Infrared spectroscopy of serum fails to identify early biomarker changes in an equine model of traumatic osteoarthritis

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A B S T R A C T

Objective: to determine the accuracy of infrared (IR)-based serum biomarker profiling to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal osteoarthritis (OA) from controls.

Method: unilateral carpal OA was induced in 9 of 17 healthy Thoroughbred fillies, while the remaining served as sham operated controls. Serum samples were obtained before induction of OA (Day 0) and weekly thereafter until Day 63 from both groups. Films of dried serum were created, and IR absorbance spectra acquired. Following pre-processing, partial least squares discriminant analysis (PLS-DA) and principal component analysis (PCA) were used to assess group and time differences and generate predictive models for wavenumber ranges 1300-1800 cm⁻¹ and 2600-3700 cm⁻¹.

Results: the overall correct classification rate when classifying samples by group (OA or Sham) was 52.7% (s.d. = 12.8%), while it was 94.0% (s.d. = 1.4%) by sampling Day. The correct classification results by group-sampling Day combinations with pre-intervention serum (Day 0) was 50.5% (s.d. = 21.7%).

Conclusion: with the current approach IR spectroscopic analysis could not differentiate serum of horses with induced carpal OA from that of controls. The high classification rate obtained by Day of sampling may reflect the effect of exercise on the biomarker profile. A longer study period (advanced disease) or naturally occurring disease may provide further information on the suitability of this technique in horses.

1. Introduction

Osteoarthritis (OA) is the leading cause of joint disease in humans and is responsible for significant morbidity, with clinical signs such as pain and disability leading to marked economic losses worldwide [1–4]. It is estimated that 100 million Europeans suffer from OA [5], with knee OA estimated to affect between 18 and 40% of 60–79 year old people [6,7]. In the U.S. over 91 million adults (37%) were affected by OA in 2015 [8]. Similarly, joint disease represents a significant clinical condition in horses. Approximately 50 ± 3.2% of U.S. multi-horse operations experience one or more lameness cases annually, with approximately 50% limb or joint related [9]. In 2007, 4 million of the 7.3 million horses in the U.S. were estimated to be affected by OA, with a negative impact on 4.6 million people (owners, service providers, and businesses) associated with the equine industry producing goods and services of US$38.3 billion [10].

Compared with advances in human orthopedic research [11], there is a lack of a validated and repeatable approach to staging clinical osteoarthritis in the horse and case definitions referable to OA in specific joints. This has led to reliance upon well-validated models of traumatic
Many different soluble biomarkers associated with joint metabolism and pathology have been investigated as potential markers of OA [18–20]. In parallel with medical advances, a significant body of research in horses has been published, and progress has been made in identifying potentially clinically useful markers of OA [12,16,21]. Despite advancements in biomarker research [22–25] and limited application to equine clinical cases [26], the wider adoption and validation within a clinical practice setting of candidate biomarkers is yet to be established [19]. The main limitations of the techniques evaluated are the lack of consistency, the limited practicality of the tests in a clinical setting, and the relatively high costs when used as a tool for disease surveillance [27].

Fourier-transform infrared (IR) spectroscopy has been used to identify a “biochemical fingerprint” of all molecules in biofluids, rather than separating single molecules or biomarkers associated with a disease or physiological state [28]. It provides a means for detecting quantitative and qualitative changes in the molecular profile of a biofluid, accounting for known and unknown markers of disease. This aspect is particularly important since it is recognized that OA may be initiated by trauma and inflammation in any or all of the multiple joint tissues, including the synovial membrane, fibrous joint capsule, subchondral bone, peri-articular ligaments or articular cartilage [29]. The advantages of IR spectroscopy as a diagnostic tool are its high sensitivity and specificity in other diagnostic applications [30,31], the low cost (no reagents are required), and the low-invasive nature of the sample collection (serum) for testing. This technique has been investigated as a potential diagnostic and screening tool for joint disease in human synovial fluid [32–34] and serum [35], focusing on rheumatoid arthritis. However, these investigations have been in clinical cases with advanced disease and not applied to early detection of OA. In a rabbit model of knee osteoarthritis, IR spectroscopy of serum proved to be a sensitive approach to differentiate between rabbits with OA and controls [36]. This model produces severe acute joint instability, rather than focal osteochondral trauma often seen in young human and equine athletes [37,38]. Serum-based IR spectroscopy is capable of differentiating dogs with naturally occurring knee OA from unmatched controls [39]. In the horse, synovial fluid IR spectroscopy has been used in the diagnosis of clinical osteochondrosis and traumatic osteoarthritis [38,40]. This method has been applied to the diagnosis of clinical OA based on spectra of equine serum, but the comparison group used in this study was not verified as free of OA [41].

