (T3) weeks. At each recheck, physical and orthopedic examinations were performed prior to radiographic imaging of knees under sedation. For dogs with unilateral CrCLR, follow-up telephone calls to owners, primary veterinarians, and examination of medical records were used to obtain information on the fate of the contralateral (stable) knee for a maximum of four years after conclusion of study.

At T1, the unstable knee was explored via medial arthrotomy [30] and presence of CrCLR as well as state of medial and lateral menisci in the joint were grossly assessed, and damaged portions of the ligament and menisci were debrided. All OA group dogs underwent either tibial plateau leveling osteotomy (TPLO) or lateral fabellotibial suture (LFTS) to stabilize the CrCL deficient knee(s) [31,32]. For bilaterally affected dogs, bilateral or staged surgeries were performed based on surgeon and owners' preference. For staged procedures, the second surgery on the contralateral knee was delayed until the dog had completed the study. Pain management postoperatively included hydromorphone (Hydromorphone hydrochloride, 2 mg/mL, Sandoz Canada Inc., Quebec) at 0.05–0.1 mg/kg IV/SC every 4–6 h for 48 h, meloxicam (Metacam®, 1.5 mg/mL, Boehringer-Ingelheim, Burlington, ON) at 0.1 mg/kg, orally, every 24 h for 7–10 days, and tramadol (Tramadol, Chiron, Compounding Pharmacy Inc., Guelph, ON) at 4–8 mg/kg, orally, every 8–12 h for 5–7 days.

Control group

Adult, systemically healthy, medium to large breed dogs (>15 kg body weight) euthanized for reasons unrelated to this project were included. The control dogs had no orthopedic or systemic abnormalities based on physical and orthopedic examinations prior to euthanasia, as well as testing negative for dirofilariasis, anaplasmosis, borreliosis or ehrlichiosis, using an ELISA-based test (SNAP® 4D® Test, IDEXX Laboratories, Westbrook, ME). Post-mortem examination of both knee joints was performed to confirm lack of gross abnormalities.

2.2. Synovial fluid collection

On the day of surgery, the OA dogs were anesthetized and both knees (irrespective of CrCL status) were prepared for aseptic arthrocentesis prior to surgical intervention. A 6 mL syringe and 1.5", 22 gauge hypodermic needle were used to aseptically aspirate SF. If less than 0.5 mL of SF was obtained, 1.5 mL of sterile water was injected into the joint. The knee joint was flexed and extended ten times to allow distribution of the infused sterile water, and re-aspirated using the previously described technique [33]. The obtained SF was stored as 0.5 mL aliquots in cryovials (Nalgene Cryogenic tubes, VWR International, Batavia, IL, USA) and frozen at −80 °C for later batch analysis. At recheck visits (i.e., T2 and T3), both knees were sampled using the same methodology under sedation after radiographs of knees were performed. In the control group, the SF samples were collected from both knee joints immediately after euthanasia using protocols identical to those utilized for the OA group.

2.3. Synovial membrane collection

In the OA group, during the medial arthrotomy of the CrCLR knee, a 1 × 2 cm synovial membrane biopsy from the edge of the medial arthrotomy incision was obtained. The same surgical approach was used to approach and recover biopsies from both knee joints of the control group dogs immediately after euthanasia. The synovial biopsies were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). These samples were examined by a board-certified veterinary pathologist. The case was excluded if there were non-OA related changes including neoplasia, pyogranulomatous inflammation or evidence of infectious organisms,.

2.4. Infrared spectroscopy

Synovial fluid samples were thawed at 22 °C and dried-films were prepared as described previously with the following modifications [19]. An internal spectroscopic control of potassium thiocyanate (KSCN, SigmaUltra, Sigma-Aldrich Inc, St Louis, MO) in a 2:1 SF to KSCN ratio (40:20 μL) was used. Six, 8 μL replicates for each sample was applied to 96-well silicon microplates [19,34]. Each microplate was dried at room temperature (20–22 °C) and then placed in the multi-sampler (HTS-XT, Autosampler, Bruker Optics, Milton, ON, Canada) attachment of an IR spectrometer (Tensor 37, Bruker Optics). Infrared absorbance spectra in the wavenumber range of 400 to 4000 cm^{-1} were recorded using the OPUS software (version 6.5, Bruker Optics, GmbH, Ettlingen, Germany). For each sample evaluation, 512 interferograms were signal averaged and were Fourier transformed to produce a nominal resolution of 4 cm^{-1} for the resulting spectrum [20,21,34].

