

1 **Concentric lamellae – novel microanatomical structures in the articular calcified cartilage of mice.**

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31 **Abstract**

32 The structure, ultrastructure and function of hyaline articular cartilage (HAC) and subchondral bone (SCB), and
33 their involvement in the pathogenesis of osteoarthritis (OA) have been extensively researched. However, much
34 less attention has been focused on the intervening tissue, articular calcified cartilage (ACC) and its role in the
35 initiation and progression of OA. Using both light microscopy (LM) and transmission electron microscopy (TEM),
36 a study of ACC in wild type (WT) mice, and mice with genetic osteoarthropathies (AKU) was undertaken to
37 further understand the role played by ACC in the early stages of OA. Tibio-femoral joints were obtained from
38 BALB/c WT and BALB/c AKU mice aged between 7 and 69 weeks. One joint was processed for routine
39 histological analysis. The tip of the medial femoral condyle (MFC), which contained HAC, ACC, and SCB, was
40 dissected from the contra-lateral joint and processed for TEM. In WT and AKU mice novel microanatomical
41 structures, designated concentric lamellae, were identified surrounding chondrocytes in the ACC. The lamellae
42 appeared to be laid down in association with advancement of the tidemark indicating they may be formed during
43 calcification of cartilage matrix. The lamellae were associated with hypertrophic chondrocytes throughout the
44 ACC. Novel microanatomical structures, termed concentric lamellae, which were present around hypertrophic
45 chondrocytes in the ACC are described for the first time. Their apparent association with mineralisation,
46 advancement of the tidemark, and greater abundance in a model of osteoarthropathy indicate their formation could
47 be important in the pathogenesis of OA and AKU.

48

49 **Keywords:** Cartilage, Osteoarthritis, Concentric Lamellae, Alkaptonuria, Chondrocytes, Calcification

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57 **Introduction**

58 The roles of hyaline articular cartilage (HAC) and subchondral bone (SCB) in the pathogenesis of osteoarthritis
59 (OA) have been widely described, along with their structure, ultrastructure and function [1-4]. Much less attention
60 has been focused on articular calcified cartilage (ACC) [5] and its significance in the initiation and progression of

61 OA has largely been ignored [6]. One possible explanation for this could be that the ultrastructure of ACC is
62 notoriously difficult to study. Silberberg and colleagues performed TEM analysis on the femoral heads of mice
63 of various ages but little attention was paid to ACC in these studies [7, 8]. Recently, Hughes and colleagues used
64 scanning electron microscopy (SEM) to describe in detail the orientation of chondrocytes and collagen fibers in
65 the territorial and interterritorial matrices of murine HAC [9]. Similar to previous ultrastructural studies on mouse
66 cartilage, there was far less detail on ACC than on HAC. Although the literature on the ultrastructure of ACC is
67 scarce, and its role in the etiology of OA is not fully understood, it is known to play a significant role in the
68 initiation and progression of the ultra-rare disease Alkaptonuria (AKU) [10, 11].

69
70 AKU is an ultra-rare autosomal recessive disorder characterized by elevated levels of homogentisic acid (HGA)
71 in plasma. The HGA becomes deposited over the lifespan as a polymerized pigment in collagenous tissues,
72 principally the cartilages of loaded joints, in a process known as ochronosis. In humans this results in an extreme
73 and very severe OA phenotype in which cartilage is lost from the joints beyond the third and fourth decades of
74 life. The pathogenesis of AKU has yet to be fully elucidated. However, Taylor and colleagues showed that pigment
75 deposition in cartilage starts in the pericellular matrix (PCM) of chondrons, deep in the ACC and progresses up
76 throughout HAC, leading to the early onset of the devastating osteoarthropathy associated with AKU [10]. It is
77 generally accepted that OA initiates due to degradation of HAC, however it is clear from the work of Taylor and
78 colleagues that ACC has more of a role than previously thought in the initiation and progression of
79 osteoarthropathies [10]. Recently, there have been two murine models of AKU described which show
80 pigmentation similar to that seen in the human condition [12, 13]. In the latter of the two models, pigmentation
81 was shown to be localized to chondrons in ACC confirming what Taylor *et al* had identified in human tissue [10,
82 12]. Until recently the only real, therapeutic option available for AKU patients was joint replacement surgery
83 which is also the gold standard treatment for patients with OA. However, several pre-clinical and clinical studies
84 have shown that a compound known as nitisinone is effective at preventing the build-up of HGA in plasma.
85 Nitisinone was also shown to prevent pigment deposition in an animal model of AKU [14-17].

