Biochemical Variation Among Normal Equine Carpal and Tarsocrural Joint Fluids are Detected by Infrared Spectral Characteristics and A Modified Approach to Linear Discriminant Analysis

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***Abstract*—Research into osteoarthritis diagnostics has evolved from traditional methods that are only useful in more advanced clinical disease, towards the discovery of biomarkers that are predictive or reflective of preclinical joint disease. The potential of Fourier transform infrared spectroscopy (FTIR) coupled with chemometrics has been demonstrated as useful for the assessment of biomolecular responses to disease. Joint fluid was collected from 105 clinically normal antebrachiocarpal (AC), midcarpal (MC) and tarsocrural (TCRL) joints. Thin films were prepared and FTIR absorbance spectra in the mid-infrared region recorded. Interferograms were signal averaged and Fourier transformed to generate spectra with a nominal resolution of 4 cm^{-1}. Comparisons among joints were made using a novel modified method similar to linear discriminant analysis, which maximized the difference of between-group variance minus within-group variance, followed by permutation testing. Differences within animal between contralateral pairs of joints were minimal. Significant differences among AC, MC and TCRL joint fluid spectra were found. The range of biomolecular differences among these normal joints as characterized by FTIR indicates that interarticular variation within the horse needs to be considered for ongoing research, especially when utilizing within-horse joints as controls.**

Keywords-infrared spectroscopy, osteoarthritis, synovial fluid, discriminant analysis

I. INTRODUCTION

Normal synovial joints contain a fluid (SF) comprised of a unique plasma filtrate augmented with hyaluronate and other biomolecules produced by type-B synoviocytes [1-3]. The SF plays critical functional roles in articular cartilage nourishment, joint lubrication and the response to disease [2]. Conventional and, more recently, novel molecular approaches have been reported for the diagnosis of arthritic conditions in veterinary and human medicine [4-8]. The former, less sensitive and specific approach consists of evaluations of color, viscosity, volume, clot formation, total protein and cytological examination [6, 8]. Specificity in the horse is limited to cases of septic arthritis, and although these parameters may provide limited information with respect to synovitis, they poorly characterize articular cartilage or subchondral bone injury in cases of osteoarthritis (OA) [3, 4]. In recent years, research into osteoarthritis diagnostics has moved from traditional methods that are only suited to more advanced clinical disease, towards the discovery of biomarkers (direct and indirect molecular markers of aberrant musculoskeletal metabolism) that are predictive or reflective of preclinical joint disease [4-11]. The qualitative and quantitative underpinning the inter-relationships among articular catabolic and anabolic activities have, to some extent, been characterized using a range of biochemical assays including radioimmunoassay or ELISA approaches [4-11]. Although they yield some promise, they suffer practical limitations for use in clinical diagnosis, in so far as complex multiple assays are required, and they generally evaluate only known biomarkers of indices of articular disease [10]. Genome based technologic methods for the assessment of joint disease risk and assessment of disease have been described but access to the technology is limited in veterinary medicine, and technical and economic barriers have impaired their wider utility outside of the research laboratory [12].

The utility of Fourier transform infrared spectroscopy (FTIR), traditionally the domain of analytical chemistry, has been confirmed for the qualitative and quantitative evaluation of biological molecules and their response to disease [13-16]. In brief, the exploitation of FTIR relies upon the principles underpinning the absorption characteristics of organic molecules when irradiated with light in the infrared portion of the electromagnetic spectrum [15]. The absorption patterns, or "biomolecular fingerprints" are highly specific with respect to the nature and distribution of molecular species within a sample, and the intensity of absorptions at different wavenumbers may be directly related to the concentration and composition of

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constituent molecules [13-15]. These absorption patterns may be correlated to the disease status of a sample or patient [13-15]. The technique of FTIR has several well recognized advantages: it is a reagent free method; repetitive analyses may be automated, and; it is economic, and requires just a few µL of sample per test [14]. With respect to biomarker discovery, it is a recognized as a powerful technique that provides a breadth of qualitative and quantitative molecular information, encompassed with the spectrum of infrared active components, including recognized and undiscovered biomarkers in a sample [14-15].