The objective of this study is to determine the feasibility and accuracy of IR-based serum biomarker profiling to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal OA from controls. Our hypothesis is that IR biomarker profiling of serum will be able to differentiate horses with induced carpal OA from controls.

2. Method

This randomized prospective experimental study was approved by the Massey University Animal Ethics Committee (MUAEC 14/18). The sample size for treatment and control groups was estimated based upon previous publications using the same model for biomarker and treatment trials [12–15].

2.1. Animals

As part of a larger biomarker study, fifteen 2-year-old and two 3-year-old female Thoroughbred horses (n = 17) not previously used or trained for any athletic activity were selected for the study. The inclusion criteria were lack of clinical abnormalities on physical examination by two equine surgical specialists, absence of lameness at the walk and trot before and after limb flexion tests, and no abnormalities on radiographs of the carpi immediately before the study. All horses were kept on grass pasture before the study. Nine horses were randomly assigned to the OA group, and eight to the Sham horse group, after blocking horses for sire and age. The order of procedures, Sham operation or surgical OA induction, was randomized. At the end of the trial the horses in the OA group were euthanised for tissue collection as part of another study (unpublished data), while Sham operated horses were rehomed.

OA group: Procaine penicillin at 22 mg/kg IM (Phoenix Pharmacillin 300, 300 mg/mL, Phoenix Pharm, New Zealand) was administered to all horses once before surgical intervention. All horses then were subjected to general anesthesia for arthroscopic surgery. Anesthesia was induced with 2.5 mg/kg of ketamine (Ceva Ketamine injection; Ceva Animal Health Pty Ltd, Australia) and 0.01 mg/kg of Diazepam (ilium Diazepam Injection USP; Troy Laboratories, Australia) intravenously. Following orotracheal intubation, anesthesia was maintained with isoflurane (Isoflurane; Bayer New Zealand Ltd, New Zealand) in 5 L/min of 100% oxygen. Following general anesthetic induction and aseptic preparation of a randomly chosen carpus, an 8 mm osteochondral fragment was arthroscopically created in the middle carpal joint using a bone gouge in the distal dorsal aspect of the radial carpal bone to induce traumatic OA as previously described [14,15]. The osteochondral fragment remained attached to the dorsal joint capsule reflection. The parent bone from where the fragment had been separated was debrided with a motorized bone burr to create a ~15 mm-wide defect (including the width of the fragment). The articular debris was left in the joint. These horses were identified as the OA horse group. All horses were administered phenylbutazone immediately after completion of the procedure at 4.4 mg/kg IV (Nabudone P, 200 mg/mL, Troy Laboratories, Australia) and for the following four days at 4.4 mg/kg PO, every 24 h (Equine Bute Paste, 200 mg/mL, Randlab, Australia). Postoperatively horses were examined twice daily to evaluate their comfort and well-being.

Sham control group: Sham horses underwent arthroscopic exploration only of one randomly selected middle carpal joint. The perioperative pharmaceutical treatment protocol and monitoring plan was identical to that of the OA group.

2.2. Post-operative exercise and clinical assessment

After a 14-day recovery period in horse stalls with 30 min of daily turnout in a 6 m × 6 m yard, a 7-week-long treadmill exercise protocol (5 days a week) was initiated. Horses were exercised daily for 2 min at a trot (4–5 m/s), then 2 min at a gallop (8–9 m/s), and then 2 min at a trot (4–5 m/s). The model mimics naturally occurring equine traumatic OA [12,13]. Scores were assigned on a weekly basis for lameness, joint effusion and response to carpal flexion and at the end of the study for radiographic changes (Supplementary Tables 1-4) as confirmation of the establishment of OA [12–16].

2.3. Serum sample collection

Blood (~10 mL) was collected by left jugular venipuncture immediately before induction of OA (or Sham surgery) (Day 0), and then weekly from all horses until Day 63. After collection, blood samples were allowed to clot in plain tubes, and 4–5 mL of serum obtained after centrifugation at 5000 rpm (3400 g) for 5 min within 60 min of sample collection. The serum was divided into 1 mL aliquots and stored at −80 °C until later analysis.