86
87 This study was undertaken to provide a detailed analysis of the ultrastructure of all regions of articular cartilage,
88 and to identify any differences between WT and AKU mice which could further the understanding of the
89 development of the severe osteoarthropathy associated with AKU.

90

91 **Methods**

92 **Mice**

93 WT and AKU mice on a BALB/c background were used for all experiments. All work was carried out in
94 accordance with the UK Home Office guidelines and regulations under the Animals (Scientific Procedures) Act
95 1986, and with approval from the University of Liverpool ethics committee. All mice were housed and maintained
96 in the Biological Services Unit at the University of Liverpool, UK.

97

98 **Light Microscopy**

99 Tibio-femoral joints was harvested from WT and AKU mice aged between 7 and 69 weeks and fixed in 10%
100 phosphate buffered formalin solution (PBFS). After 24 hours tissues were transferred to 12% EDTA to decalcify.
101 Once decalcification was complete tissues were washed several times with PBS and processed for histological
102 analysis using a Leica TP1020 processor (Leica, Germany). Following processing, tissues were embedded for
103 coronal sectioning in paraffin wax. Tissue blocks were sectioned using a Leica RM2245 microtome (Leica,
104 Germany), sections stained with H&E and Schmorl's stain, and images captured using a Nikon Eclipse *Ci*
105 microscope (Nikon, UK). Image analysis was performed using NIS Br elements software (Nikon, UK).

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107 **Transmission Electron Microscopy**

108 Following fixation in either 10% PBFS or 2.5% glutaraldehyde, the tip of the MFC, encompassing the HAC, ACC
109 and SCB, was removed and post-fixed in 1% osmium tetroxide for 3 hours at RT, on bloc stained with 1% uranyl
110 acetate for 24 hours at RT, dehydrated in ethanol and embedded in Agar 100 resin (Agar Scientific, UK). 70nm
111 sections were cut using a diamond knife (Diatome, Switzerland) on a Leica EM UC6 ultra-microtome (Leica,
112 Germany). Sections were collected on formvar coated 100 mesh copper grids (TAAB, UK) and post-stained with
113 uranyl acetate (5% by weight in 50% ethanol and 50% distilled water) followed by lead citrate. Grids were
114 examined using a FEI 120kV Tecnai G2 Spirit BioTWIN electron microscope, and all images captured with an
115 SIS Megaview III camera.

116

117 **Results**

118 **Histological analysis of BALB/c AKU mice**

119 Sections from the tibiofemoral joints of AKU mice of varying ages were stained with H&E and Schmorl's stain,
120 and analysed using LM to determine if any hallmarks of OA, along with signs of ochronosis were present. At 31

121 weeks remodelling of the SCB was visible, along with what appeared to be concentric lamellar-like structures
122 around a chondrocyte deep in ACC (Fig. 1a). Analysis of a 60 week old AKU mouse also showed a similar feature
123 of concentric lamellae around a chondrocyte located along the SCB plate (Fig. 1b). Ochronotic pigmentation of
124 chondrocytes and their surrounding matrices, located in ACC, was also visible (Fig. 1c). Hallmark signs of OA
125 were observed in the mice, including loss of the articular surface and vertical clefts extending deep into the zones
126 of HAC (Fig. 1d).

