

1 Optical photothermal infrared spectroscopy can differentiate equine osteoarthritic plasma  
2 extracellular vesicles from healthy controls

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24 Synovial fluid

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25 **Abstract-**

26 *Background* – Equine osteoarthritis is a chronic degenerative disease of the articular joint,  
27 characterised by cartilage degradation resulting in pain and reduced mobility and thus is a prominent  
28 equine welfare concern. Diagnosis is usually at a late stage through radiographic examination, whilst  
29 treatment is symptomatic not curative. Extracellular vesicles are small nanoparticles that are involved

30 in intercellular communication. The objective of this study was to investigate the feasibility of Raman  
31 and optical photothermal infrared spectroscopy to detect osteoarthritis using plasma-derived  
32 extracellular vesicles.

33 *Methods* – Plasma samples were derived from thoroughbred racehorses. A total of 14 samples were  
34 selected (control; n= 6 and diseased; n=8). Extracellular vesicles were isolated using differential  
35 ultracentrifugation and characterised using nanoparticle tracking analysis, transmission electron  
36 microscopy, and human tetraspanin chips. Samples were then analysed using Raman and optical  
37 photothermal infrared spectroscopy.

38 *Results* –Infrared spectra were analysed between 950-1800  $\text{cm}^{-1}$ . Raman spectra had bands between  
39 the wavelengths of 900-1800  $\text{cm}^{-1}$  analysed. Bands below 900  $\text{cm}^{-1}$ . Spectral data for both Raman and  
40 optical photothermal infrared spectroscopy was used to obtain a classification model and confusion  
41 matrices, characterising the techniques ability to distinguish diseased samples. Optical photothermal  
42 infrared spectroscopy could differentiate osteoarthritic extracellular vesicles from healthy with good  
43 classification (93.4%) whereas Raman displayed poor classification (64.3%). Plasma-derived  
44 extracellular vesicles from osteoarthritic horses contained increased signal for proteins, lipids and  
45 nucleic acids.

46 *Discussion/ conclusion* – For the first time we demonstrated the ability to use optical photothermal  
47 infrared spectroscopy to interrogate extracellular vesicles and osteoarthritis-related samples. Optical  
48 photothermal infrared spectroscopy was superior to Raman in this study, and could distinguish  
49 osteoarthritis samples, suggestive of its potential use diagnostically to identify osteoarthritis in equine  
50 patients. This study demonstrates the potential of Raman and optical photothermal infrared  
51 spectroscopy to be used as a diagnostic tool in clinical practice, with the capacity to detect changes in  
52 extracellular vesicles from clinically derived samples.

### 53 Introduction

54 Osteoarthritis (OA) is a common degenerative disease of the synovial joint, characterised by catabolic  
55 processes observed in articular cartilage, and a notable imbalance in bone remodelling. It results in  
56 pain, inflammation and reduced mobility (Mustonen and Nieminen, 2021). OA is the most prevalent  
57 cause of equine lameness, with over 60% of horses developing OA within their lifetime; a significant  
58 welfare concern (McIlwraith *et al*, 2012). It is a complex heterogeneous condition of multiple causative  
59 factors, including mechanical, genetic, metabolic and inflammatory pathway involvement (Clarke *et*  
60 *al*, 2021). OA pathophysiology is conserved across species, resulting in synovitis, cartilage degradation,  
61 osteophyte formation, subchondral bone sclerosis, fibrosis and reduced elastoviscosity of synovial

62 fluid found within the joint capsule (Kuyinu *et al*, 2016). The horse is both of interest as a target species  
63 for veterinary equine medicine as well as a model organism to study osteoarthritis due to these  
64 established similarities, as well as comparable anatomic structures of the human carpal joint with  
65 equine carpal and metacarpophalangeal joint (McIlwraith *et al*, 2012).

66 Extracellular vesicles (EVs) are nanoparticles enveloped in a phospholipid bilayer membrane, secreted  
67 by most mammalian cells, that transport biologically active cargo, such as proteins, RNAs, DNAs, lipids,  
68 and metabolites (Boere *et al*, 2018). EVs are divided into subgroups as determined by their size and  
69 biogenesis. EVs elicit their effects through paracrine signalling, proving fundamental in intercellular  
70 communication (Herrmann *et al*, 2021). EVs have been implicated in the propagation of OA, and have  
71 been shown to be released and enter chondrocytes, synoviocytes and inflammatory cells (Esa *et al*,  
72 2019; Withrow *et al*; 2016 and Lin *et al*, 2021). Interestingly EVs can serve as disease propagators;  
73 promoting an increased expression of cytokines, chemokines and matrix degrading proteinases, or  
74 disease preventing; increasing cellular differentiation and reducing apoptosis.