2.5. Non-spectral data analysis

Analyses of non-spectral data were performed using SPSS software (IBM SPSS Statistics, version 23. Armonk, NY: IBM Corp.). Variables without a normal distribution were reported by their median and interquartile range (IQR). For inter- and intra-group comparison of control and OA group variables (i.e., age, gender, weight), Mann-Whitney U, Fisher's exact test or Pearson Chi-square test were used. Friedman's test was used. Statistical significance was set at $P \leq 0.05$, while reporting non-significant trends less than $P = 0.1$. Significant values less than 0.001 were presented as $P < 0.001$.

2.6. Spectral data analysis

After conversion of absorbance spectra into printable (PRN) format (GRAMS/AI 7.02, Thermo Galactic, Salem, NH), the data was imported into the software (MATLAB®, MathWorks R2015b (8.6.0.267246), Natick, MA) utilizing in-house written scripts for processing. At first, data were smoothed using the Savitsky-Golay filter [35] (2nd degree polynomial functions and 11-point smoothing window) [36]. Standard normal variate transform (SNV) was utilized for spectral normalization [37]. At each time point if more than one sample per joint was available for a given dog, selection of the spectra prioritized pure SF samples over blood contaminated over diluted samples for analysis. The effect of sample dilution was deemed negligible due to the qualitative approach of this analytical method in which the effect of water is eliminated due to the drying method used in sample preparation. Principal component analysis [38]was then applied separately to the data from each of the investigated categories and the optimal number of components in each PCA model was estimated as the one leading to the lowest root mean square error in an 8-fold cross-validation procedure. Verification of whether an observation was an outlier or not relied on the values of the two statistics T^2 and Q, for which both the null hypothesis was tested at a 95% confidence level [39]. Statistical significance was set at $P < 0.05$. Replicates of each sample were averaged prior to analysis.

Classification and predictive model development

To verify whether IR analysis of SF could provide for reliable discrimination among different groups (i.e., OA, contralateral, and control), supervised pattern recognition (classification) models were built and validated [40]. Due to its ability to deal with highly collinear predictors (e.g., spectral variables), partial least squares discriminant analysis (PLS-DA) was selected [41,42]. To validate the resulting models and spectroscopic markers, repeated double cross-validation (rDCV) procedure coupled with permutation tests was adopted [43,44]. Permutation tests were used to non-parametrically evaluate the null distributions and calculate corresponding P value to estimate the significance of the observed discrimination [43]. The quality of the predictive models was evaluated on the test set (samples not used for model development) by calculating sensitivity, specificity, overall classification accuracy and the area under the ROC curves (AUC).

To evaluate the potential effect of clinically relevant covariates multivariate PLS regression models were utilized. The discriminant abilities of these covariates were also separately calculated using the rDCV strategy for validation. The evaluated covariates included gender, age, weight, status of the menisci in the CrCLR knee at T1, and reported chronicity of CrCLR. Chronicity was based on whether the signs of lameness were first documented; less than 2 months (acute) or greater than 2 months (chronic).

Multivariate analysis of longitudinal data

Analysis of temporal changes was carried out by ANOVA-simultaneous component analysis (ASCA) [45]. This method was selected due to the multicollinearity of the data in this study that cannot be subjected to the traditional multivariate analysis of variance (MANOVA).

To identify the minimum set of predictors that would provide the same discrimination accuracy as the previous models that included the entire spectral range, the covariance selection (CovSel) algorithm was utilized [46]. Summary of steps of preprocessing and analyses are presented in Fig. 1.