2.4. Infrared spectroscopy

Serum samples were thawed at room temperature and replicate (x 6) dry films made for each sample on a silicon 96-well microplate [17,38,39]. The microplate was mounted on a multi-sampler accessory (XY Microtitter Plate Accessory, PIKE Technologies, Madison, WI, USA) interfaced with an IR spectrometer (Tensor 27, Bruker Optics, Preston,
Spectral file import: The references to colour in this figure (right). The median (thick line) and 2.5%- and 97.5%-quantiles (thin lines) for both groups are shown: osteoarthritis (OA) in red, Sham in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.5. Analyses and model development for classification of spectral data

Spectral files were imported into proprietary spectral manipulation software (The Unscrambler Xv10.5.1, Camo Software, Oslo, Norway) and converted into delimited csv files for further analyses. All subsequent analyses were performed in R (R V3.6.3, R Core Team, Auckland, New Zealand; ChemoSpec and MixOmix packages V 4.3.34 and V6.8.5 respectively).

Spectral pre-processing: Savitzky-Golay filtering was applied to all spectra with a 1st-order derivative of the signal, a 1st order polynomials function and a smoothing window of width 5; parameters were tuned to maximize spectral separation by Day. An analysis of sensitivity was performed, and similar performances were also retrieved for other combinations of filter parameters. Then, standard normal variate (SNV) transformation was used to normalize spectra and remove baseline effects, reducing within-class variance [42]. Spectra values in the “fingerprint” regions between 1300 and 1800 cm\(^{-1}\) and 2600-3700 cm\(^{-1}\) were selected for further analyses. We did not remove any outlier samples as their number did not exceed the threshold of extreme PCA scores expected by chance (5%), and further inspection did not reveal any anomaly with the samples.

Classification model development: a series of predictive models were built to predict the horse group (task i; OA vs Sham), the sampling Day (task ii; 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63) and to assign samples a day-group class label, except Day 0 (i.e. prior to interventions) for which OA and Sham groups were pooled (task ii; n = 19 classes). Partial least squares discriminant analysis (PLS-DA) [43] was initially used in multi-level mode to account for repeated measures. Predictions were made using the first ten (10) principal components (PC) of the PLS-DA as no significant performance improvement was observed with >10 PCs and more than 50% of the variance of the design matrix was accounted for in all explored scenarios. More specifically, scores along each PC were computed for each new sample. Then new samples were assigned to the class with the closest centroid, relying on the Mahalanobis metric, as it was observed to lead to the most accurate class prediction [44].

The overall correct classification rate when classifying sample spectra according to their treatment group (OA or Sham) was 52.7% (s.d. = 12.8%) using 10 PC, with 50.2% of the total variance explained. Fig. 2 shows the lack of clear separation for the first 2 components in the first sample space. The classification rates obtained overall and for each group (OA or Sham) for a number of PC comprised between 1 and 10 is shown in Supplementary Fig. 1. Although the total variance associated with the model increases with the number of PCs, the classification accuracy is not improved by using more PCs.

3. Results

3.1. Spectral pre-processing

The normalized spectra of serum from OA and Sham groups are shown in Fig. 1a (left). There was no visually apparent difference in the pattern between the two groups. The greater peaks reflecting infrared absorption are associated with proteins: bands centred at 1650 cm\(^{-1}\) (amide I) and 1545 cm\(^{-1}\) (amide II) correspond to stretching and bending vibrations on the amide C=O and N-H groups, respectively; the broadband at 3300 cm\(^{-1}\) corresponds to the N-H group as well, but is a stretching vibration called the amide A mode [45]. The image of the pre-processed spectra is shown in Fig. 1b (right).

3.2. Classification of IR spectra from OA versus sham horses

The overall correct classification rate when classifying sample spectra according to their treatment group (OA or Sham) was 52.7% (s.d. = 12.8%) using 10 PC, with 50.2% of the total variance explained. Fig. 2 shows the lack of clear separation for the first 2 components in the first sample space. The classification rates obtained overall and for each group (OA or Sham) for a number of PC comprised between 1 and 10 is shown in Supplementary Fig. 1. Although the total variance associated with the model increases with the number of PCs, the classification accuracy is not improved by using more PCs.

Classification of IR spectra at different sampling times (Days).

The overall correct classification rate for samples according to their sampling Day (Days 0–63) was 94.0% (s.d. = 1.4%) when using 10 PC, with 53.2% of the total variance explained. The classification rates obtained overall and for each sampling Day for a number of PC comprised
between 1 and 10 are reported in Supplementary Fig. 2. Fig. 3a–c are plots of all samples in the first 3 PC defined by the PLSDA performed with sampling Day as the outcome (not applying the leave-one-horse-out scheme).