75 Vibrational spectroscopic methods, including Raman and infrared spectroscopies, provide molecular  
76 information about the main molecular constituents commonly found in biological samples such as  
77 proteins, lipids, nucleic acids, and carbohydrates based on bond-specific chemical signatures in a non-  
78 invasive, non-destructive, and label-free manner (Paraskevaidi *et al*, 2021). In Raman spectroscopy,  
79 photons from a monochromatic source interact with the sample and a small fraction of them are  
80 inelastically scattered with either higher or lower energies compared with the excitation wavelength.  
81 The energy difference between incident and scattered photons corresponds to a Raman shift and it is  
82 associated with the chemical structure of molecules in the sample (Lima *et al*, 2021). Raman  
83 spectroscopy can discriminate between cell and tissue types and detect chemical alterations prior to  
84 morphological changes in various pathological states. It has previously been used to assess the purity  
85 of EV preparations (Gualerzi *et al*, 2019), as well as identify cellular origin of mesenchymal stem cell  
86 (MSC)-derived EVs (Gualerzi *et al*, 2017). Infrared spectroscopy is based on the absorption of infrared  
87 radiation by molecular vibrations from bonds that possess an electric dipole moment that can change  
88 by atomic displacement (Baker *et al*, 2014).

89 Although Raman and infrared spectroscopies provide molecular information about the overall  
90 biochemistry of samples by monitoring the internal motion of atoms in molecules, each method has  
91 advantages and disadvantages for different types of samples due to their different working principles.  
92 Thus, having both techniques combined in one single technology is a powerful tool with promising  
93 applications. More recently, a novel far-field optical technique has been developed in order to acquire  
94 Raman and infrared signatures simultaneously. In this method, the infrared signatures are collected

95 via optical photothermal infrared spectroscopy (O-PTIR), which is based on a pump-probe  
96 configuration that couples a tuneable infrared quantum cascade laser (QCL) acting as pump and a  
97 visible laser to probe the thermal expansion resulting from the temperature rise induced by the QCL.  
98 The probe laser also acts as excitation source for acquiring Raman spectrum simultaneously with  
99 infrared data at the same spatial resolution. This scheme has been used to interrogate tissue samples,  
100 mammalian cells (Banas *et al*, 2021; Spadea *et al*, 2021) and bacteria (Lima *et al*, 2021) but this is the  
101 first study, to our knowledge, to use it in EV or OA work.

102 We hypothesised that Raman and O-PTIR can be used to identify potential biomarkers of OA using  
103 plasma-derived EVs.

## 104 Materials and Methods

### 105 Sample Selection

106 Plasma samples were collected in accordance with the Hong Kong Jockey Club owner consent  
107 regulations (VREC561). All samples were from thoroughbred racehorses, with the donor cohort having  
108 a mean age (+/-SEM) of 6.57 +/- 0.45. Horses were selected based on histological scoring of OA severity  
109 using a modified Mankin score (McIlwraith *et al*, 2010). A total of 14 samples were selected (control;  
110 n= 6 (mean score +/- SEM = 1.83 +/-0.48) and diseased; n=8 (mean score +/- SEM= 16.25 +/- 1.15).

### 111 Extracellular Vesicle Isolation – Differential Ultracentrifugation

112 Equine plasma samples underwent differential ultracentrifugation (dUC) in order to isolate EVs.  
113 Samples were subjected to a 300g spin for 10 minutes, 2000g spin for 10 minutes, 10,000g spin for 30  
114 minutes in a bench top centrifuge. Samples were then transferred to Beckman Coulter thick wall  
115 polycarbonate 4ml ultracentrifugation tubes, and spun at 100,000g for 70 minutes at 4°C (Optima  
116 XPN-80 Ultracentrifuge, Beckman Coulter, California, USA) in a 45ti fixed angle rotor, with the use of  
117 a 13mm diameter Delrin adaptor. Sample pellets were suspended in 50µl of filtered phosphate  
118 buffered saline (PBS) (Gibco™ PBS, pH 7.4 - Fisher Scientific, Massachusetts, USA), resulting in 50µl  
119 plasma EV (P-EV) samples.

### 120 Extracellular Vesicle Characterisation

#### 121 Nanoparticle Tracking Analysis

122 Nanoparticle tracking analysis (NTA) was used to quantify EV concentration and size of all samples,  
123 using a NanoSight NS300 (Malvern, UK). All samples were diluted in filtered PBS 1:50 (10µl of sample  
124 used), to a final volume of 500µl. For each measurement, three 1-min videos were captured, (at a  
125 screen gain of 4 and detection threshold of 12. After capture, the videos were analysed by the in-build

126 NanoSight Software NTA 3.1 Build 3.1.46. Hardware: embedded laser: 45 mW at 488 nm; camera:  
127 sCMOS.

#### 128 Transmission Electron Microscopy

129 EV presence and morphology were characterised using transmission electron microscopy. 10 µl of  
130 each sample was placed onto a carbon coated glow discharged grid and incubated at room  
131 temperature for 20 minutes. Samples were then subject to a negative staining protocol. EVs were fixed  
132 onto the grid with 1% glutaraldehyde for 5 minutes. The sample grids were incubated on 1% aqueous  
133 uranyl acetate (UA) (ThermoFisher Scientific, Massachusetts, USA), for 60 seconds, followed by  
134 4%UA/2% Methyl Cellulose (Sigma Aldrich, Gillingham, UK) at a 1:9 ratio on ice for 10 minutes. Grids  
135 were then removed with a 5mm wire loop and dried. The prepared grids were then viewed at 120KV  
136 on a FEI Tecnai G2 Spirit with Gatan RIO16 digital camera.

