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1 **Microstructural analysis of collagen and elastin fibres in the**
2 **kangaroo articular cartilage reveals a structural divergence**
3 **depending on its local mechanical environment**

4
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21 **SUMMARY**

22

23 *Objective:* To assess the microstructure of the collagen and elastin fibres in articular
24 cartilage under different natural mechanical loading conditions and determine the
25 relationship between the microstructure of collagen and its mechanical environment.

26 *Method:* Articular cartilage specimens were collected from the load bearing regions of
27 the medial femoral condyle and the medial distal humerus of adult kangaroos. The
28 microstructure of collagen and elastin fibres of these specimens was studied using
29 laser scanning confocal microscopy (LSCM) and the orientation and texture features
30 of the collagen were analysed using ImageJ.

31 *Results:* A zonal arrangement of collagen was found in kangaroo articular cartilage:
32 the collagen fibres aligned parallel to the surface in the superficial zone and ran
33 perpendicular in the deep zone. Compared with the distal humerus, the collagen in the
34 femoral condyle was less isotropic and more clearly oriented, especially in the
35 superficial and deep zones. The collagen in the femoral condyle was highly
36 heterogeneous, less linear and more complex. Elastin fibres were found mainly in the
37 superficial zone of the articular cartilage of both femoral condyle and distal humerus.

38 *Conclusions:* The present study demonstrates that the collagen structure and texture of
39 kangaroo articular cartilage is joint-dependent. This finding emphasizes the effects of
40 loading on collagen development and suggests that articular cartilage with high
41 biochemical and biomechanical qualities could be achieved by optimizing joint
42 loading, which may benefit cartilage tissue engineering and prevention of joint injury.

43 The existence of elastin fibres in articular cartilage could have important functional
44 implications.

45 *Key words:* Collagen, Articular cartilage, Elastin fibre, Mechanical environment,
46 Orientation, Texture.

47

48 **Introduction**

49

50 Collagen is an important component of the matrix of articular cartilage and plays
51 an important role in the function of articular cartilage in diarthrodial joints. It accounts
52 for about two-thirds of the dry weight of articular cartilage and is distributed in a
53 zonal pattern with tissue depth¹. Generally, the collagen is parallel to the articular
54 surface in the superficial zone, more random in the middle zone and perpendicular to
55 the articular surface in the deep zone¹. The collagen fibres in the superficial zone
56 contribute to the tensile and shearing strength of the articular cartilage. Furthermore,
57 they integrate with the collagen fibres in the middle and deep zones to form a
58 three-dimensional collagenous framework, which entraps the hydrated proteoglycans
59 (PGs) and constrains the expansion of the PGs so that the articular cartilage is
60 afforded loading capacity^{1, 2}. Disruption of the collagenous framework results in
61 unconfined expansion of PGs, increased water concentration, softening of the articular
62 cartilage, and hence mechanical failure of the matrix with less capacity to support
63 load³. The structure and integrity of the collagen network are believed to be one of the
64 key factors in maintaining the normal functions of articular cartilage^{1, 3}.

65 Despite the highly organized network of collagen in adult cartilage, articular
66 cartilage at birth is biochemically and biomechanically homogenous⁴⁻⁸. The
67 mechanical environment to which the articular cartilage is subsequently exposed is
68 thought to play a crucial role in the development of the biochemical and
69 biomechanical constitution of articular cartilage⁵⁻⁷. During growth and development,
70 the articular cartilage in different joints is subjected to different mechanical forces. By
71 regulating biosynthetic activities, these mechanical forces shape the heterogeneous
72 composition and microstructure of articular cartilage, which is crucial to the
73 mechanical function of articular cartilage^{9, 10}.

74 Although many previous studies have tested the effects of mechanical forces on
75 the structure and composition of articular cartilage, the effects of mechanical stimuli
76 are not fully understood, particularly in the early stages of articular cartilage
77 development. In addition, the complex mechanical environment of articular cartilage
78 cannot be simulated in experiments. In this study, we used kangaroos as an animal
79 model in which the elbow and knee were exposed to significantly different
80 mechanical regimes during their activities. By studying the collagen structure in
81 kangaroo articular cartilage, we assessed the role of mechanical forces in the
82 development of the collagen structure, and the relationship between the collagen
83 structure and mechanical function of articular cartilage. As recent studies have
84 revealed the presence of elastin fibres in the superficial zone of articular cartilage of
85 bovine^{11, 12} and equine¹³, the current study aimed to verify the presence of elastin
86 fibres in the extracellular matrix (ECM) of kangaroo articular cartilage. The present

87 study could also provide valuable information regarding the level and influence of
88 mechanical forces on the growth and structure of engineered articular cartilage, and
89 also with regard to prevention of cartilage injury.

90

91 **Materials and Methods**

92

93 *Specimen preparation*

94

95 Three elbow and three knee joints from three male kangaroos aged approximately
96 5 years were collected from a local butcher (King River International Company, Perth,
97 Australia). The cartilage surfaces were checked for the absence of osteoarthritis (OA)
98 (Fig. 1). Cylindrical articular cartilage samples connected to the subchondral bone
99 were then harvested using 5 mm diameter punches from the weight-bearing areas of
100 the medial femoral condyle and the medial distal humerus (dash square in Fig. 1).
101 After removal, each cylindrical sample was cut in half from the surface of the
102 cartilage to the subchondral bone. One semi-cylindrical cartilage sample was used to
103 assess elastin fibres near the cartilage surface. The other half was used to assess the
104 zonal arrangement of collagen and was chemically fixed in 10% buffered formalin
105 solution (BFS) for 24h. After being processed and embedded, it was longitudinally
106 sliced into 5- μ m-thick sections for full-thickness analysis.