### 3.3. Classification of group-sampling day combinations with serum from day 0

For task iii, the overall classification rate is 50.5% (s.d. = 21.7%) using 10 PC, with 53.2% of the total variance explained. All class-specific classification rates for this task are reported in Supplementary Table 5 and Supplementary Fig. 3.

The specificity for a class (group x Day) identification varies. Sampling Day identification was still performed accurately, with classification rates similar to those shown in Supplementary Fig. 2. However, within sampling Days, the correct identification of groups (OA vs Sham) was limited, with classification rates ranging from 94.1% for samples collected prior to interventions (Day 0) to 85.0% (Day 35, Sham) and 20.0% (Day 42, OA). Lastly, Fig. 4(a, b, c) shows all samples for the first three PC of the PLSDA for this task (not applying the leave-one-horse-out scheme) and confirms the lack of separation by group. Incidentally, the separation of spectra by days is observed for a few samples.

### 4. Discussion

This study is the first to attempt evaluation of IR spectroscopy and spectral analyses to discriminate between serum from horses with traumatically induced OA and strictly characterized controls. However, our results showed that the approach described here failed to differentiate serum spectra of horses with early traumatically induced osteoarthritic changes (OA) from those of controls (Sham). Significant differences in

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**Fig. 2.** Sample score plots by group for the first 2 components of partial least squares discriminant analysis (PLSDA) (task i). Osteoarthritis (OA) horses in red circles, Sham in blue triangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Fig. 3.** a, b, c. Sample plots of the partial least squares discriminant analysis (PLSDA) for sample day identification (task ii) in the first three principal components (PC) (a, PC2 vs PC1, top left; b, PC3 vs PC1, bottom left; and c, PC2 vs PC 3, top right).
the spectroscopic profile of serum were detected at different time points during a postoperative exercise program in both groups.

In a previous experimental study in rabbits using a model of knee OA (cranial cruciate ligament transection inducing marked instability) IR spectroscopy on serum allowed differentiation of affected animals from controls at biweekly intervals between 2 and 12 weeks post injury [36]. The joint instability created with this model results in early onset of OA, with a severe and debilitating burden of disease of all articular structures within 2 weeks, sufficient to contribute to detectable changes in the IR spectrum of rabbit serum [46]. Similarly, IR spectroscopy was able to identify serum of dogs with naturally occurring knee OA associated with cranial cruciate ligament rupture from controls, with high sensitivity and specificity [39]. In this prospective controlled study, the dogs recruited had clinically evident naturally occurring OA due to joint instability that was sufficiently advanced to warrant surgical intervention. The equine model used in the current study does not create marked joint instability. In agreement with other reports using the model, clinical signs attributed to the osteochondral fragments were minimal [12,14,15]. This model has been sufficient to demonstrate biochemical differences based on ELISA biomarkers [12]. However, a more severe equine model of joint injury that includes instability, or prolonged follow-up period, may be required to exploit the discriminatory potential of IR spectroscopy of serum.

In contrast with our study, others utilizing the same equine model and one of naturally occurring OA in similarly aged horses of the same breed have demonstrated significant differences in specific ELISA based biomarkers (e.g. epitope CS846 and type II collagen carboxy-propeptide) between OA and control groups [12,26]. Although an IR spectroscopy-based approach provides for a broad-based “fingerprint” of known and unknown biomarkers, few IR based assays of biofluids rely upon the detection of a single molecular species. Where such assays are successful, they are typically for biomolecules in high concentrations (e.g. immunoglobulins) that overshadow contributions to serum IR spectra of solutes in lower concentrations [30,31,47]. In the case of previously established ELISA-based soluble biomarkers for this equine model, concentrations in the serum are 25–1000 fold lower than in synovial fluid, and these levels approach or are below the detection threshold for a single molecular species with mid-IR transmission spectroscopy [12,26,47]. In contrast, IR spectroscopy is effective at identifying synovial fluid from horses affected by osteochondrosis and traumatic arthritis from controls, presumably because disease-related IR active biomolecules are in sufficient concentrations for IR based detection and discrimination [38,40]. To improve the quantification of proteins with low serum concentrations (e.g. immunoglobulin A) an ultrafiltration method has been described to separate molecules based on their molecular size [48]. In future studies, pre-processing of serum samples with techniques such as ultrafiltration may enhance the relative contribution of specific proteins in the IR spectral profile but is unlikely to separate specific peptides or glycosaminoglycan-based biomarkers.