The use of FTIR to classify human arthridities was first reported almost two decades ago [16, 17]. Following on from this novel approach, FTIR-based diagnostic techniques have been explored as a diagnostic approach to equine joint disease [18]. Although this shown promise, there a need to explore those physiological factors which might reasonably be expected to influence the molecular diagnosis of OA and other articular disorders in the horse. Among these factors are the intrinsic variations among joints with respect to anatomy, mechanical environment, and the biochemical composition of its constituent tissues and fluids. To the authors knowledge there are no published studies comparing the FTIR profiles among normal joints, or identifying the effect of anatomic location on spectral variables used to characterize joint disease in any species. The advancement of the FTIR technique as a biomarker discovery or diagnostic tool requires that those differences that are physiologic variations of "normal" be differentiated and accounted for when investigating OA from different comparative anatomic locations within the animal species being studied. Using other approaches, previously published works have described significant mechanical and biochemical differences among joints from different anatomic locations within an animal species (e.g. the horse) [7, 8, 19-24]. These differences most likely reflect the different locomotory requirements of these joints, and notably may result in significant variations in the molecular responses to injury, irrespective of any similarity in the inciting cause [7, 8, 23]. Thus intrinsic sources of variance (i.e. sources other than those due to disease), attributable to biological variation, need to be differentiated and accounted for when exploring changes in SF composition attributable to disease.

In the current study, the authors hypothesized that the anatomic location from which SF is sampled may affect the mid-infrared (MIR) spectral features of the biological fluid. The objective of this study was to determine whether and to what extent the IR absorption spectral features differ among clinically normal antebrachiocarpal (AC), midcarpal (MC) and tarsocrural (TCRL) joints, as a consequence of natural biochemical variation among these high motion joints.

II. MATERIALS AND METHODS

A. Study Design and Sample Population

This study was approved by the Animal Care Committee in accordance with the University of Prince Edward Island policy and the Guide to the care and Use of Experimental Animals prepared by the Canadian Council on Animal Care.

Synovial fluid from high motion joints (AC, MC, and TCRL) and side (left versus right) in were used to study the effect of anatomical location on mid-infrared (MIR) absorbance spectra (wave number range 400-4000 cm^{-1}) in a complete block experimental design The SF was collected from 22 clinically normal horses (105 joints) that met the criteria for inclusion. Inclusion criteria were: no history of lameness or orthopedic problems; not lame at the walk or trot or on the lunge line; no palpable abnormalities of the joints or limbs; negative responses to joint flexion tests, and; an absence of radiographic findings on survey films of the carpi and tarsi. Additional criteria included an absence of gross pathological lesions of the synovium and articular cartilage on necropsy, and normal routine synovial fluid analysis (cytology, total protein concentration) [22,24]. An initial pool of ~ 60 horses was examined to arrive at the final 22 animals; exclusion was mostly due to abnormal radiographic findings (despite a normal clinical examination). Breeds represented included Standardbred (n=18), Quarter horse (n=1), and mixed breeds (n=3). There were 10 females and 12 males aged between 2-12 years (median 3.75 years). Of these 22 horses, 13 provided bilateral and 9 provided unilateral ipsilateral SF samples from AC, MC, and TCRL joints. Samples were stored at -80^{o}C in plain cryovials for later batch analysis by FTIR.

B. Fourier Transform Infrared Spectroscopy (FTIR)

Synovial fluid samples were thawed at 22^{0}C and centrifuged at 2700 g for 10 minutes. Sample preparation was a modification of a previously described technique [25, 26]. Briefly, for each sample, an aliquot of the centrifuged supernatant was drawn and diluted in aqueous 4 g/L potassium thiocyanate (KSCN, Sigma-Aldrich Inc., St. Louis, MO) solution in the ratio 3 parts SF: 1 part KSCN solution.

Dry films of the SF were made in triplicate for each sample by spreading 8 µL aliquots evenly onto 5 mm diameter circular islands within a custom made, adhesive masked, 96 well-silicon microplate [26]. The adhesive mask adhered to the surface of the microplate served to spatially define and separate the 5 mm islands on the microplate, aligning samples within the microplate multisampling FTIR accessory. SF aliquots from each horse were randomly assigned to well positions on the microplate, and left to dry at room temperature for 12 h. Once dry the microplate was mounted within the multisampler (HTS-XT, Bruker optics, Milton, ON) interfaced with the Fourier transform infrared spectrometer (Tensor 37, Bruker optics, Milton, ON) to for the acquisition of spectra.