107

108 *Picrosirius red staining*

109

110 A method described by Miller¹⁴ was used to stain the collagen. Following
111 de-waxing, rehydration and a 2-min incubation in 0.2% phosphomolybdic acid (PMA),
112 the 5- μ m-thick sections were stained with a solution of 0.1% picosirius red (PSR;
113 Sirius Red F3B and saturated picric acid) for 90min. The samples were rinsed for 2
114 min in 0.01 N HCL (hydrogen chloride), followed by 1-min rinses in 70% ethanol,
115 100% ethanol (3 times), and toluene (3 times). Samples were then mounted for LSCM
116 imaging.

117

118 *Sulforhodamine B staining*

119

120 Sulforhodamine B (SRB) is a low-molecular-weight polar fluorescent molecule,
121 belonging to the xanthenes fluorescent dye family. The maximum absorption and
122 maximum emission wavelengths are 565 nm and 590 nm, respectively¹⁵. The
123 specificity of SRB staining to elastin was testified by the strong colocalization
124 observed between the SRB staining and immunostaining of elastin¹⁵. In the present
125 study, SRB powder (Sigma-Aldrich) was dissolved in 0.9% saline water and 1mg/ml
126 SRB solution was used for staining of kangaroo articular cartilage for 1min. After
127 thorough washing in phosphate buffed saline (PBS, pH 7.2), articular cartilage
128 samples were immersed in PBS to maintain tissue hydration and mounted between a
129 coverslip and a glass slide.

130

131 *Imaging*

132

133 All images of collagen and elastin were collected using LSCM (inverted TCS SP2,
134 Leica) with a plan apochromat $\times 63 / 1.4$ oil-immersion objective lens. For PSR
135 stained slides, a 514 nm argon ion laser was used and the emission was recorded
136 through a 570-700 nm bandpass filter; collagen images were taken in a longitudinal
137 view of femoral condyle and distal humerus articular cartilage. For the SRB stained
138 articular cartilage sample, a 561 nm DPSS laser was used and the emission signal was
139 recorded at 565-590 wavelengths; images of elastin fibres were taken in a transverse
140 view of articular cartilage plugs. Laser power and detector sensitivity were adjusted to
141 provide optimum image quality without excessive dye bleaching or pixel saturation.
142 For noise reduction, images with 1024 \times 1024 pixel were obtained using a 1- μ m step
143 and frame averaging 4 scans per image.

144

145 *Image analysis*

146

147 Orientation analysis

148

149 Digital image analysis software ImageJ (NIH, Maryland, USA) was used to
150 conduct image analysis. The orientation of the collagen fibres was analysed using
151 OrientationJ (an ImageJ-plugin) which was validated for study of collagen
152 orientation in a previous study¹⁶. The angles of the oriented structures could be

153 characterized by hue-saturation-brightness (HSB) colour coded image outputs in
154 which the colours indicated the orientation of the collagen. To quantitatively evaluate
155 the local organization and isotropic properties of the collagen fibres, orientation and
156 energy were selected as output parameters. Five regions of interest (ROIs) for every
157 zone of each of three kangaroos were investigated. By quantitatively evaluating every
158 pixel of the image, the degree of collagen fibre orientation could be determined from
159 -90° to 90° . Pixels with higher energy values correspond to less isotropic and more
160 clearly oriented structures.

161

162 Texture Analysis

163

164 Texture in an image refers to the distribution of brightness and darkness within the
165 image and describes a group of image properties related to intuitive notions of
166 coarseness, smoothness, and similar properties^{17,18}. It contains important information
167 about the structural arrangement of surfaces and their relationship to the surrounding
168 environment¹⁷. Texture analysis methods evaluate the spatial location and signal
169 intensity characteristics of the fundamental structural elements (pixels) of digital
170 images¹⁹. In order to quantify the contrast and spatial distribution of selected ROIs,
171 images were firstly converted to gray-scale, and the gray-level co-occurrence matrix
172 (GLCM), the texture analyzer ImageJ plug-in was then used to calculate texture
173 features in the X, Z plane. The GLCM is a statistical approach of texture analysis and
174 has been widely used to analyse medical images²⁰. In the present study, five regions of

175 interest (ROIs) for every zone of each of three kangaroos were investigated. Three
176 most important parameters were selected for characterizing collagen structure:
177 angular second moment (ASM), correlation, and entropy. ASM is a measure of
178 homogeneity of an image and its value increases with texture homogeneity^{17, 19, 21}.
179 Correlation is a measure of gray tone linear dependencies in the image region, where
180 high values (i.e., close to unity) imply a linear relationship between the gray levels of
181 pixel pairs^{17, 19}. The correlation value is 1 or -1 for a perfectly positively or negatively
182 correlated^{17, 19}. Entropy is a measure of disorder or complexity of intensity
183 distribution and its value is large when the image is not texturally uniform^{17, 19}.

184

185 *Statistical Analysis*

186

187 Differences in orientation parameters (orientation and energy) and texture features
188 (ASM, correlation and entropy) between the zones of articular cartilage from the
189 femoral condyle and distal humerus were statistically examined. The linear mixed
190 effects model was chosen for statistical comparison. The benefit of this model is that
191 samples with potential interrelations can be reliably compared²². In the model, joints
192 and zones of articular cartilage were set as fixed variables, and the animal was coded
193 as the random variable. Multiple comparisons were achieved by Least Significant
194 Difference (LSD) post-hoc analysis. Dot plots were used to compare the differences
195 of texture features between the femoral condyle and distal humerus. Estimated means
196 of orientation and energy values for the different zones were obtained and the main

197 effects between the zones were compared. 95% confidence intervals with LSD
198 adjustment were finally presented. Differences were considered significant at *P* values
199 less than or equal to 0.05. All statistical analyses and graphs were performed using
200 statistical package for the social sciences (SPSS), version 16.0 (SPSS Inc., Chicago,
201 IL, USA).