3. Results

There were 104 dogs in the OA group; 50 of these dogs were re-examined at T2 and 46 at T3 time points. The control group included 50 dogs that met the inclusion criteria. The median age in the OA and control groups were 4.8 years (IQR: 3.8 years) and two years (1.1 years), respectively. The median body weight of OA and control groups were 37 kg (IQR: 11.7 kg) and 24 kg (5.1 kg), respectively. Dogs in the OA group were older ($U = 672$, $P < 0.001$) and heavier ($U = 443.5$, $P < 0.001$) than the control group. There were 50 female (48 spayed, 2 intact) and 54 male (51 neutered, 3 intact) dogs in the OA group. The control group included 22 female (2 spayed, 20 intact) and 28 males (2 neutered, 26 intact) dogs. There was a significant difference between the intact status of OA and the control groups with the latter having a significantly higher number of intact dogs compared to the OA group dogs ($P < 0.001$). Labrador Retriever (n = 34) and mixed breed dogs (n = 28) were the most common breeds in the OA group and control group respectively (Table 1).

There were 118 knee joints diagnosed with CrCLR in 104 dogs; 90 unilateral (43 right, 47 left) and 14 bilateral. In 5/14 bilaterally affected dogs, only one knee was operated. Therefore, of the 118 CrCLR joints, 113 were operated (i.e., nine dogs (n = 18 knees) as bilateral single-stage procedures and 95 dogs (n = 95 knees) as unilateral procedures). There were 53 partial and 60 complete CrCLRs noted in the operated joints, and concurrent lateral meniscal tears were observed in 2/58 knees with medial meniscus tears. The relationship between CrCL status and medial meniscus is reported in Table 2. There was a significant difference between the status of the medial meniscus between partial and complete CrCLR ($\chi^2 = 9.572$, $P < 0.001$). Knees with complete CrCLR were 3.3 times more likely to have a medial meniscal tear than knees with partial

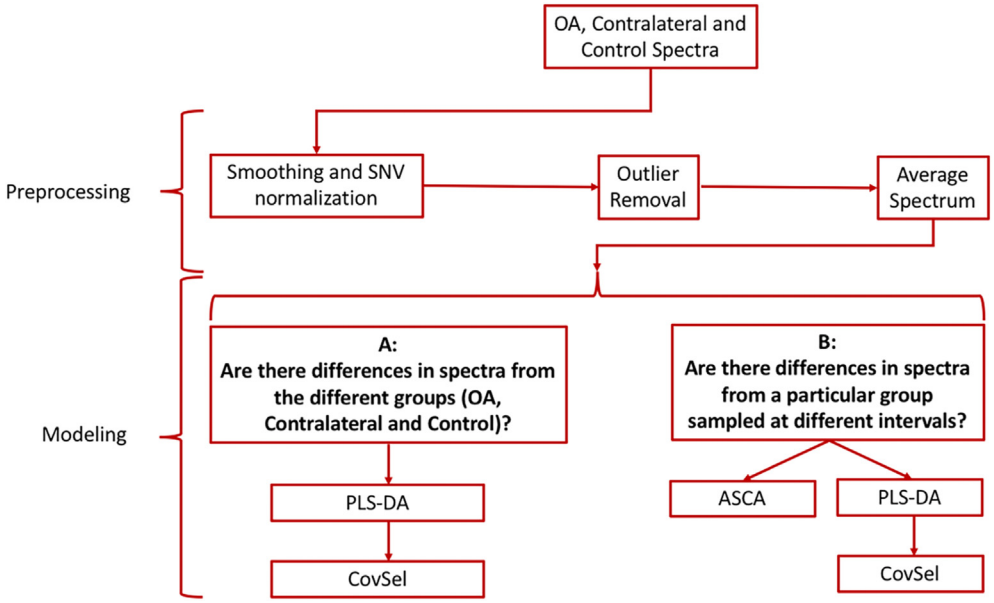

Fig. 1. Work flow of data preprocessing steps of spectral data of synovial fluid samples from the OA (CrCL deficient knees) and contralateral knee of the affected group and knees of control group dogs. The data underwent spectral preprocessing prior to evaluation of differences amongst the three sample groups at the initial visit and within the OA and contralateral groups over time. SNV: standard normal variate. PLS-DA: Partial least square discriminant analysis, ASCA: ANOVA-simultaneous component analysis, CovSel: Covariance selection.