MIR (400-4000 cm^{-1}) absorbance spectra were recorded by a FTIR spectrometer equipped with a deuterium tryglycine sulphate detector. For each spectrum, 512 interferograms were signal averaged and Fourier transformed, resulting in a spectrum with a nominal resolution of 4 cm^{-1} [18, 26].

C. Data Preprocessing

The triplicate primary spectra of each SF sample were averaged and preprocessed, resulting in a second order derivative spectra (Savitsky-Golay method via a second degree polynomial function, with 7 points) resolve and enhance weak spectral features, and remove the variation in baselines, using spectral manipulation software (GRAM/AI 7.02 Thermo Galactic, Salem, NH) [27]. Scripts were written by the authors

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in MATLAB® (MATLAB 6.5, The Math Works Inc., Natick, MA) to performed vector normalization to reduce signal noise due to light scattering. Briefly, for each second order derivative spectrum, the sum of the squares of absorption intensities for all data points (1 data point corresponded to ~1 wave number) was calculated, within the spectral basis range of 800-1450 cm-^{1} [28]. The square root of this sum of squares was calculated for each individual spectrum and used as the normalization factor for that same spectrum by dividing each wavenumber by the factor.

D. Statistical Analysis

Within- horse comparisons of left and right joint fluid spectra. Twenty-six samples from 13 horses yielded the 78 preprocessed spectra of bilateral SF samples from AC, MC, and TCRL joints that were used for this part of the analysis. The relative (normalized) intensities at each data point (wave number) in the second derivative spectra were assigned as dependent variables. Analyses of variance for a randomized block model using each horse as a blocking factor were performed (PROC MIXED, SAS 8.02, SAS institute Inc., Cary, NC) on each wave number-intensity value for the entire MIR wavenumber region. The effect of limb side (left versus right) was considered significant for any wave number within the MIR region if $p < 0.01$. More advance modeling of the differences was not requires (see results).

Comparison of the FTIR spectra among high motion joints. The preprocessed second order derivative spectra were initially compared to identify the scope of differences (if any) among SF samples from the different anatomic locations (PROC MIXED; SAS 8.02, SAS institute Inc., Cary, NC). Analyses of variance were performed as described above to detect possible significant effects of anatomic joint type (p<0.01) (i.e. AC-MC, AC-TCRL, and MC-TCRL). The differences between joints for any wave number within MIR region were considered significant for p<0.01. A large number of variables were found to differ significantly at the univariate level. Therefore, a modified linear discriminant analysis approach (MDA) was employed to classify the second order derivative spectra among the SF samples from different joints. This novel approach was similar to linear discriminant analysis (LDA) [29] in that it was designed to maximize the difference of between-group variance minus within-group variance. The mathematical approach was as follows:

If for a spectral data matrix $\mathbf{X}$ consisting of samples from g groups,

$$\mathbf{X} = \begin{bmatrix} \mathbf{X}_1 \\ \mathbf{X}_2 \\ \vdots \\ \mathbf{X}_g \end{bmatrix} \tag{1}$$

and for each group there is a matrix $\mathbf{X}_i$ containing n_i samples,

$$\mathbf{X}_i = \begin{bmatrix} \mathbf{x}_{i1}^{\mathrm{T}} \\ \mathbf{x}_{i2}^{\mathrm{T}} \\ \vdots \\ \mathbf{x}_{in_i}^{\mathrm{T}} \end{bmatrix} \tag{2}$$

then the pooled within-group covariance matrix ($\mathbf{W}$) and between-group covariance matrix ($\mathbf{B}$) can be expressed as equations (3) and (4) respectively,

$$\mathbf{W} = \frac{1}{n}\sum_{i=1}^{g}\sum_{j=1}^{n_i}(\mathbf{x}_{ij} - \bar{\mathbf{x}}_i)(\mathbf{x}_{ij} - \bar{\mathbf{x}}_i)^{\mathrm{T}} \tag{3}$$

$$\mathbf{B} = \frac{1}{n}\sum_{i=1}^{g} n_i(\bar{\mathbf{x}}_i - \bar{\mathbf{x}})(\bar{\mathbf{x}}_i - \bar{\mathbf{x}})^{\mathrm{T}} \tag{4}$$