#### 137 Exoview Characterisation

138 The exoview platform (NanoView Biosciences, Malvern Hills Science Park, Malvern) was used to  
139 determine EV concentration, surface marker identification and to perform fluorescent microscopy and  
140 tetraspanin colocalization analysis. We had previously tested both the human and murine chips on  
141 equine samples and demonstrated the human chips were more compatible (data not shown). ExoView  
142 analyses EVs using visible light interference for size measurements and fluorescence for protein  
143 profiling. Samples were analysed in triplicate using the ExoView Tetraspanin Kit (NanoView  
144 Biosciences, USA) and were incubated on the human ExoView Tetraspanin Microarray Chip for 16  
145 hours at room temperature. Following this sample chips were incubated with tetraspanin labelling  
146 antibodies, namely anti-CD9 CF488, anti-CD81 CF555 and anti-CD63 CF647 and the MIgG negative  
147 control. The antibodies were diluted 1:500 in PBST with 2% BSA. The chips were incubated with 250  
148 µL of the labelling solution for 1 hour. The sample chips were washed and imaged with the ExoView  
149 R100 reader ExoView Scanner v3.0. Data was analysed using ExoView Analyzer v3.0. Fluorescent cut  
150 offs were set relative to the MIgG control. Total EVs were determined as the number of detected  
151 particles bound to tetraspanin antibodies (CD9, CD81, CD63) and normalised to MIgG antibody.

#### 152 Raman Spectroscopy and Infrared Spectroscopy (O-PTIR)

153 For all samples O-PTIR measurements were acquired on single-point mode using a mIRage infrared  
154 microscope (Photothermal Spectroscopy Corp., Santa Barbara, USA), with the pump consisting of a  
155 tuneable four-stage QCL device, while the probe beam is a continuous wave (CW) 532 nm laser.  
156 Spectral data were collected in reflection mode using a 40×, 0.78 NA, and 8 mm working distance  
157 Schwarzschild objective. Single-point spectral data were acquired over a spectral region of 930–1800

158  $\text{cm}^{-1}$ , with  $2\text{cm}^{-1}$  spectral resolution and 10 scans per spectrum. Raman data were acquired using a  
159 Horiba Scientific iHR-320 spectrometer coupled to mIRage, using a grating of 600 l/mm, 10 s as  
160 acquisition time, spectral region of 500–3400  $\text{cm}^{-1}$ , with 2  $\text{cm}^{-1}$  spectral resolution and 10 scans per  
161 spectrum.

## 162 Statistical Analysis

163 Raman and O-PTIR spectroscopic data was analysed using principal component analysis (PCA) to  
164 determine each techniques ability to identify diseased samples from healthy. Spectral data was also  
165 used as input for partial least squares discriminant analysis (PLS-DA) in order to generate a  
166 computerised model. Further analysis involved using a classification model and confusion matrices,  
167 whereby bootstrapping was performed 10000 times to permute whether the EV spectrum was  
168 classified as OA or control.

## 169 Results

### 170 EV Characterisation

#### 171 Nanoparticle tracking analysis

172 Particle size and concentration characterisation was performed using NTA. NTA determined the  
173 average plasma sample concentration to be  $2.02 \times 10^9$  particles/ml. Analysis was suggestive of a  
174 heterogeneous population of EVs, ranging from exosomes to microvesicles (Figure 1A).

#### 175 Transmission electron microscopy

176 To confirm the particles isolated from plasma samples were indeed EVs we negatively stained and  
177 visualised them using transmission electron microscopy. Spherical structures within EV size ranges  
178 (30nm-100nm (exosomes) and 100nm-1000nm (microvesicles)) were identified with a clearly defined  
179 peripheral membrane as shown in Figure 1B.

180

#### 181 Exoview

182 Exoview was used on a representative pool of plasma samples. The EVs extracted from plasma had  
183 the highest particle counts on the CD9 capture spots, equating to a concentration of around  $1.4 \times 10^8$   
184 CD9 positive particles/ml. CD81 ( $7 \times 10^7$  particles/ml) and CD63 ( $6 \times 10^7$  particles/ml) positive particles  
185 were also detectable (Figure 2A). It was observed that most EVs detected were less than 100nm  
186 (Figure 2B). It was also found that with plasma EV samples, the greater the expression of CD9 the  
187 greater the expression of CD81, whereby a distinct positive correlation can be observed (Figure 2C).