CrCLR ($P = 0.002$, 95% CI 1.54–7.2). Long-term follow-up data was available for 67/90 dogs with unilateral CrCLR; 31 (46.3%) developed subsequent degenerative CrCLR in the contralateral knee. Mean (range) overall follow-up time and time to subsequent CrCLR were 567 (43–2291) days and 350 (48–949) days respectively.

3.1. Radiographic findings

Complete follow up recheck radiographs were available for 42 OA group dogs. There were significant differences between CrCLR and contralateral joints in radiographic effusion, osteophytes and global scores at all three time-points, with CrCLR knees having higher scores ($P < 0.001$). Knees with a meniscal tear had significantly higher effusion, osteophyte, intraarticular mineralization and global scores compared to knees without meniscal tears at T1 only ($P < 0.05$). There was a statistically significant increase in osteophyte score between T1 and T3 recheck in both CrCLR and contralateral knees ($P < 0.05$). The increase in the global score was statistically significant only between the initial and 12-week recheck time point in the CrCLR knees ($P < 0.05$).

Table 1
Frequency of breeds of dogs sampled in osteoarthritis and control groups.

OA Group (n = 104)		Control Group (n = 50)	
Dog Breed	Count	Dog Breed	Count
Labrador Retriever	34	Mixed breed	28
Mixed breed	13	Pit Bull Terrier	11
Golden Retriever	10	German Shepherd	4
Boxer	6	Labrador retriever	3
Bernese Mountain Dog	5	Australian Cattle Dog	2
Mastiff	5	Pointer	1
German Shepherd	4	Rottweiler	1
Newfoundland Dog	4		
Pitt Bull Terrier	3		
Rottweiler	3		
Chesapeake Bay Retriever	2		
Valley Bulldog	2		
Airedale Terrier	1		
American Bulldog	1		
Australian Shepherd	1		
Beauceron	1		
Bouvier des Flanders	1		
Brittany Spaniel	1		
Doberman Pinscher	1		
Great Dane	1		
Leonberger	1		
Poodle	1		
Shiloh Shepherd	1		
Staffordshire Terrier	1		
Wheaten Terrier	1		

3.2. Spectral data results

After outlier removal from the raw data, 3072 of 3462 spectra were retained. After normalization and averaging replicates, there were 475 SF samples available for analysis (100 control and 375 from OA group). The OA group included 223 spectra from the CrCLR subset (T1: 125, T2: 49, and T3: 49 samples) and 152 spectra from contralateral subset (T1: 84, T2: 34, and T3: 34 samples).

An initial discriminatory model demonstrated correct classification rates for control, CrCLR knees and contralateral knees of 90.5 ± 1.9%, 77.6 ± 1.7% and 63.1 ± 2.7%, respectively. Permutation tests indicate that the observed discrimination was statistically significant ($P < 0.001$). There were also statistically significant spectral differences when pairwise comparisons amongst CrCLR and contralateral knees in the OA group at T2 and T3, and control group knees were performed (Table 3). Based on the predictive models for these comparisons (Table 3), the CrCLR knees were accurately discriminated from control samples at all time points. Similar predictive abilities were observed when comparing contralateral knees with control samples, despite a slightly lower correct classification rate at T2 and T3 time points. Although statistically significant, the classification accuracy of the models discriminating between CrCLR and contralateral knees were in general lower than in the other pairwise comparisons. The subsequent CrCLR tear in the contralateral knee could not be predicted based on the spectra due to poor discriminatory ability of the model.

Considering the disparity in distribution of sexually intact versus neutered in females and males in both groups, statistical analysis of the

Table 2
Intraoperative status of cranial cruciate ligament rupture and medial meniscus in operated knees in OA group.