127

128 Ultrastructural analysis of articular cartilage

129 Detailed TEM micrographs from an area of the MFC highlighted the ultrastructure of HAC and ACC, and the
130 cells and collagenous matrices contained within them (Figs. 2a & 2b). Flattened chondrocytes in the superficial
131 zone lay parallel to the articular surface while chondrocytes in the transitional zone appeared larger and more
132 spherical (Fig. 2a). Higher powered images of chondrocytes in both the superficial and transitional zones showed
133 the presence of collagen fibres in the pericellular matrix (PCM), and increased cellular detail with the nucleus and
134 rough endoplasmic reticulum both visible (Figs. 3a & 3b). The tidemark, which is the boundary between calcified
135 and non-calcified cartilage, still generates much discussion as to its composition [18-20]. It is highlighted to show
136 the differences between the matrices and cells in non-calcified and calcified articular cartilage (Fig. 2a).
137 Hypertrophic chondrocytes were localised to the ACC (Fig. 2a). Chondroptotic cells, showing chromatin
138 condensation, cellular disintegration and empty lacunae were visible deep in the ACC adjacent to the cement line
139 (Figs. 2b & 3c). Surrounding several of these cells we observed the appearance of novel concentric lamellar
140 structures (Figs. 2b & 3c, dashed arrows). The lamellae initially looked as if they formed part of the pericellular
141 matrix however upon further examination they could be seen to extend into the territorial matrix.

142

143 Identification of concentric lamellae in the articular calcified cartilage of BALB/c AKU and WT mice

144 As described above our analysis of the cartilage in BALB/c Hgd^{-/-} and WT mice led to the identification of
145 distinct patterns of concentric circles, which we have termed concentric lamellae, surrounding chondrocytes in
146 the ACC. The lamellae were visible both around viable cells, located towards the mineralisation front (Figs. 4a, b
147 & c) and around hypertrophic and chondroptotic cells located deeper in the ACC, close to the boundary with the
148 SCB (Fig. 4d). Chondrocytes located adjacent to the mineralisation front appeared to be partially engulfed by the
149 lamellae before progressing deeper into the ACC and becoming completely surrounded (Figs. 4a & b). This
150 process appeared to show an apparent opening and closing of the tidemark as the cells became surrounded and

151 embedded in the ACC. The lamellae appeared to be laid down around the chondrocytes in a periodic-like manner
152 (Figs. 4c & d). Chondrocytes located deeper in the ACC had more defined lamellae which enabled us to quantify
153 the lamellae and determine if they became more or less frequent with age, and whether their size was affected by
154 the age of the mice. Eleven samples were subjected to quantitative analysis, three from mice aged 9 weeks and
155 younger, including two AKU and one WT, and a further eight from mice aged 53 weeks and older, including
156 seven AKU and one WT. It was clear from the images that the lamellae found in young AKU mice were fewer in
157 number and thicker in width (Fig. 5a) than in aged AKU mice where they were more frequent but narrower (Figs.
158 5b & c). Along with more lamellae being present in aged AKU mice there were also more cells affected than in
159 young mice. Although the lamellae were visible in WT mice (Fig. 5d) there did appear to be fewer affected
160 chondrocytes. While the number of lamellae surrounding chondrocytes also appeared to decrease in WT mice, the
161 width remained consistent to those seen in AKU mice of similar age (Figs. 5b & c). This appears to confirm that
162 an increase in the age of the mice leads to a decrease in the width of the lamellae present in the cartilage.

163

164 Once the lamellae had been identified and quantified we wanted to identify their composition. In a large number
165 of the images it was difficult to ascertain what the lamellae were composed of. However, on further inspection at
166 higher magnification we were able to identify the presence of collagen fibres in the lamellae on a number of the
167 aged AKU mice (Figs. 6a & b). It is clear from both images that collagen fibres were located in the lamellae,
168 particularly those which were located closer to the cell.

169

170 **Discussion**

171 TEM was used to detail the ultrastructure of the HAC and ACC of BALB/c AKU mice. These mice are a model
172 of experimental OA due to the osteoarthritic phenotype they show including cartilage degeneration, SCB
173 remodelling and increasing amounts of calcification. The OA phenotype associated with AKU mice provided an
174 opportunity to use TEM to identify any ultrastructural changes in cartilage between AKU and WT mice with the
175 aim of further understanding the role played, particularly that of ACC, in the initiation and progression of OA.