188 Co-localisation analysis was also performed and identifying that 91% of plasma-derived EVs were  
189 positive for the CD9 surface tetraspanin, followed by 5% expressing CD81, and 3% expressing both  
190 CD81 and CD9. CD63 expression was lowest at 0.8% (Figure 2D). Finally, plasma EVs were visualised  
191 using fluorescent microscopy, as shown in Figure 2E.

192

### 193 Raman Spectroscopy and Infrared Spectroscopy (O-PTIR)

194 Infrared and Raman spectral data collected from healthy and diseased plasma-derived EV samples  
195 displayed similar biochemical features with subtle changes in signal intensity, while appearance or  
196 disappearance of peaks were not observed (Figure 3A and B).

197 Infrared signatures acquired by O-PTIR spectroscopy were recorded from 950-1800  $\text{cm}^{-1}$ , however,  
198 bands peaking below 1200  $\text{cm}^{-1}$  were removed from the analysis due to interference from bands  
199 attributed to minerals from PBS (Mihaly *et al*, 2017). The band peaking at 1743  $\text{cm}^{-1}$  arose from C=O  
200 ester groups from lipids including phospholipids, triglycerides and cholesterol (Mihaly *et al*, 2017)  
201 Amide I vibration, peaking at 1647  $\text{cm}^{-1}$  was associated mainly to C=O stretching vibration from  
202 peptide bonds in proteins (Mihaly *et al*, 2017; Barth, 2007; Paolini *et al*, 2020). Amide II band  
203 absorption was found in 1544  $\text{cm}^{-1}$  and is attributed to the out-of-phase combination of the N-H in-  
204 plane bend and C-N stretching vibration with smaller contributions from the C-O in-plane bend and  
205 the C-C and N-C stretching vibrations of peptide groups (Mihaly *et al*, 2017; Barth, 2007; Paolini *et al*,  
206 2020). The band observed at 1240  $\text{cm}^{-1}$  results from the coupling between C-N and N-H stretching  
207 from proteins (amide III) (Barth, 2007), but it was also influenced by  $\text{PO}_2^-$  asymmetric stretching from  
208 phosphodiester bonds in nucleic acids (Paraskevaidi *et al*, 2021). The peak at 1451  $\text{cm}^{-1}$  corresponded  
209 to bending vibration (scissoring) of acyl  $\text{CH}_2$  groups in lipids (Mihaly *et al*, 2017; Barth *et al*, 2007;  
210 Paolini *et al*, 2020), whereas the band peaking at 1395  $\text{cm}^{-1}$  arises from  $\text{COO}^-$  symmetric stretching  
211 from amino acid side chains and fatty acids (Mihaly *et al*, 2017; Barth, 2007; Paolini *et al*, 2020).  
212 Spectral signatures from minerals were also observed in Raman spectrum acquired from healthy and  
213 diseased samples in the low wavenumber region (below 900  $\text{cm}^{-1}$ ), therefore, only bands peaking  
214 between 900-1800  $\text{cm}^{-1}$  were analysed. In Raman spectra, peaks associated to amide I, II, and III from  
215 peptide bonds were observed peaking at 1672, 1556, and 1241  $\text{cm}^{-1}$  respectively (Zhang *et al* 2020;  
216 Gualerzi *et al*, 2019). The band peaking at 1450  $\text{cm}^{-1}$  originated from  $\text{CH}_2/\text{CH}_3$  bending vibrations from  
217 lipids and proteins, while the peak at 1004  $\text{cm}^{-1}$  was attributed to the phenylalanine ring breathing,  
218 and the peak at 1340  $\text{cm}^{-1}$  was associated to nucleic acids (Zhang *et al* 2020; Gualerzi *et al*, 2019).

219 Infrared and Raman were subjected to principal component analysis (PCA) in order to examine the  
220 ability of both techniques to discriminate healthy and diseased plasma-derived EV samples (Figure 4).

221

222 PC scores plot obtained from O-PTIR (infrared signatures) as input data showed satisfactory  
223 discrimination between healthy and diseased samples (Figure 4A), with scores from control samples  
224 grouping on the negative side of PC-1 axis while scores related to OA clustered on positive side of the  
225 PC-1 axis. The loadings plot (Figure 4B) revealed positive loadings to all bands displayed in Figure 4a,  
226 indicating higher amount of the molecular constituents associated with these vibrations, i.e. proteins,  
227 lipids and nucleic acids, in samples derived from plasma EVs in OA. The scores plot obtained by  
228 subjecting Raman signatures to PCA displayed poor discrimination between healthy and OA samples  
229 (Figure 4).

230 Raman and O-PTIR spectral data were used as input for PLS-DA in order to generate a model for  
231 classifying OA and control samples based on EV composition. The classification models and confusion  
232 matrices obtained by PLS-DA using O-PTIR and Raman data are shown in Figure 5. Bootstrapping was  
233 performed 10000 times to permute whether the EV spectrum was classified as OA or control. PLS-DA  
234 model obtained from O-PTIR data demonstrated good correct classification rates (CCR) of an average  
235 of 93.41%, while PLS-DA model generated by using Raman signatures as input data presented a poor  
236 CCR (64.63%). These findings agree with the results obtained by PCA, indicating superior ability of O-  
237 PTIR to discriminate healthy from OA samples.