where $\mathbf{x}_{ij}$ denotes the jth sample in the ith group, $\bar{\mathbf{x}}_i$ represents the sample mean vector of the ith group, $\bar{\mathbf{x}}$ designates the overall mean, and n is the total number of samples. The superscript "T" denotes the transpose operator. When the high-dimensional data (preprocessed spectra) are projected onto a unit vector $\mathbf{v}$, this method maximizes the difference of between-group variance minus the within-group variance. Mathematically this can be expressed as

$$\text{maximize } \mathbf{v}^{\mathrm{T}}(\mathbf{W}-\mathbf{B})\mathbf{v} \tag{5}$$

$$\text{subject to } \|\mathbf{v}\| = 1 \tag{6}$$

Because $\mathbf{W}$ and $\mathbf{B}$ are both symmetric matrices, $\mathbf{W}-\mathbf{B}$ is also a symmetric matrix. Based on spectral and principal axis theorems, $\mathbf{W}-\mathbf{B}$ has orthogonal eigenvectors and the maximum of $\mathbf{v}^{\mathrm{T}}(\mathbf{W}-\mathbf{B})\mathbf{v}$ is obtained when the data are projected onto the eigenvector corresponding to the largest eigenvalue. Thus, the solution to the optimization problem in this work was obtained by finding the eigenvectors of $\mathbf{W}-\mathbf{B}$. The between-group variance minus the within-group variance projected on the eigenvectors of $\mathbf{W}-\mathbf{B}$ is equal to the corresponding eigenvalues.

For the current study, spectral wave number regions 597-1850 cm^{-1} and 2815-3972 cm^{-1} were employed as the magnitudes the FTIR signals in these bands had higher relative intensities. Data were auto-scaled (mean-centered and scaled) and the pooled within-group (**W**) and between-group (**B**) covariance matrices were calculated. The high-dimensional data was projected onto a unit vector, maximizing the differences between the between-group variance and the within-group variance. Sample scores were obtained by projecting the original data onto the eigenvector corresponding to the largest eigenvalue. Script written by the authors in MATLAB® was used to find the chemometric solution that best served to classify AC, MC and TCRL spectra. This approach was applied to: (1) separation of all the three classes; (2) separation between AC and TCRL classes; (3) separation between MC and TCRL classes; and (4) separation between AC and MC classes. For each of the analyses, the MDA method was applied to detect the separation of the samples. For the three-class classification (separation), the first and second scores were used; while for the paired comparisons, only the first score was used since only one dimension is required to separate two classes. Permutation tests were carried out in which sample class labels were randomly re-assigned 10,000 times to evaluate the validity of the separation of joint classes [30]. The eigenvalue (equal between-group

variance minus the within-group variance) was employed to measure the degree of separation. For the three-class classification (AC, MC and TCRL), the sum of the first two eigenvalues was calculated, while for paired-class comparisons (e.g. AC versus MC, etc.), only the first eigenvalue was used.

III. RESULTS

Analyses of variance performed on 3731 wave number data points from the set of derivative spectra found little difference between left and right joints at the horse level, but a wide scope of changes among anatomically distinct joints. When the spectra of matched contralateral joints were evaluated, the only significant difference were found at wave numbers 2568 cm^{-1} (p = 0.008) and 2699 cm^{-1} (p = 0.005). Among the AC, MC and TCRL joints there were significant differences at the univariate level, prior to MDA analysis, which encompassed a range of spectral bands (Table 1)

TABLE I. SCOPE OF INFRARED SPECTRAL DIFFERENCES AMONG JOINTS

Comparison[a]	Wavenumber differences	
	Wavenumber bands cm^{-1}	*Number* [b]
AC-TCRL	560-650, 790-840, 890-900, 940-1240, 1400-1440, 1650-1700, 2260-2270, 3000-3020, and 3620-3830	384
MC-TCRL	600-740, 810-900, 950-1050, 1070-1090, 1120-1140, 1160-1800, 2030-2200, and 2830-3400	995
AC – MC	1000-1100, 1550-1600, and 3500-3650	193

a. AC = antebrachiocarpal; MC – midcarpal; TCRL – tarsocrural joints spectra. b. Significant at P <0.01