202

203 **Results**

204

205 *Zonal arrangement of collagen*

206

207 In order to reveal the zonal arrangement of collagen in kangaroo articular cartilage,
208 LSCM and HSB colour coded images were first employed to compare the collagen
209 structures of femoral condyle and distal humerus. Collagen fibres in the articular
210 cartilage of the femoral condyle showed a clear zonal organization [Fig. 2(A)–(C)]. In
211 the superficial zone, symbolized by the discoid chondrocytes within the top 40-50 μm
212 depth, the collagen fibres aligned predominantly parallel to the cartilage surface [Fig.
213 2(A)]. In the middle zone, symbolized by the round chondrocytes, no apparent
214 orientation of collagen was observed [Fig. 2(B)]. In the deep zone, symbolized by the
215 vertical chondrocyte columns, the collagen fibres were oriented predominantly
216 perpendicular to the cartilage surface [Fig. 2(C)]. In contrast, the collagen in the
217 articular cartilage of distal humerus was relatively fine and did not show an apparent
218 zonal organization from visual assessment [Fig. 2(D)–(F)].

219 To quantitatively evaluate the orientation of collagen, OrientationJ was used to
220 generate HSB colour coded images based on the LSCM images and the orientation
221 and energy values were calculated. In the superficial zone, collagen in the femoral
222 condyles [Fig. 3(A)] was more clearly parallel to the articular surface than in the
223 distal humerus [Fig. 3(D)], as indicated by the predominant green colour displayed in
224 the images of the femoral condyle. In the middle zone, both femoral condyle [Fig.
225 3(B)] and distal humerus samples [Fig. 3(E)] displayed a randomly organized
226 collagen network, indicated by a mix of colours. In the deep zone, the predominant
227 red colour in both femoral condyle [Fig. 3(C)] and distal humerus samples [Fig. 3(F)]
228 indicated that collagen fibres were perpendicular to the articular surface. Quantitative
229 orientation values further confirmed the results from HSB colour coded images (Table
230 I). Both femoral condyle and distal humerus samples showed a sharp increase of
231 collagen orientation from 0 to 15 degree in the superficial zone to more than 80
232 degree in the deep zone. Comparison of energy values revealed less isotropic and
233 more clearly oriented collagen fibres in the superficial zone ($P < 0.0001$), middle zone
234 ($P < 0.0001$) and deep zone ($P < 0.0001$) of femoral condyle articular cartilage than in
235 the respective zones of distal humerus articular cartilage.

236

237 *Texture features of collagen in different zones*

238

239 To further characterize the collagenous differences, texture analysis was applied to
240 reveal the textural features of the collagen fibres. Collagen in the superficial, middle

241 and deep zones of cartilage was more homogenous in the distal humerus than in
242 femoral condyle, as indicated by higher ASM values in distal humerus cartilage [Fig.
243 4(A)]. No zonal variation of homogeneity was found between zones of the femoral
244 condyle cartilage [Fig. 4(B)]. However, distal humerus articular cartilage showed a
245 more homogenous collagen structure in the deep zone than in the superficial and
246 middle zones [Fig. 4(B)]. Higher correlation values were found in the superficial,
247 middle and deep zones of distal humerus articular cartilage [Fig. 5(A)], which implied
248 a higher correlation of collagen fibres in the distal humerus than in the femoral
249 condyle. Within the femoral condyle cartilage, the superficial zone differed with the
250 middle and deep zones, but no statistically difference was found between the middle
251 and the deep zones [Fig. 5(B)]. Within the distal humerus, gray-tone linearity of
252 collagen structure differed between zones [Fig. 5(B)] and the deep zone had the
253 highest correlation [Fig. 5(A)]. Higher entropy values were present in all three zones
254 of femoral condyle cartilage [Fig. 6(A)], indicating that the collagen structure was
255 more complex in the femoral condyle than in the distal humerus cartilage. No zonal
256 variation of entropy values was found in the femoral condyle, but differences were
257 found between zones of distal humerus articular cartilage [Fig. 6(B)]. These texture
258 parameters demonstrated that the collagen was more homogeneous and linear in the
259 distal humerus cartilage than in the femoral condyle cartilage, and that distal humerus
260 cartilage had a zonal variation with respect to texture parameters while femoral
261 condyle was more consistent with respect to texture characteristics.

262

263 *Organization of elastin fibres*

264

265 Elastin fibres indicated by SRB florescence were revealed in kangaroo articular
266 cartilage. From a femoral condyle articular cartilage sample, large elastin fibres
267 bundles were found in the ECM [Fig. 7(A)–(C)]. These bundles were large and
268 mainly linear [arrow head in Fig. 7(A)–(C)] with minor waviness in the form of the
269 elastin fibres [arrow in Fig. 7(A)]. However, in the chondrocyte surface or the
270 pericellular matrix, only fine elastin but no resolvable bundle was found [Fig.
271 7(D)–(F)]. This fine elastin surrounded the chondrocyte and provided a
272 microenvironment for the entrapped cells.

273 To further characterise the elastin fibre and the fine elastin, articular cartilage were
274 assessed in terms of different zones and a comparison was made between femoral
275 condyle and distal humerus. In the most superficial layer of femoral condyle articular
276 cartilage, which was acellular layer of about 10 µm in thickness, dense elastin fibres
277 were found [Fig. 8(A)]. These fibres were lightly corss-linked with general direction.
278 In the superficial zone of femoral condyle articular cartilage, elastin fibres were
279 highly oriented to the longitudinal direction of chondrocytes [Fig. 8(B)]. However, it
280 could not be determined whether chondrocytes determined the orientation of elastin
281 fibres. In the deep zone of femoral condyle articular cartilage, elastin fibres were not
282 observed and only fine elastin was found around the chondrocytes [Fig. 8(C)]. In the
283 distal humerus, dense and relatively short elastin fibres were found within the most
284 superficial zone [Fig. 8(D)]; highly oriented elastin fibres were found in the

285 superficial zone [Fig. 8(E)]; and only fine elastin was found around chondrocytes in
286 the deep zone [Fig. 8(F)]. It appeared that the most obvious difference between
287 femoral condyle and distal humerus was in the most superficial zone.