238

## 239 Discussion

240 This study, for the first time investigated whether O-PTIR could be used successfully to interrogate  
241 plasma-derived EVs in control and OA equine samples. We demonstrated that indeed the novel  
242 spectroscopy technique O-PTIR was able to determine differences in EV content attributed to disease  
243 pathology. Thus O-PTIR could serve as a platform for as future biomarker studies in equine OA.

244 Plasma EV concentration ( $2.02 \times 10^9$  particles/ml) was quantified using NTA, an accepted method of EV  
245 evaluation known for its repeatable and reproducible (Vestad *et al*, 2017). The representative plasma  
246 sample shown reflect the biological sample heterogeneity, showing a range of EV sizes indicative of  
247 both exosome and microvesicles subgroups. The concentration determined was similar to those  
248 reported for plasma EVs in other species within literature. For example, human plasma EVs identified  
249 by Palviainen *et al* (2020)  $2.46 \times 10^9$ -  $1.10 \times 10^{10}$  particles per ml. However, a study analysing plasma EVs  
250 across the duration of equine endurance racing found that baseline plasma EV concentration was  
251  $5.6 \times 10^{12}$  particle per ml (de Oliveira Jr *et al*, 2021). Exoview analysis identified EVs that were positive  
252 for the tetraspanins CD81 and CD9, however a low percentage of EVs were positive for the surface



253 marker CD63. This may a result of poor protein homology between equine and humans. Although, a  
254 recent paper using intracellular trafficking demonstrated that CD63 expression in HeLa cells was  
255 specific to exosomes, and often a lack of CD63 expression may be due to small microvesicle  
256 production, referred to as ectosomes, and that this type of EV is far more prolific than CD63 positive  
257 exosomes (Mathieu *et al*, 2021).

258

259 Infrared spectroscopy techniques have been used to probe EVs previously in order to identify  
260 structural components (Kim *et al*, 2019) as well as proteins, lipid and nucleic acid components, as  
261 found in our study (Kim *et al*, 2019). This is the first paper to our knowledge to probe OA EVs using  
262 Raman spectroscopy and O-PTIR spectroscopy. In other work, Zhai *et al* (2019) found that in bone,  
263 mineral and carbonate content varied significantly with OA stage, with carbonate increasing with OA.  
264 They also identified using Fourier transform infrared spectroscopy that acid phosphate, collagen  
265 maturity and crystallinity varied with OA. In addition, the use of infra-red spectroscopy is compounded  
266 by the findings of Afra *et al* (2017) in a study utilising an experimental model of OA in rats. Here the  
267 spectral differences between control and OA samples could be correlated to Mankin score and  
268 glycosaminoglycan content. A previous study by our group used attenuated total reflection Fourier-  
269 transform infrared (ATR-FTIR) spectroscopy on OA equine serum. This infrared spectroscopy study  
270 found separation between groups with 100% sensitivity and specificity, with the six most significant  
271 peaks between groups being attributed to proteins and lipids. Similarly, this was observed within our  
272 study, with increased abundance found within our OA group. The stated study postulated these  
273 observations may be associated to increased lipid and protein expression including increased  
274 expression of type 1 collagen, and decreased expression of type 2 collagen characteristic of OA  
275 (Paraskevaidi *et al*, 2020).

276

277 Previously, changes in plasma and lipid concentration in plasma and serum derived from OA patients  
278 has been described. One study utilised serum samples from horses to discriminate proteomic changes  
279 due to exercise or the development of early OA (Frisbie *et al*, 2008). Researchers identified six  
280 biomarkers with the ability to discriminate OA from exercise groups. For example, the concentration  
281 of serum C1,2C (reflective of type 1 and 2 collagen degradation fragments) and collagen 1 was found  
282 to significantly increase in OA groups compared with exercise alone (Frisbie *et al*, 2008). In addition, a  
283 multiplexed proteomic study on human OA serum identified a panel of 14 candidate biomarkers for  
284 OA (Fernandez-Puente *et al*, 2017), utilising cartilage, synovial fluid, chondrocytes and serum. These  
285 prospective biomarkers include von Willebrand factor (inflammation and haemostasis) and  
286 haptoglobin (an inflammation inducible plasma protein) (Fernandez-Puente *et al*, 2017).

287 Furthermore, a previous study in plasma identified different lipid profiles in OA using a destabilisation  
288 of the medial meniscus model (Pousinis *et al* 2020). Altered lipids included classes of cholesterol  
289 esters, fatty acids, phosphatidylcholines, N-acylethanolamines, and sphingomyelins and some were  
290 attributed to cartilage degradation.