	Meniscal status		Total
CrCL status	Torn	Intact	
Full tear	39	21	60
Partial tear	19	34	53
Total	58	5	113

Table 3
Pairwise comparisons between synovial fluid samples in the two osteoarthritis (OA) subgroups (OA affected knee with CrCLR and contralateral knee) and control group at different time points: initial preoperative (T1), 4 weeks after surgery (T2), and 12 weeks after surgery (T3).

Pairwise comparisons		Sensitivity (%)		Accuracy (%)	AUROC	P-value
Group1	Group 2	Group1[a]	Group 2[a]			
OA (T1)	Control	97.6 ± 1.1	99.7 ± 0.6	98.6 ± 0.7	0.993 ± 0.002	<0.001
Contralateral (T1)	Control	93.3 ± 1.4	92.4 ± 2.6	92.8 ± 1.6	0.978 ± 0.007	<0.001
OA (T1)	Contralateral (T1)	75.8 ± 2.1	75.5 ± 2.2	75.7 ± 1.5	0.824 ± 0.011	<0.001
OA (T2)	Control	99.3 ± 1.0	95.6 ± 1.1	96.8 ± 0.8	0.998 ± 0.003	<0.001
Contralateral (T2)	Control	95.9 ± 2.6	86.8 ± 2.1	89.1 ± 1.8	0.973 ± 0.015	<0.001
OA (T2)	Contralateral (T2)	85.9 ± 3.1	81.9 ± 3.0	84.2 ± 2.6	0.869 ± 0.017	<0.001
OA (T3)	Control	97.6 ± 0.8	93.9 ± 1.7	95.1 ± 1.3	0.977 ± 0.002	<0.001
Contralateral (T3)	Control	95.4 ± 2.5	87.0 ± 2.1	89.1 ± 1.8	0.971 ± 0.016	<0.001
OA (T3)	Contralateral (T3)	74.0 ± 4.9	59.2 ± 4.8	67.9 ± 3.9	0.679 ± 0.039	0.017

Statistical significance is $P < 0.05$.
[a] Due to the symmetry of the binary classification problem, Group 1 sensitivity corresponds to Group 2 specificity, while Group 2 sensitivity corresponds to Group 1 specificity.

effect of gender could not be performed. When considering the possibility of building regression models for predicting age and weight based on the IR spectroscopic fingerprint, a poor correlation was found between the spectral profiles and the two covariates; the mean values for the validation R^2 being 0.157 and 0.193 for age and weight, respectively. When discriminant ability of age and weight of dogs was evaluated using the same methodology, the percent correct classification rates for each pairwise comparison obtained using these descriptors were always at least 20% lower than those reported in Table 3 for the spectroscopic data and, in many cases, statistically insignificant.

The predictive model based on SF spectra for detecting meniscal tear in CrCLR knees in the OA group had sensitivities for knees with and without meniscal tear and overall accuracy of 53.4 ± 6.0%, 53.9 ± 4.0% and 53.7 ± 3.8%, respectively. The corresponding AUROC for this predictive model was only 0.519 ± 0.039 and did not show a significant difference between the spectra from the CrCLR knees with and without meniscal tear ($P = 0.367$).

When evaluating overall temporal changes in the OA group, the differences in spectra of CrCLR and contralateral knees over time were statistically significant ($P = 0.0004$ and $P = 0.0001$, respectively). Based on presence of overall significant differences amongst the three time points, pairwise comparisons between T1, T2 and T3 time points for SF spectra of the OA knees in the CrCLR and contralateral groups were performed and correct classification rates for each comparison are reported in Table 4. Differences in spectra were significant among the three time points for the CrCLR knee with high percent correct classification. Similar pattern was observed in the contralateral knees at the three time points. However, the percent correct classification rate was lower in the contralateral compared to the CrCLR knees and the differences in spectra between T2 and T3 time points were not significant. Evaluation of the chronicity of CrCLR and its effect on group comparisons or temporal changes did not show a significant effect. The minimum set of predictive wavenumbers for each predictive model is presented in Table 5.