A recent study utilizing IR spectroscopy of serum claimed success in discriminating between horses with naturally occurring OA from controls [41]. The study compared 15 horses with OA and 48 designated controls. Serum from horses affected with metacarpophalangeal, metatarsophalangeal or carpal joints were included. However, conventional and IR-based comparisons of equine synovial fluid from different normal...
joints differ significantly biochemically [49,50]. Therefore, in our study a single joint was evaluated to eliminate possible variability arising from different anatomic locations. The control group in the report by Paraskevaidi et al. (2020) included 12 different breeds, ranging in age from 1 day to 26 years, and the clinical OA group consisted only of Thoroughbred racehorses ranging in age from 3 to 10 years. Age has been recognized to play an effect on clinical disease and on the performance of IR-based spectroscopy techniques in horses affected by osteochondrosis and dogs with cranial cruciate ligament rupture associated OA [39,40]. Therefore, in agreement with the approach taken by others [12,26], we report the narrow inclusion criteria of unarced 2 to 3-year-old female Thoroughbreds to minimize the possible confounding effects of age or gender on biomarker profiles. Nevertheless, the narrow selection criteria included in the current study did not improve the ability of IR spectroscopy to detect changes associated with induced OA.

Excellent classification rates by sampling Day were obtained when using 10 PC of the PLSDA. There was clear separation of classes for samples collected at Day 0 and 7. Lameness, flexion test, and effusion scores were significantly different between these days for the OA group (Supplementary Tables 1–3) providing clinical evidence of inflammation. For the Sham group only lameness and effusion scores were significantly different, suggesting perhaps a less severe inflammatory response. However, differences in these scores between OA and Sham groups were not significant. This may be attributed to the clinical response to the use of a non-steroidal anti-inflammatory drug for five days following arthroscopy in both groups (as dictated by the animal ethics protocol).

The inability to reliably classify the treatment group (OA or Sham) at the remaining sampling times may reflect the effect of exercise on the biomarker profile contained within the serum spectra rather than disease [12,51]. This finding is in agreement with previous works using the same equine model that found significant changes in the concentrations of specific soluble biomarkers (epitope CS846, epitope CPII, glycosaminoglycans, osteocalcin, type 1 and 2 collagen degradation fragments, and bone specific type I collagen) in serum in response to the same exercise protocol [12]. Adaptations to exercise are complex and encompass the musculoskeletal, cardiovascular, respiratory, and other systems. The IR-active biomolecular contributions to each spectrum reflect both physiologic (i.e. exercise induced or natural temporal variations) and disease (OA) contributions. In the current study, the exercise-related changes in the spectra may have obscured the detection of those associated with early disease-related responses in the equine OA model.

The comparison of group-sampling day combination with serum from Day 0 was performed in an attempt to further explore the poor results observed for the OA-Sham analysis. However, the results were highly variable with performance best at Day 35. Although the precise reasons for these results are unknown, the limitations discussed above are likely to apply similarly.

A limitation of our study was the lack of an age-matched unexercised control group, which did not allow the determination of the role of exercise on IR spectra. Moreover, although the selection criteria of the animals in our study (young age, female gender and Thoroughbred breed) were chosen to limit variability, differences may exist in serum spectra of animals of different age, gender or breed, which were not explored in this study. In addition, the IR spectra obtained in horses with induced OA may differ significantly from those in horses with naturally occurring OA.

In conclusion, this is the first study to investigate the use of IR spectroscopy on serum from horses with traumatically induced OA. This technique did not facilitate the early discrimination of horses affected by OA from controls. A prospective study using horses affected by naturally occurring OA with precise case definitions and appropriately matched controls may provide more useful information on the suitability of this technique in horses.

Author contributions

The authors’ contributions included concept and study design (LP, CBR), data analysis and interpretation (LP, MV, KED, MRW, CBR), statistical analysis (MV, CBR), animal recruitment (CWR, CBR), serum sampling (LP), surgical induction of OA (LP, CWM), provision of general anesthesia (HS), radiographic assessment (SP, MO), treadmill exercise (LP), obtaining funding (CBR), preparation of draft article (LP, MV, KED, MRW, CBR). All authors provided feedback and approved the final version of the article.

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This study was funded by the New Zealand Equine Trust. The Chair of New Zealand Equine Trust (C.W. McIlwraith) was involved in surgical creation of OA for his expertise with the model of equine OA used in this study. He has also reviewed the manuscript prior to submission, but was not involved in study design, data collection or analysis.

Declaration of competing interest

One of the authors (C.W. McIlwraith) is the Chair of the New Zealand Equine Trust.

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Appendix A. Supplementary data

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