291

292 Raman spectroscopy has been used to characterise EVs and their composition. It has been used to  
293 interrogate EVs undergoing autophagy (Chalapathi *et al*, 202), as well as distinguishing EVs derived  
294 from bovine placenta and mononuclear cells (Zhang *et al*, 2020), identifying differential features in  
295 Parkinson's disease pathology (Gualerzi *et al*, 2019) and sporadic Amyotrophic Lateral Sclerosis  
296 (Morasso *et al*, 2020). Additionally, it has previously been used to successfully discriminate between  
297 healthy and diseased joint tissues in order to identify subtle molecular and biochemical changes as a  
298 result of disease. Buchwald *et al* (2017) utilised Raman spectroscopy to identify compositional and  
299 structural changes in bone from the hip joints of OA patients demonstrating that subchondral bone  
300 from OA patients was less mineralised due to a decrease in hydroxyapatite. Furthermore, a study  
301 performed by de Souza *et al* (2014) using two *in vivo* experimental rat models of knee OA (treadmill  
302 exercise induced and collagenase induced) established molecular signatures unique to OA. Raman  
303 ratios relating to mineralization and tissue remodelling were significantly higher in OA groups.  
304 Specifically, the ratio between phosphate and amide III has been shown to reflect the degree of  
305 mineralisation and carbonate/ amide III is indicative of bone remodelling. De Souza *et al* (2014) also  
306 commented on the lack of literature available with regard to the use of Raman spectroscopy for OA  
307 research and the importance of this developing field. More recently, Hosu *et al* (2019) reviewed the  
308 importance of Raman spectroscopy in identifying pathologically associated crystals such as  
309 monosodium urate and calcium pyrophosphate dihydrate in rheumatoid diseases.

310 We recognise a number of limitations in our study. We were restrained by the number of samples  
311 available to us, and our findings need validation in a larger cohort. In addition, large sample volumes  
312 were necessary to have an adequate number of EVs for analysis, providing an appropriate signal to  
313 noise ratio. In our future studies minimum sample volume required will be optimised. Additionally,  
314 we used a single time point 'snap shot' of disease. Further work is needed to determine if O-PTIR is  
315 sensitive enough to determine differences in a range of OA phenotypes and severities, and correlate  
316 differences to specific biological functions of EVs.

317 Overall this study demonstrates the potential of Raman and OPTIR spectroscopy to be used as a  
318 diagnostic tool in clinical practice. Further work is required to identify if OA-related changes in plasma-  
319 derived EVs is related to the pathogenesis in the joint. We are currently quantifying the EV cargo using

320 NMR metabolomics, mass spectrometry proteomics and sequencing platforms in order to provide  
321 complete characterisation of EVs in OA and determine their contribution to disease propagation.

### 322 Conclusion

323 In conclusion, EVs derived from equine plasma in OA were probed using Raman and O-PTIR  
324 spectroscopy. O-PTIR spectroscopic data was found to be superior in classifying samples from OA  
325 patients compared to Raman spectroscopy. O-PTIR spectroscopy is an exciting platform for bedside  
326 analysis of plasma to diagnose OA.