4. Discussion

FTIR spectroscopy of SF as described here distinguished among samples from knees with CrCLR and the contralateral (stable) joint (OA group), and the knees of control dogs; the first null hypothesis was rejected. The predictive model based on SF had improved correct classification rates compared that based on serum samples from the same cohort of dogs [34]. Direct sampling from the joint reduces the biomolecular influence of other joints, and concurrent systemic physiologic processes that may affect samples such as serum and urine. When evaluating the radiographic changes, significant changes over time were only noted between the initial and 12-weeks recheck for osteophyte and global scores in the CrCLR knees, and osteophyte score only in the contralateral knees. However, the spectral pattern differences were statistically significant amongst all three time points. These differences can be attributed to changes in the characteristics of complex range of molecules within the SF sample responsible for the spectral fingerprint. Radiographic scores were as sensitive as the spectra in detecting changes that occurred in the 4-week postoperative period and less sensitive to changes in the contralateral knees. This finding may be influenced by the lack of validated radiographic scoring systems in knee OA in dogs, or presence of biochemical changes in the SF due to surgical intervention are not accompanied by a radiographic manifestation. Further evaluation of the specific biomarkers contributing to the spectral differences between knees with OA and normal knees in dogs is warranted. Identification of such correlations can be a first step towards using FTIR spectroscopy as a surrogate for validated biomarkers of OA or as an initial screening test to improve trial enrichment strategies. Meniscal tear occurs in 20–77% of dogs diagnosed with CrCLR and is a contributing factor to the OA in the knee joint [47]. Unlike the radiographic scores, FTIR spectroscopy was not able to discriminate between dogs with and without meniscal tear.

FTIR spectroscopy was able to distinguish between samples from the three time points in both the CrCLR and the contralateral (stable) knees

Table 4
Pairwise comparison between initial (T1), 4-week (T2), and 12-week (T3) time points for synovial fluid spectra of both cranial cruciate ligament rupture (unstable) and contralateral (clinically stable) knee joints in the osteoarthritis group.

Pairwise comparisons		%Correct classification rates				
Group1	Group 2	Group1	Group 2	Overall	AUROC	*P*-value
OA (T1)	OA (T2)	86.1 ± 3.1	93.5 ± 3.0	89.8 ± 2.3	0.965 ± 0.011	<0.001*
OA (T1)	OA (T3)	95.5 ± 2.2	96.9 ± 2.4	96.2 ± 1.6	0.994 ± 0.007	<0.001*
OA (T2)	OA (T3)	84.4 ± 3.0	85.1 ± 2.4	84.8 ± 2.3	0.907 ± 0.020	<0.001*
Contralateral (T1)	Contralateral (T2)	76.2 ± 6.8	90.6 ± 3.8	83.4 ± 3.9	0.918 ± 0.028	<0.001*
Contralateral (T1)	Contralateral (T3)	84.1 ± 4.7	85.5 ± 3.8	84.8 ± 3.6	0.905 ± 0.025	<0.001*
Contralateral (T2)	Contralateral (T3)	63.4 ± 6.6	59.3 ± 7.0	61.3 ± 4.6	0.665 ± 0.046	0.076

*Statistical significant n was $P < 0.05$.

Table 5
Minimum set of predictive wavenumbers (cm^{-1}) leading to the same discriminant accuracy as that obtainable with the full spectral data set. Identification is based on the covariance selection (CovSel) algorithm. CrCLR: samples from the OA group from knees with cranial cruciate ligament rupture, Contralateral: samples from the OA group from knees that were stable at the time of unilateral CrCLR diagnosis, Control: samples from stable knees of clinically healthy dogs.