288

289 **Discussion**

290

291 This current study is the first to analyse kangaroo articular cartilage in the femoral
292 condyle and the distal humerus to determine the collagen and elastin structure in the
293 superficial, middle and deep zones. It was found that the structure, orientation and
294 texture features of the collagen varied significantly between zones and joint types.
295 Collagen in the femoral condyle was more clearly oriented, and more heterogeneous
296 and irregular in texture when compared to collagen in the cartilage of the distal
297 humerus. Collectively, these results suggest that the pattern and magnitude of
298 mechanical forces play a crucial role in the development of collagen fibres and overall
299 articular cartilage architecture.

300 This work used kangaroos as an animal model as it has several fundamental
301 characteristics that are advantageous compared to other animal models^{6, 8, 23}.
302 Generally, conventional studies involve variations in individual animals with different
303 genetic and environmental background. In contrast, the kangaroo can provide two
304 significantly different types of loading in one individual. The knee joints of kangaroos
305 are subjected to both dynamic and static loading during movement, while the elbow
306 joints are rarely used for jumping and are mainly subjected to static loading²⁴. By

307 comparing these two types of joints from the same individual kangaroo, intrinsic
308 experimental errors could be avoided and experiments were expected to be more
309 accurate and consistent than other animal models.

310 The present study revealed zonal variation and joint-dependent variation in the
311 collagen network of kangaroo cartilage. Zonal organization of collagen was observed
312 both in the femoral condyle and in the distal humerus, although zonal arrangement of
313 collagen in the distal humerus was slight. Compared with the distal humerus, the
314 collagen fibres in the femoral condyle were more clearly oriented and less
315 homogenous. Previous studies have shown that articular cartilage responds to varied
316 mechanical stimuli by functional adaptation during cartilage development^{5-7, 25}. In this
317 process, the heterogeneity of chondrocytes and PGs in the articular cartilage of mature
318 individuals was developed from a homogeneous composition of a less mature status⁶,
319 ²⁶. The present study shows that, compared with elbow joint, dynamic loading in the
320 knee joint assists in establishing the heterogeneous organization of collagen, with
321 more clearly oriented fibres in the superficial and deep zones. The collagenous
322 difference due to mechanical stimuli demonstrated by this study provides an
323 implication of how to optimize the loading in articular cartilage engineering, which is
324 a promising strategy for the treatment of cartilage diseases such as OA. This work
325 also emphasises loading to be a crucial variable in collagen development, and
326 necessitates the optimization of joint loading during early life to create optimal
327 biomechanical characteristics of articular cartilage, which may contribute to
328 prevention of OA later in life.

329 Texture analysis has traditionally been applied in fields such as material science,
330 geography and satellite image analysis. As the mathematically quantified “texture
331 features” are very sensitive, texture analysis is increasingly being used in the medical
332 area ranging from diagnostic²⁷⁻²⁹ to prognostic applications³⁰. A recent study
333 examined the influence of mechanical loading on the organization of collagen in
334 articular cartilage and found that the structural modification of collagen in the ECM
335 of articular cartilage could be distinguished by texture analysis³¹. The present study
336 confirms that the texture analysis is a valuable and quantitative tool for the study of
337 collagen in articular cartilage. Further application of texture analysis could promote
338 more future work to compare textural differences in the ECM of diseased and health
339 cartilage.

340 Collagen structural alterations have been observed in OA by laboratory methods,
341 but cannot be clinically diagnosed. Generally, OA can be diagnosed by X-rays, MRI
342 (magnetic resonance imaging) or arthroscopy at the more advanced stages, at which
343 time the options for therapeutic intervention without surgery are limited. It is
344 therefore crucial to develop new diagnostic strategies targeting the early stages of OA.
345 As damage of collagen fibrils or collagen network disorganization is one of the early
346 signs of cartilage injury and OA^{32,33}, testing of alteration in collagen structure could
347 be a valuable tool for early OA diagnosis. Texture analysis of the collagen could be
348 used to discover the textural differences which may not be detected by conventional
349 methods. The changes in textural features of collagen during early degenerative
350 changes are a subject for future investigations. Combining texture analysis with

351 non-invasive imaging techniques (such as laser scanning confocal arthroscopy)³⁴
352 could be promising to diagnose cartilage diseases such as OA at early stage.

353 Early histological results indicated that little elastin fibres or elastin existed in
354 articular cartilage³⁵⁻³⁷. However, recent studies carried out with more sensitive
355 methods have observed the existence of elastin fibres in the superficial zone of
356 articular cartilage¹¹⁻¹³. The present study revealed that an extensive elastin fibre
357 network was present within the kangaroo cartilage surface, including most superficial
358 layer and superficial zone, and that fine elastin surrounded chondrocytes throughout
359 the whole cartilage depth. Due to its significant volume, elastin fibres and fine elastin
360 should no longer be ignored in the future studies regarding articular cartilage. The
361 mechanical function of elastin fibres is believed to endow critical properties of
362 elasticity and resilience to tissues, such as skin, lung, blood vessels and elastic
363 cartilage³⁵. Investigation of the mechanical functions of elastin fibres in articular
364 cartilage and their relationship with other components of the ECM would be
365 important, but is beyond the scope of the current study.

366 In conclusion, the present work assessed microstructural and textural
367 characteristics of collagen and revealed the presence of elastin fibres in articular
368 cartilage under different natural mechanical environments. Significant differences
369 observed between the knee and elbow with respect to the structural, orientation and
370 textural features of collagen suggest that the type and magnitude of mechanical forces
371 play a crucial role in collagen structure development. The existence of elastin fibres
372 and fine elastin around chondrocytes suggests it as another important component for

373 articular cartilage besides collagen and PGs. This work suggests that articular
374 cartilage with optimal biochemical and biomechanical qualities could be achieved by
375 optimizing mechanical forces, which may benefit cartilage tissue engineering and
376 prevention of joint injuries. Our findings also promote the application of texture
377 analysis as a promising method for future collagen structural studies and early
378 diagnosis of OA.