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333

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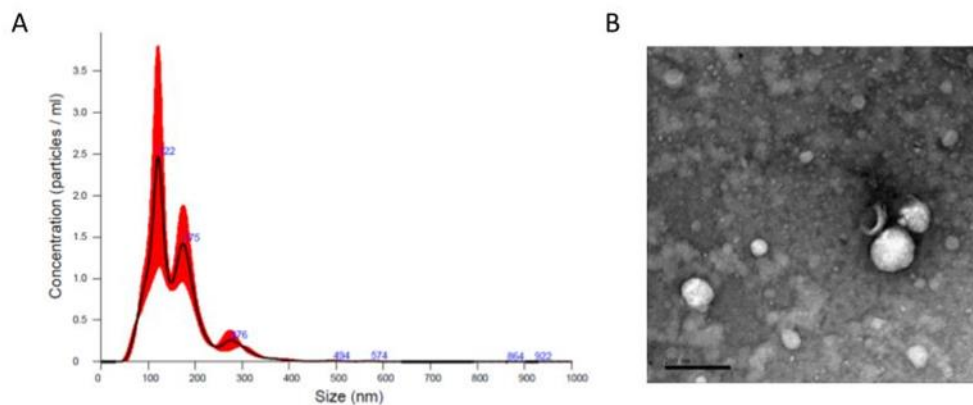
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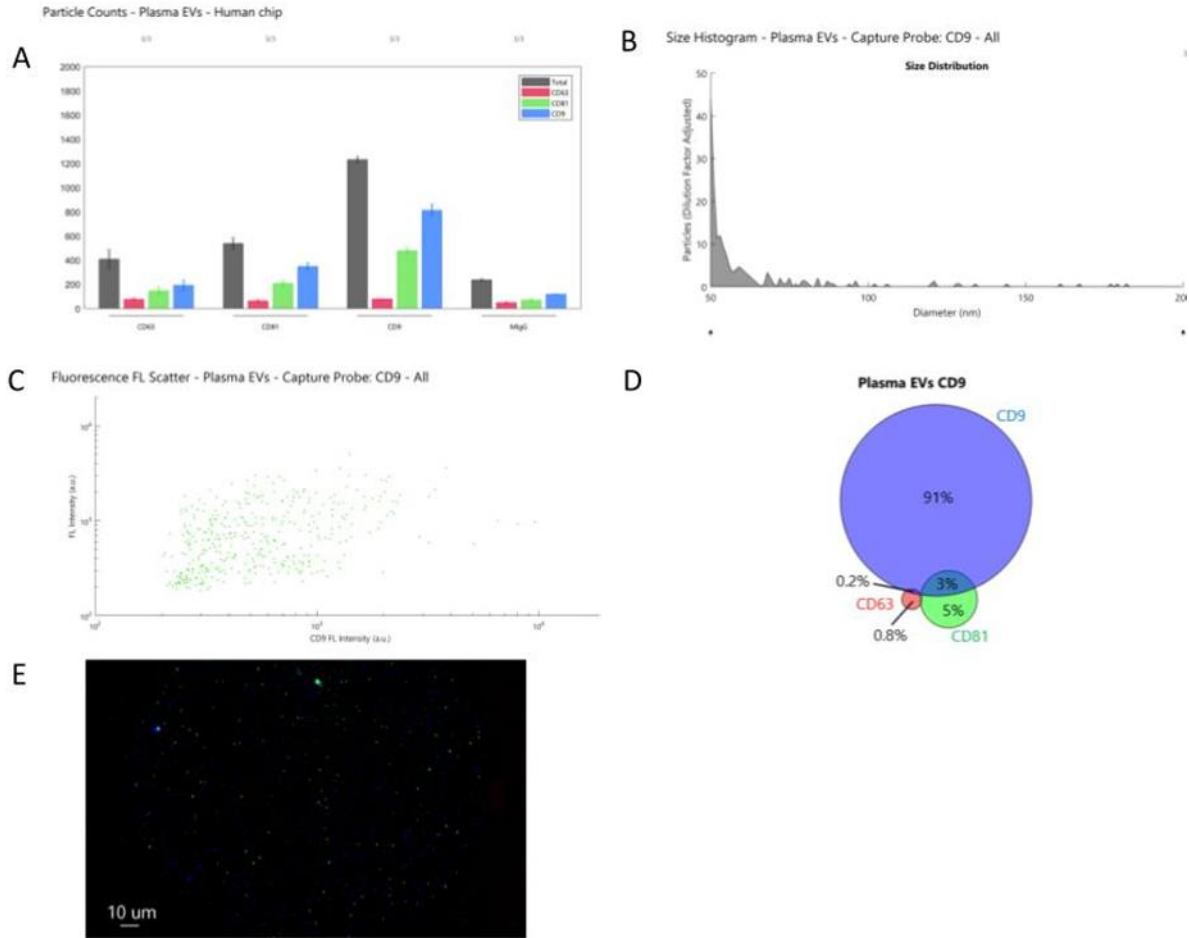


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476 Figure 1. Characterisation of EVs using Nanoparticle Tracking. 1A) Nanoparticle tracking analysis (NTA)  
477 of a representative sample of plasma derived EVs. Concentrations of EVs in particles/ml and particle  
478 size measured in nm, all measurements recorded using NanoSight NS300, and data analysed by the  
479 in-built NanoSight Software NTA 3.1 Build 3.1.46. Hardware: embedded laser: 45 mW at 488 nm;  
480 camera: sCMOS. 1B) Transmission electron microscopy (TEM) micrograph of negatively stained  
481 representative P-EV samples. Samples fixed to grids were visualised using a FEI Tecnai G2 Spirit with  
482 Gatan RIO16 digital camera.

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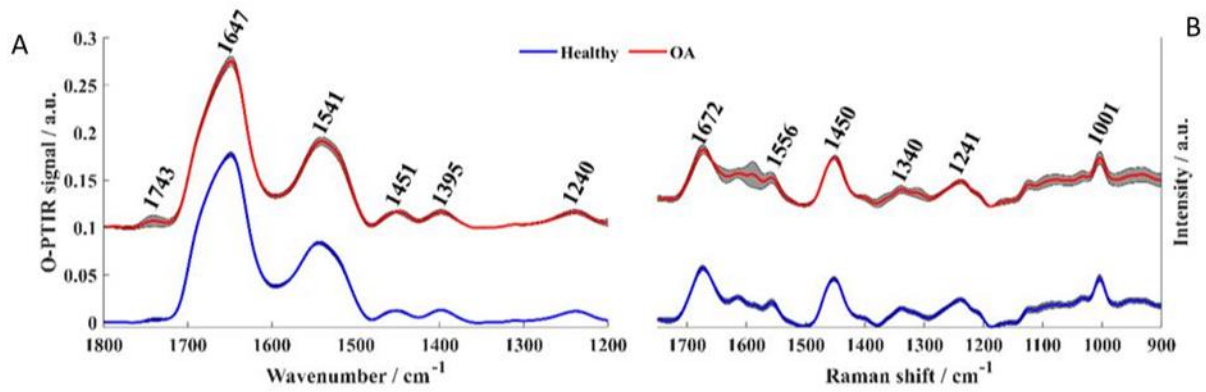