176

177 Initial histological examination of AKU mice revealed the presence of concentric ring like structures around
178 chondrocytes deep in ACC at both 31 and 60 weeks of age. Using LM it was not possible to determine what these
179 structures were or to gain any detailed knowledge of their ultrastructure. Further analysis of AKU and WT with
180 TEM also revealed the presence of these concentric ring structures and led to a more robust analysis of the cartilage

181 to try and determine the nature of these structures. Initial signs of typical OA including remodelling of the SCB
182 and protrusion of SCB into ACC were also observed under LM (Figs. 1a & b). Pigmented chondrocytes, a
183 hallmark of AKU, were visible with both H&E and Schmorl's stain (Figs. 1b & c).

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187 TEM analysis of ACC in AKU mice revealed the presence of concentric lamellae around the majority of
188 chondrocytes scattered throughout this zone of cartilage. The lamellae were also present around chondrocytes in
189 the ACC of WT mice but to a much lesser extent. On initial examination it was unclear if the concentric structures
190 observed under TEM were identical to the ones seen under LM, however the fact they were both present deep in
191 ACC, and not in HAC, suggested possible structural similarities between the two and provided a basis for an in-
192 depth analysis. Extensive literature searches revealed that the presence of these lamellae is a novel finding in the
193 ACC. The structures identified may be related to the lamellae detected using SEM by Hirotsu et al [21], who
194 proposed the existence of a lamellar system around chondrocytes in the deep zone of the articular cartilage in
195 patients with secondary OA. It must be noted however, that these were found only in the HAC and not in the
196 ACC. No definitive reasoning is given by Hirotsu *et al* [21] for this system of lamellae in the cartilage, although
197 it is suggested it may be as a result of shrinkage from tissue preparation. With no other literature describing this
198 phenomenon, the mechanism behind their formation is not clearly understood. There is evidence, both from the
199 work described in this paper and the results gained by Hirotsu that the lamellae are related to the pathogenesis of
200 OA. The lamellae appeared around both viable chondrocytes towards the tidemark, which appeared to be partially
201 engulfed by lamellae, and to a much higher degree around hypertrophic chondrocytes located deep in the ACC
202 (Fig. 4). The fact that they appear much more regularly around hypertrophic chondrocytes may be significant as
203 to the origins of their formation. Hypertrophic chondrocytes are known to express type X collagen [22, 23], and
204 release increased levels of alkaline phosphatase [24] leading to cartilage calcification [25, 26]. Cartilage
205 calcification has been associated with both ageing of tissues [27, 28] and OA pathogenesis [25, 29]. Calcification
206 of cartilage associated with OA pathogenesis leads to thinning of HAC [30] and thickening of ACC [31], and can
207 be identified by advancement and duplication of the tidemark [32, 33] as mineralisation progresses towards the
208 surface. Thinning of the ACC can also occur during OA if the rate of subchondral remodelling is quicker than the
209 rate of tidemark advancement [34]. The lamellae identified in the ACC appeared to be laid down in association
210 with the advancing tidemark, which would indicate they may be formed during cartilage calcification. Viable

211 chondrocytes at the mineralisation front could be seen to be partially surrounded by the lamellae (Figs. 4a & b).
212 This suggests that chondrocytes in the HAC, which are close to the tidemark, are surrounded by ACC and the
213 lamellae are then laid down during calcification of the cartilage.

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219 The lamellae were identified in both young and aged AKU and WT mice. The greater abundance of lamellae in
220 AKU mice, which are a model of OA, suggests that they may have a role in the pathogenesis of OA. Lamellae
221 were present in young AKU mice (Fig. 5a) suggesting that cartilage calcification and OA initiation begins at a
222 young age in OA mice. There were fewer individual lamellae surrounding the chondrocytes in young AKU mice
223 and they appeared thicker than those seen in aged AKU mice. As the mice increased in age the lamellae became
224 thinner and more frequent around the chondrocytes. This correlates with the increased calcification and cartilage
225 thinning seen in aged mice. Increased calcification which is associated with OA progression [6], appears to be
226 linked to increasing amounts of lamellae formation around chondrocytes in ACC of aged AKU mice.