379

380 **Author contributions**

381

382 All authors significantly participated and were involved in the (1) conception and
383 design (2) drafting the manuscript or revising it critically for intellectual content, and
384 (3) all authors approved the final version of the paper before submission.

385

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387

388 The sponsor had no role in the study design, collection, analysis and interpretation
389 of data; in the writing of the manuscript; and in the decision to submit the manuscript
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391

392 **Conflict of interest**

393 The authors declare that they have no competing interests.

394

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403

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495 **Table I.** Mean (95% confidence interval, CI) orientation and energy values of collagen in articular
 496 cartilage. A linear mixed effects model was used to perform statistical comparison.
 497

Zones	Cartilage	Orientation (95% CI)	Energy
Superficial	Femoral condyle	-2 (-6 - 2)	134 (125 - 142)
	Distal humerus	11 (7 - 15)	44 (35 - 52)
Middle	Femoral condyle	67 (63 - 71)	86 (77 - 94)
	Distal humerus	80 (76 - 84)	63 (54 - 71)
Deep	Femoral condyle	86 (82 - 90)	126 (117 - 134)
	Distal humerus	84 (80 - 88)	21 (12 - 29)

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502 **Fig. 1.** A photograph of a kangaroo femoral condyle and distal humerus showing sampling of articular
503 cartilage. The femoral condyle was connected by the anterior cruciate ligament (ACL) and the posterior
504 cruciate ligament (PCL) to the femoral-tibial joint (A). Articular cartilage samples were collected from
505 the central load bearing area of the medial femoral condyle (dashed square in B) and distal humerus
506 (dashed square in C).

507

508 **Fig. 2.** LSCM images of picosirius red stained collagen in the articular cartilage of the femoral
509 condyle and the distal humerus (longitudinal view). In the femoral condyle, collagen was mainly
510 horizontally oriented in the superficial zone (A), randomly oriented in the middle zone (B) and
511 vertically oriented in the deep zone (C). In the distal humerus, no apparent organization of collagen was
512 found from visual assessment in the superficial zone (D), middle zone (E) and deep zone (F). Scale
513 bar=10 μm .

514

515 **Fig. 3.** HSB colour coded images of collagen in the articular cartilage of the femoral condyle and the
516 distal humerus. In the femoral condyle, the predominant green colour indicated horizontally oriented
517 collagen in the superficial zone (A); a mix of colour indicated randomly oriented collagen in the middle
518 zone (B); and the predominant red colour indicated vertically oriented collagen in the deep zone (C). In
519 the distal humerus, the horizontally oriented collagen in the superficial zone (D) and randomly
520 organized collagen in the middle zone (E) and perpendicular collagen in the deep zone (F) were also
521 found but not as obvious as in the femoral condyle. Scale bar=10 μm .

522

523 **Fig. 4.** A comparison of homogeneity of the collagen structure indicated by ASM values. Compared
524 with the femoral condyle articular cartilage, the distal humerus articular cartilage showed more

525 homogenous structure of collagen in the superficial, middle and deep zones, as indicated by higher
526 ASM values in respective zones (A). Within the femoral condyle, no zonal variation was observed in
527 terms of homogeneity of collagen (B). Within the distal humerus, the homogeneity of collagen differs
528 between zones, and the collagen structure is significantly more homogenous in the deep zone than in
529 superficial and middle zones (B).

530

531 **Fig. 5.** A comparison of correlation of collagen in the femoral condyle articular cartilage and the distal
532 humerus articular cartilage. Higher correlation of collagen in cartilage were found in the distal humerus
533 than in the femoral condyle (A). Within the femoral condyle, the superficial zone differed with the
534 middle and deep zones in terms of correlation, but there was no significant difference between the
535 middle and deep zones. (B). Within the distal humerus, correlation of collagen differed between zones
536 (B) and the collagen in the deep zone was most highly correlated (A).

537

538 **Fig. 6.** A comparison of complexity of collagen structure indicated by entropy values. Collagens of the
539 superficial, middle and deep zones were more complex in femoral condyle than in distal humerus, as
540 indicated by higher entropy values in femoral condyles (A). Within the femoral condyle, no zonal
541 variation was observed in terms of complexity of collagen (B). Within the distal humerus, collagen
542 differed between zones (B), and the middle zone was the most complex layer as indicated by higher
543 entropy value (A).

544

545 **Fig. 7.** Sulforhodamine B staining revealed the existence of elastin fibres in kangaroo femoral condyle
546 articular cartilage (transverse view). Both straight elastin fibres (arrow head in A, B and C) and wave
547 elastin fibres (arrow in A) were observed in the extracellular matrix. Fine elastin was observed in

548 pericellular matrix (D, E and F). Scale bar = 10 μ m.

549

550 **Fig. 8.** Comparison between femoral condyle and distal humerus with respect to sulforhodamine B

551 stained elastin fibres in different zones (transverse view). Dense elastin fibres were found in the most

552 superficial zone of articular cartilage from femoral condyle (A) and distal humerus (D). Less dense