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485 Figure 2. Characterisation of EVs using Exoview. A) Particle counts for plasma EVs from the human  
486 tetraspanin chip. B) A size histogram of plasma EV samples as captured on the CD9 human tetraspanin  
487 chip. C) Scatter diagram demonstrating correlation between the number of CD9 positive EVs (X axis)  
488 and CD81 positive EVs (Y axis). D) colocalization analysis of the presence of surface tetraspanins on  
489 equine plasma EVs. E) fluorescent microscopy visualising plasma-derived EVs, with colour denoting  
490 surface tetraspanin positive identification (red – CD63, blue- CD9, and green -CD81).

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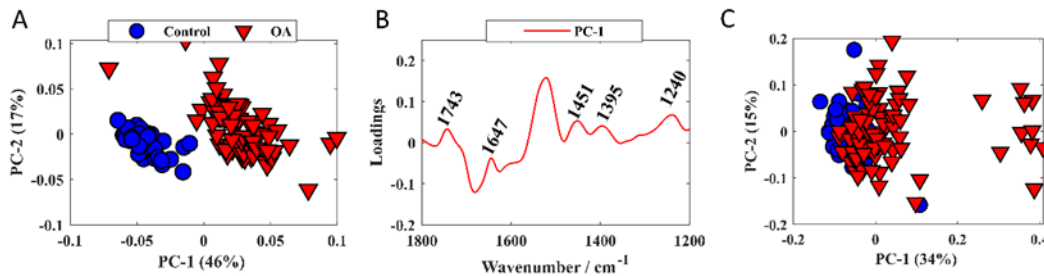
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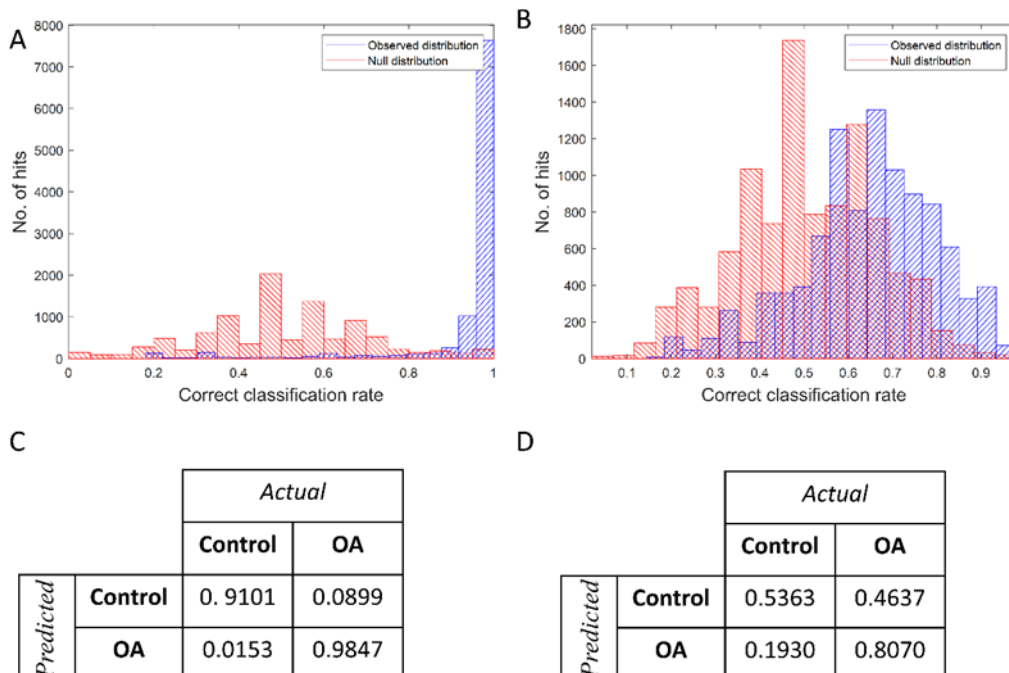
494 Figure 3. Fingerprint region of averaged spectra. A) Infrared and B) Raman spectra collected from  
495 healthy (blue line) and diseased (red line). Plots are offset for clarity.

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498 Figure 4. PCA scores and loadings plots. A) PCA score plot from infrared data. B) PCA loadings plot  
499 from infrared data. C) PC scores plot of Raman data; values in parentheses are the percentage total  
500 explained variance.



501

502 Figure 5. Classification model for PLS-DA of infrared and Raman spectra. A) Infrared and B) Raman  
503 spectra showing classification rates for real (blue) and random (red), with a correct classification rate  
504 of 93.41% for infrared and 64.63%. C) and D) are the respective average confusion matrices with rows  
505 representing predicted classification and columns representing experimental.

506