CrCLR vs Control	CrCLR vs Contralateral	Contralateral vs Control	CrCLR Group			Contralateral Group		
			T1 vs T2	T1 vs T3	T2 vs T3	T1 vs T2	T1 vs T3	T2 vs T3
3420.8	3477.7	3444.9	3649.3	2959.8	1644.3	3386.0	3296.3	3400.5
3291.5	1735.9	3289.6	3409.2	2360.9	1619.2	2926.0	3209.5	3296.3
2924.1	1654.9	2985.8	3283.8	1683.8		2510.3	2925.0	3206.7
2361.8	1635.6	2926.0	2593.3	1651.0		2360.9	2561.5	2979.0
1690.6	1595.1	2360.9	2360.9	1627.9		1674.2	2361.8	2922.1
1656.8	1538.2	1749.4	1740.7	1582.6		1656.8	1683.8	2433.2
1626.9	1041.5	1718.5	1683.8	1081.1		1640.4	1654.9	2360.9
1596.1		1677.1	1654.9	915.2		1597.0	1639.5	1750.4
1572.9		1656.8	1639.5			1544.0	1595.1	1709.9
1541.1		1637.5	1581.6			1041.5	1540.1	1681.9
1486.1		1614.4	1546.9			666.4	1476.5	1664.5
1184.3		1592.2	1523.7			609.5	1082.0	1655.9
1159.2		1557.5	1399.3				928.7	1639.5
1123.5		1540.1	1181.4				667.4	1593.2
1095.5		1508.3	1109.0				619.2	1558.5
1049.2		1414.8	1079.1					1542.1
972.1		1184.3	856.4					1527.6
693.4		1122.5	619.2					1410.9
608.6		1089.7						1203.5
		1047.3						1186.2
		855.4						1174.6
		667.4						1099.4
		619.2						1042.5
								946.1
								616.3
								506.3

in the OA group; the second null hypothesis was rejected. Ability of FTIR spectroscopy in detecting changes in the contralateral knees that were stable at the time of initial diagnosis of unilateral CrCLR confirms the previously documented abnormalities (e.g., synovitis and early OA) in the contralateral knees of dogs with unilateral CrCLR [9,10]. Early detection of these at-risk contralateral knees using a minimally invasive approach such as FTIR spectroscopy may be a valuable modality worth exploring in trials evaluating preventative treatments in dogs. It was not possible to predict subsequent CrCLR in the contralateral knees based on spectral patterns that may have been due to the wide range of disease burdens, differences in age, and chronicity of early changes in the contralateral knee at the time of enrollment.

The impact of age and weight of dogs as covariates on the ability of FTIR spectroscopy to detect differences between groups was not significant in this study. In previous studies evaluating IR spectroscopy of SF in clinical OA, one in humans did not evaluate the effect of age [23], one in traumatic OA in horses [21] did not show any age-related effect, but one evaluating osteochondrosis in young horses showed an impact on performance of the IR-based algorithm when age was included [20]. Obesity is an established risk factor in development of OA in dogs [48,49]. In the current study, weight of dogs and not body mass index measurements were used which may have affected findings. Additionally, the heterogenicity of duration of clinical signs attributed to CrCLR in the OA group may have been responsible for the lack of effect for chronicity on the spectral results. The disparity of breeds between groups in this study may have attributed to some of the observed differences. However, the effect of breed on SF biomarker profile has not been investigated previously.

In conclusion, this is the first study using FTIR spectroscopy as potential SF-based screening test for this CrCLR clinical model of OA in dogs. Prospective studies evaluating the predictive model's performance in larger population of dogs with more strict selection criteria and in comparison to other forms of canine arthritis is warranted.

Contributions

Authors' contribution included conception and design (SM, CBR, RB, SAM), analysis and interpretation of the data (SM, FM, CBR, SAM, GW), statistical expertise (FM), patient recruitment and sample procurement (SM, MR, RB), obtaining of funding (SM, CBR, MR, RB), collection and assembly of data (SM), administrative support (CBR, GW, RB). All authors contributed to the preparation and approval of the article.

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Declaration of competing interest

None to report. The funding sources had no influence on the study design, data collection, analyses and interpretation, or in the writing of the manuscript or the decision to submit it.

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