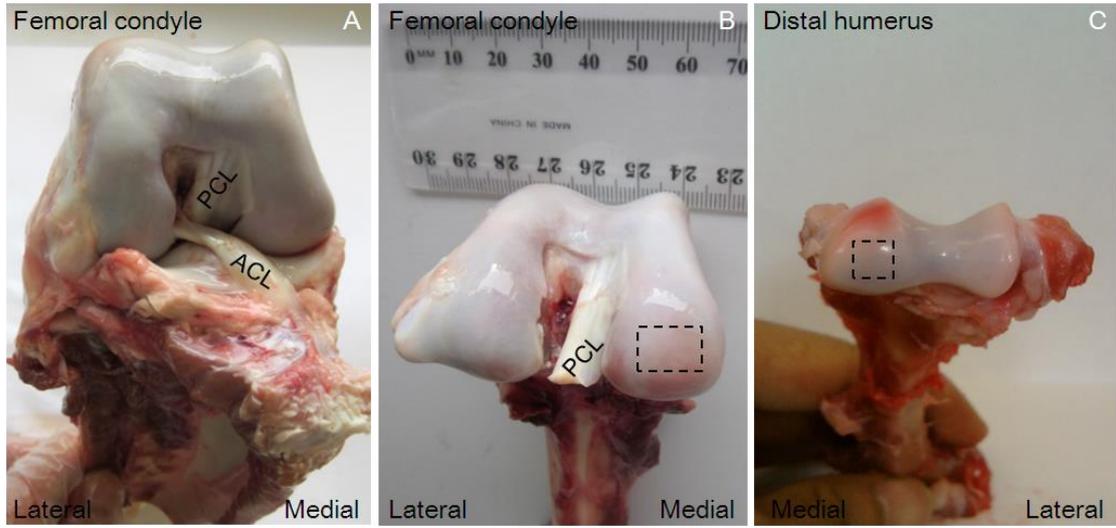
553 elastin fibres were observed to parallel to the adjacent chondrocytes in the superficial zone of articular

554 cartilage from femoral condyle (B) and distal humerus (E). Only fine elastin was found in the deep

555 zone of articular cartilage from femoral condyle (C) and distal humerus (F). Scale bar = 30 μ m.

556

557 Figure. 1

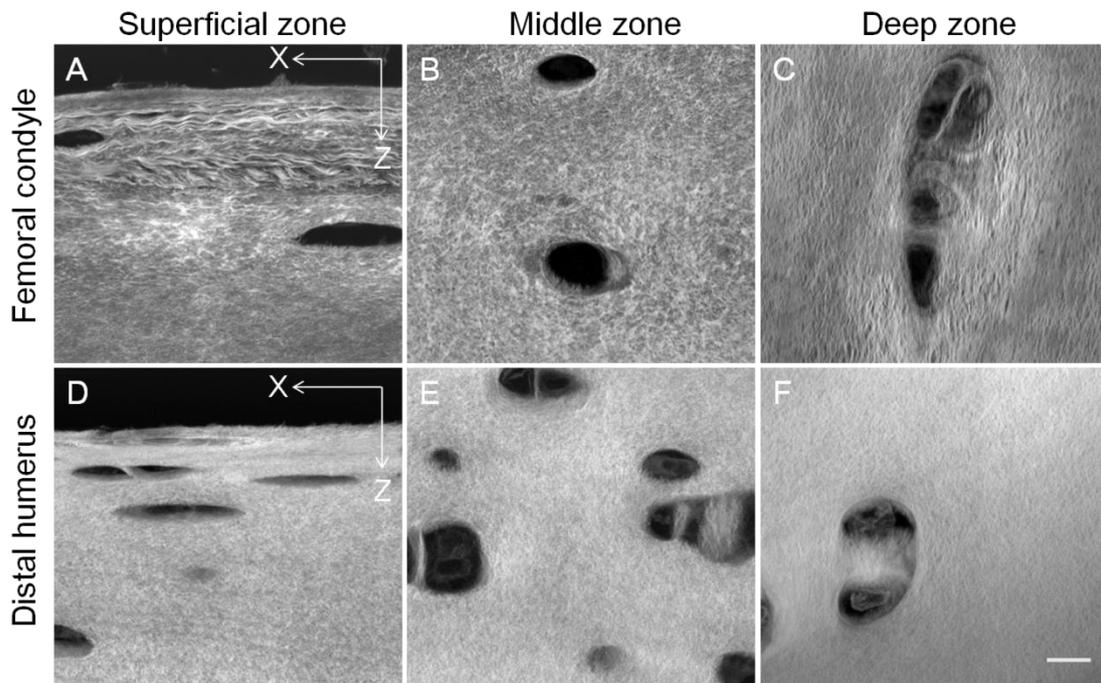


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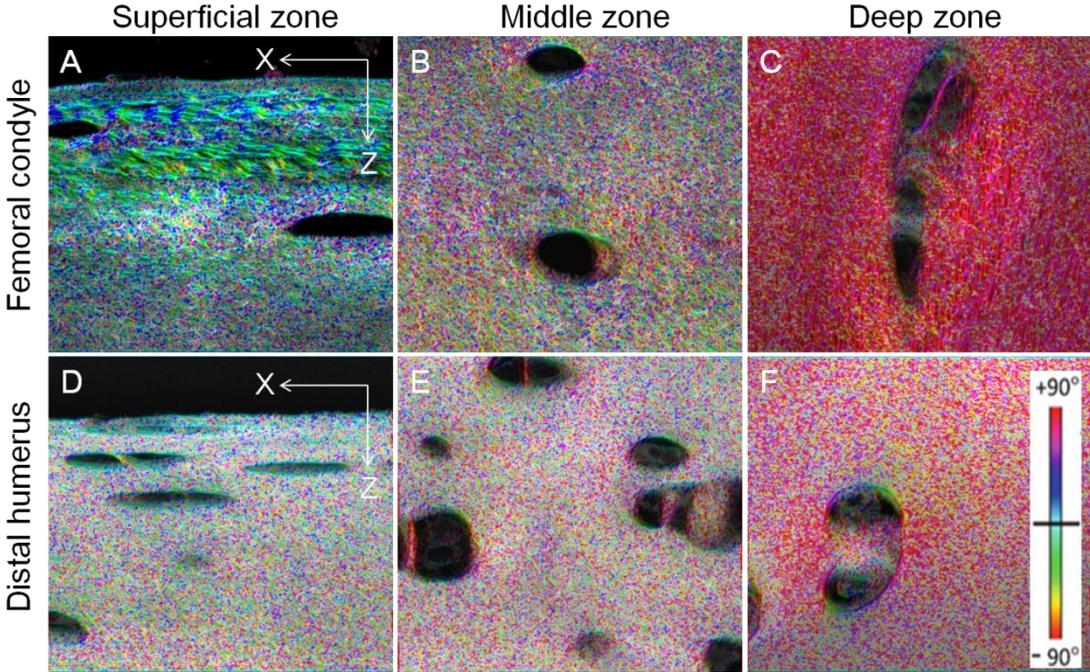
561 Figure. 2