When the MDA method was applied to the second order derivative spectra, clear separation of the three classes was revealed in the scores plot [Fig. 1(a)], whereas poor separation was observed using principal component analysis (PCA), partial least squares discriminant analysis (PLSDA), and projection pursuit (PP) [31] (Figures not shown). Furthermore a permutation test found the separation corresponding to the correct class labels was statistically significant [p<0.001; Fig. 1(b)]. Histogram plots show that the TCRL spectra are clearly distinguishable from those of other joints based on the first order scores [Figs. 2(a) and (c)] and permutation tests showed these were statistically significant separations [p<0.001, Figs. 2(b) and (d)], identifying distinctly biochemically different populations of spectra. However, the permutation test did not detect statistical significance between AC and MC spectra [Fig. 2(f)] despite the histogram plot from the MDA method revealing apparent separation [Fig. 2(e)].

IV. DISCUSSION

This work indirectly confirmed the findings of others that the biochemical composition of synovial fluids differs among normal joints [21-25]. The technique of FTIR spectroscopy paired with MDA, attained sufficient sensitivity to successfully classify SF among different high motion joints, indicative of their molecular heterogeneity However, the differences between contralateral joints were minimal, and given their scarcity and the number of variable examined (3731 wavenumbers), it is highly like that these findings were due to chance, and not reflective of significant biochemical differences. Therefore, based upon the approach described in the current study, the authors suggest that left paired contralateral AC, MC and TCRL joints may be considered to be biochemically equivalent in paired non-diseased joints [12,14]. These findings are supported, in part, by other workers that have found no significant difference in oncotic pressure between contralateral joints, providing indirect evidence that similar concentrations of molecular solutes are present in joint pairs [2, 35-37]. These previous reports, and the findings of the current work, support the practice of using contralateral joints as within-subject controls, with the caveat they be limited to those studies where the effects to be examined have been demonstrated to be 1localized to the joint of interest [22,23,38,39]. This view should be weighed against the findings of other pharmacologic and mechanical studies that have provided evidence that sympathetic changes may occur in contralateral control joints, presumably associated with alterations in load bearing of the unaffected limb [40,41]. As molecular normality is frequently assumed when joints are found to free of clinical disease, the authors suggest that the use of FTIR spectroscopy of SF may in future provide an economic and objective means to confirm this, and therefore provide an additional tool for the exclusion of those joints with subclinical disease as controls, and to identify intended or unintended biomolecular effects in other joints [40,41].

. The dissimilarities in FTIR absorption patterns of SF observed among ipsilateral high motion joint fluids were marked, particularly between more widely anatomically distant joints (e.g. AC and TCRL joints). There were, however, fewer differences in absorption patterns between adjacent AC and MC joint samples. Furthermore, these were of insufficient magnitude and scope to permit successful differentiation or classification using a one-dimensional plot based on the MDA method described. The biomechanical and functional differences between the anatomic regions have been well characterized [19, 20]. The authors therefore consider it reasonable to infer that that given the scope of biomolecular analysis encompassed by FTIR of SF, differences would be more marked for functionally dissimilar joints than those occurring between similar ones (e.g. AC and MC joints). The findings of the current study contrast with earlier works that did not find significant differences in total protein concentration (TP) between hocks and carpi despite the known biomechanical differences of the joints, (the precise joints sampled were not described) [41]. However, these workers did find that TP was higher in the fetlock; we did not have similar samples available for study. Lactate and creatinine kinase concentrations have been found to differ between normal tarsocrural and femoropatellar joints [24]. Differences in the concentrations of total glycosaminoglycans, keratan sulfate and cartilage oligomeric matrix protein between the interphalangeal joint and metacarpophalangeal joints have also been reported in normal horses [21-23, 25]. These findings provide corroborating data that glycosaminoglycans may contribute the differences detects by FTIR, as absorptions associated with the molecules are typically found in the 1800 – 400 cm wavenumber range; further investigation is warranted (13,14). Different values for total glycosaminoglycans and keratan sulphate concentrations in SF between AC and TCRL joints have also been reported, but statistical conclusions could not be drawn as the data were of insufficient power[40].