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564 Figure. 3

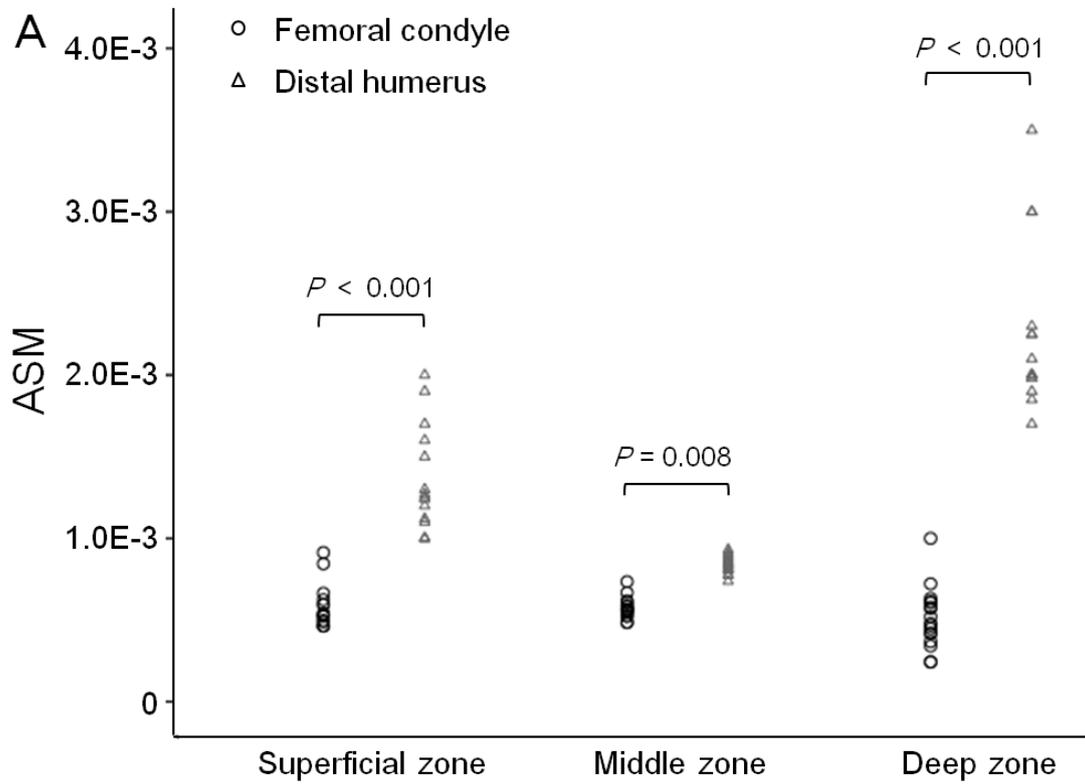


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568 Figure. 4



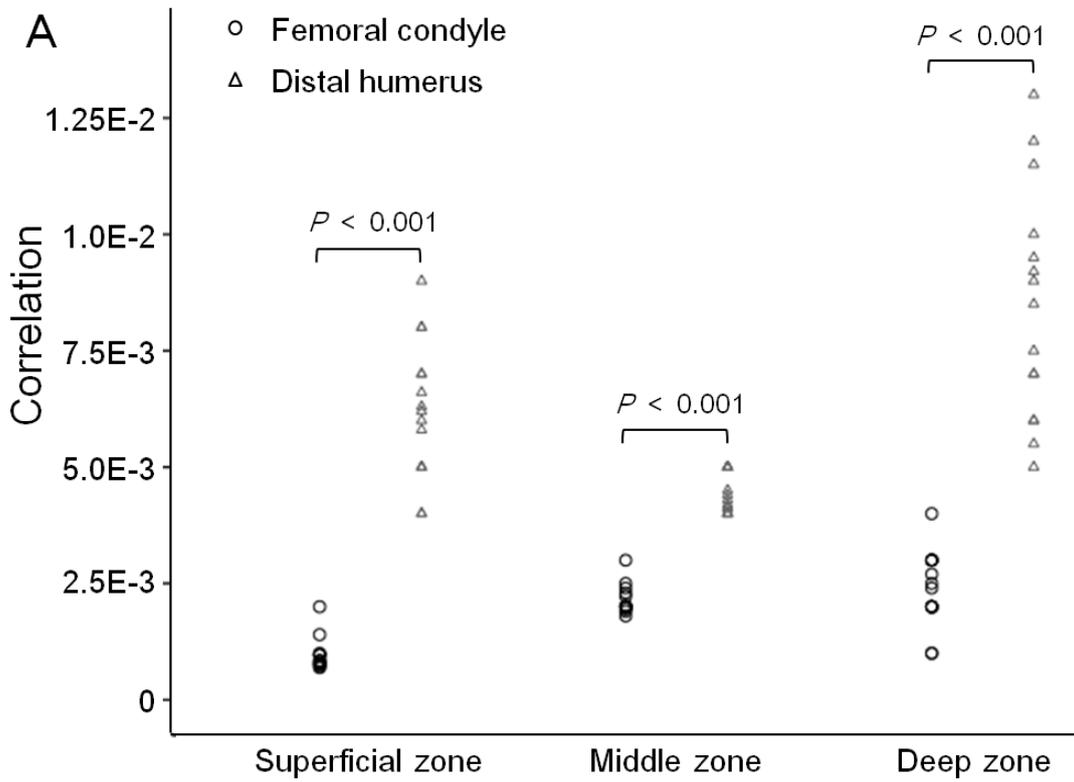
B

Articular cartilage	zones	<i>P</i> values
Femoral condyle	Superficial] 0.934] 0.439
	Middle	
	Deep	
Distal humerus	Superficial] <0.0001] <0.0001] <0.0001
	Middle	
	Deep	

569

570

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B

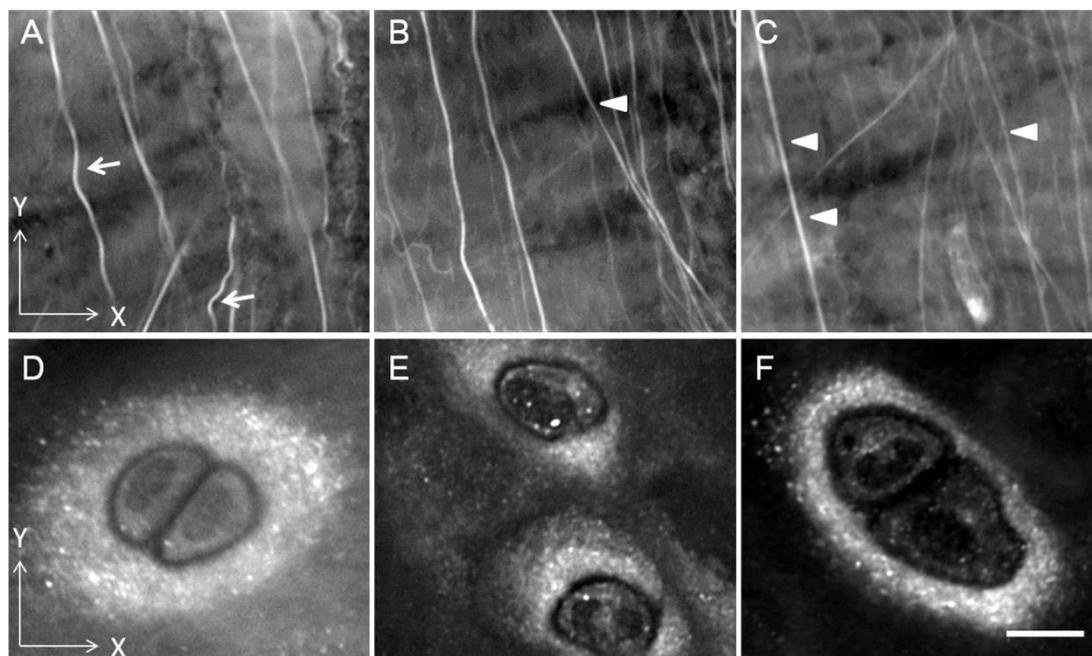
Articular cartilage	zones	<i>P</i> values
Femoral condyle	Superficial] 0.010] 0.001
	Middle	
	Deep	
Distal humerus	Superficial] <0.0001] <0.0001] <0.0001
	Middle	
	Deep	

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580 Figure. 7

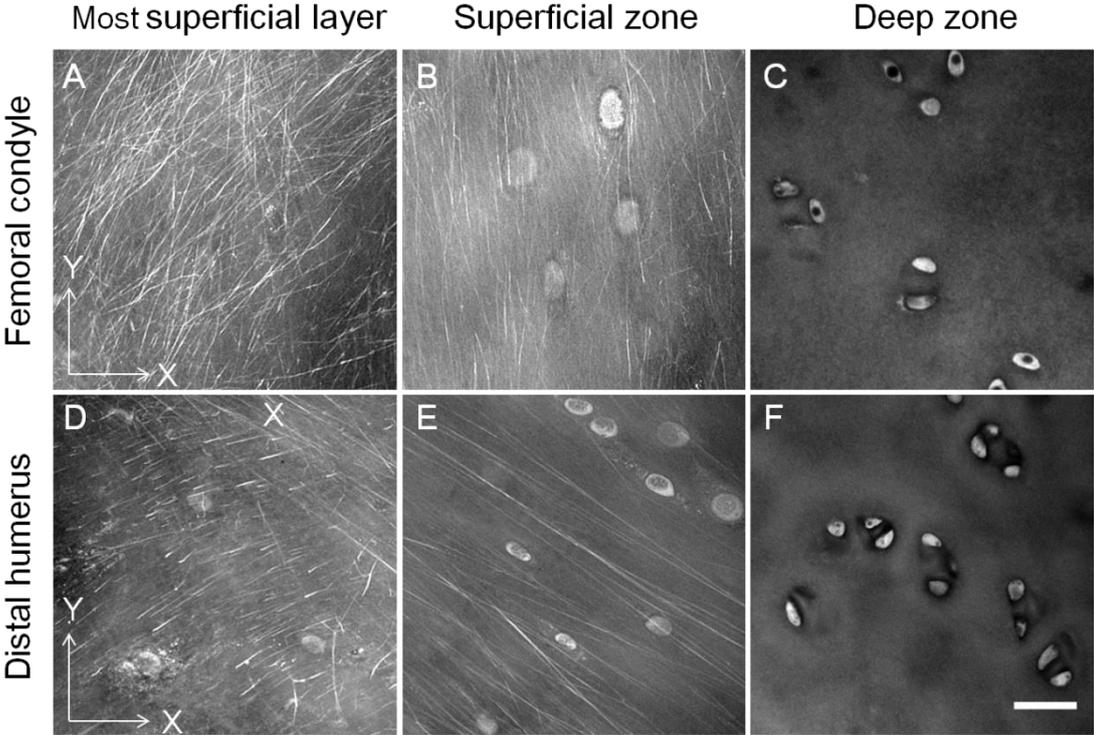


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583

584 Figure. 8



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