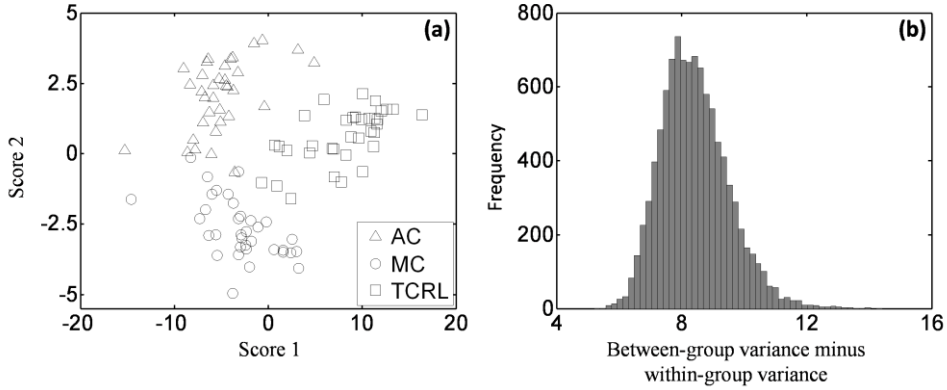

Fig. 1 (a) Scores plot for the samples from the three classes [antebrachiocarpal (AC), midcarpal (MC), and tarsocrural (TCRL) joints]. (b) Histogram of the between-group variance minus within-group variance for 10,000 random assignments of the class labels to the samples. The variance difference for the correct class labels was significant [22.08 (p<0.001)].

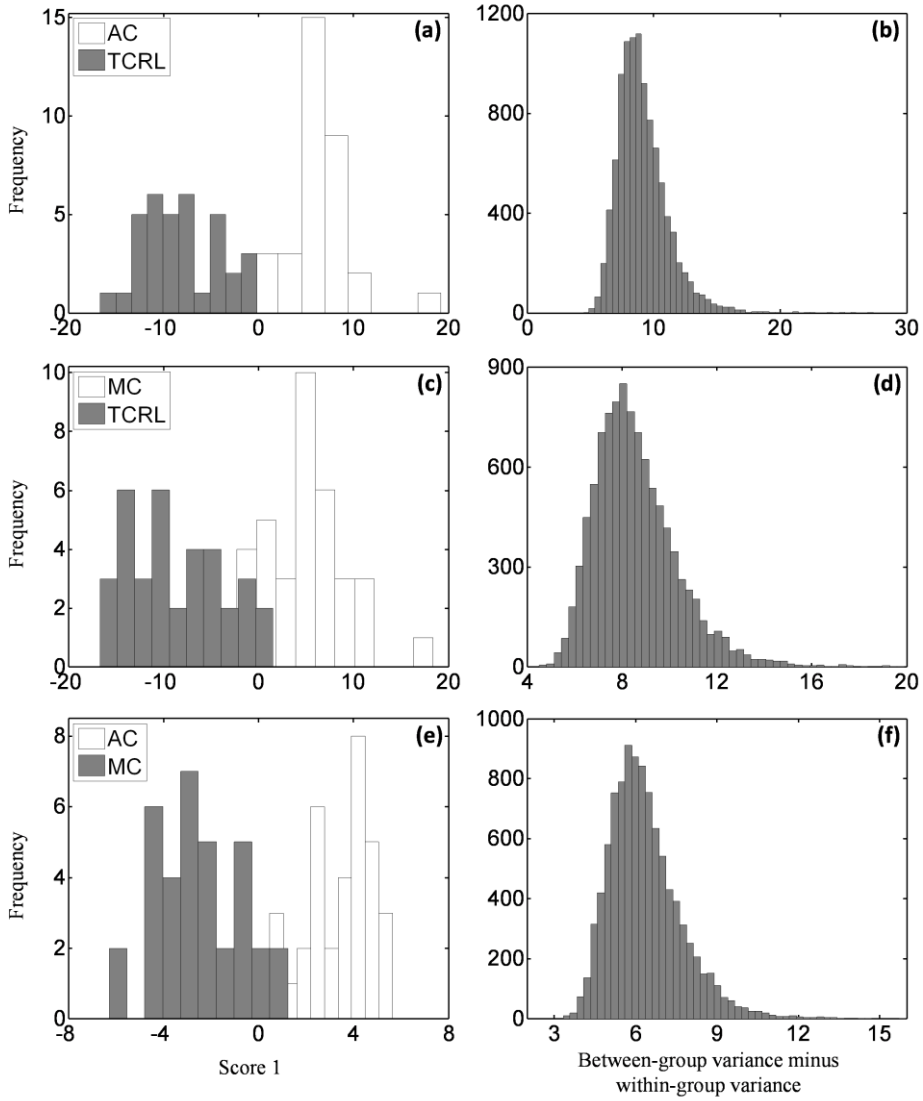

Fig. 2 (a), (c), and (e) Histograms of score 1 for antebrachiocarpal (AC) vs. tarsocrural (TCRL), midcarpal (MC) vs. TCRL, and AC vs. MC, respectively. (b), (d), and (f) Histograms of between-group variance minus within-group variance for AC vs. TCRL, MC vs. TCRL and AC vs. MC respectively, based on 10,000 random assignments of the class labels to the samples. The variance differences corresponding to correct class labels were significant for the comparisons illustrated in (a) and (c) [34.21 (p<0.001) and [25.23] (p<0.001) respectively], but not for (e) [5.98 (p=0.554)].

The number of horses available for the current study was comparable to that used in similar reports examining synovial fluid constituents, [21,38,39]. However the large number of variables examined increases the risks of cumulative type I error. As the ratio of variables to subjects is markedly unbalanced in work of this type, the use of traditional LDA to successfully model and classify the significant spectral differences characterizing each joint, is unlikely to be a successful approach (as indeed was found in the current study). For that reason, data reduction strategies were employed and a high significance level set,[17,18], followed by a more novel approach to classification as described in the current paper.

The novel classification strategy of MDA was critical in demonstrating the significance of these spectral differences. In discriminant analysis the projection of high-dimensional data, as alluded to above, into a low-dimensional subspace (e.g. two or three dimensions) is often required so that the samples (objects) from different classes can be objectively separated [42]. Typical methods include Fisher's discriminant analysis (FDA), LDA, PLSDA, and support vector machine (SVM). Various discriminant methods may give similar results for some data sets, but the results may also dramatically vary because they have different objective (cost) functions. It is also widely acknowledged that no method is suitable for all types of data and that one method may better distinguish among study groups than others, depending on the data structure. In the current study each of these other methods was explored, but with the current data structure, MDA with appropriate validation, was found to be the most useful and successful in classify the joint specific data.

In summary, FTIR spectroscopy in combination with, a novel approach to LDA, was successful tool to classify anatomically distinct high motion joints based on the detection of IR-active components in SF. Comparable FTIR absorption patterns of SF derived from contralateral joints support their use as within-subject controls. The breadth of biomolecular differences among joints indicates that inter-articular variation within the animal should be considered in prospective study design, and comparative studies of naturally occurring joint disease [21,22,39]. Further normal samples should be evaluated to better characterize the range and significance of changes detected, and their magnitude relative to those joint disorders.

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Following military service in the Air Force, Dr Riley received degrees in Physics and Veterinary Medicine from the University of Melbourne, Australia. After an internship and private practice in Australia, he completed a surgical residency at the University of Saskatchewan in Canada. Concurrently he completed M.Sc. and Ph.D. degrees in the fields of tendon in-vitro biology and biochemistry. Dr Riley then worked at briefly at Iowa State University and in private practice during which time he became a Diplomate in the American College of Veterinary Surgeons. He joined the faculty at the Atlantic Veterinary College, Canada in 1999 rising to the rank of Professor, and completed MBA course in Innovation Management at the University of Melbourne in 2007. He commenced his current position at Massey University in 2013 and has focused his research on the development of biomedical tests for animal diseases using the emerging technologies of infrared spectroscopy (FTIR), optoacoustics and bioinformatics. Dr Riley has a special interest in biomarkers for orthopedic disease, and humoral immunity, but is also interested exploring the full potential of emerging technologies as they apply to veterinary and comparative medicine. Dr Riley partnered with the Prof Wayne McIlwraith from the Orthopaedic Research Center, Colorado State University, and the Institute for Biodiagnostics, National Research Council of Canada, to develop the first FTIR test for equine traumatic arthritis and osteochondrosis. More recently he has collaborated with Prof Sheila Laverty at the University of Montreal to examine and characterize this technology further in rabbit and canine models of orthopaedic disease.