227

228 Although it is possible the lamellae may be involved in the development and progression of OA, it cannot be
229 discounted that they may be linked to the ageing process. Lamellae were present in both young and aged AKU
230 mice; the number of lamellae around chondrocytes increased in aged AKU mice (Fig. 5). The lamellae were also
231 identified in young and aged WT mice which showed very little cartilage degeneration, suggesting that their
232 formation may have been as a result of the ageing process. Increasing the number of mice examined, over a wide
233 range of ages, should help determine whether the lamellae are linked to either the development of OA or the
234 process of ageing.

235

236 Analysis of both AKU and WT mice revealed the appearance of novel concentric lamellae-like structures
237 surrounding hypertrophic chondrons in the ACC. Their possible association with mineralisation and advancement
238 of the tidemark, and their greater abundance in AKU mice indicate that the formation of these lamellae may be
239 involved in the pathogenesis of OA, since thinning of articular cartilage due to advancing mineralisation is
240 reported to be a characteristic of joints undergoing OA. Further work identifying the underlying mechanism(s) by

241 which the lamellae are formed, including immunohistochemistry and Energy Dispersive Spectroscopy (EDAX),
242 should provide a better understanding of the function and regulation of the ACC, and the role of the lamellae in
243 the initiation and progression of OA.

244 **Contributors**

245 Craig M Keenan designed the study and prepared the first draft of the paper. He is guarantor. James A Gallagher
246 designed the study and contributed to the analysis and interpretation of the data. Alison J Beckett, Hazel
247 Sutherland, Lakshminarayan R Ranganath, Jonathan C Jarvis, and Ian A Prior contributed to the analysis and
248 interpretation of the data. All authors revised the paper critically for intellectual content and approved the final
249 version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy
250 and integrity of the paper are investigated and properly resolved.

251

252 **Compliance with Ethical Standards**

253 Conflict of Interest: The authors declare that they have no conflict of interest.

254 Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of
255 animals were followed.

256 Ethical approval: This article does not contain any studies with human participants performed by any of the
257 authors.

258

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336

337 **Figure legends**

338 **Fig. 1 Histological examination of BALB/c AKU mice** (a) H&E staining of a 31 week old BALB/c AKU mouse
339 showed the appearance of a concentric ring like structure around a chondrocyte in the articular calcified cartilage
340 (ACC) of the medial femoral condyle (arrowed). Remodelling of the subchondral bone (SCB) was also seen which
341 is an indication of osteoarthritis (OA) (*). (b) H&E staining of a 60 week old BALB/c AKU mouse also showed
342 the appearance of concentric ring structures around a chondrocyte in ACC of the lateral femoral condyle (LFC)
343 (arrowed), along with protrusion of the SCB into ACC. (c) Schmorl's staining of a 60 week old BALB/c AKU
344 mouse showed large numbers of pigmented chondrocytes, a hallmark of AKU, present throughout ACC of the
345 LFC (arrowed). Pigmented chondrocytes were also visible in the H&E stained section (b) where they can be seen
346 deep in ACC (*). (d) Analysis of a 49 week old BALB/c AKU mouse showed complete loss of the articular
347 surface and vertical clefts running through the medial tibial plateau (arrowed), illustrating the severity of OA in
348 these mice. Scale = 20µm.

349

350 **Fig. 2 TEM micrographs of the medial femoral condyle from a 53 week old BALB/c AKU mouse** (a) HAC
351 and ACC with the tidemark, which separates the two types of articular cartilage, have been labelled. A
352 hypertrophic chondrocyte can be seen deep in the ACC. (b) Chondrocytes undergoing chondroptosis were visible
353 in the ACC. Concentric lamellae were also visible surrounding the cells (dashed lines). The cement line which
354 separates the ACC from the underlying SCB is highlighted (x1250). Tissue fixed in glutaraldehyde. Scale = 10µm.

355

356 **Fig. 3 Ultrastructural examination of chondrocytes from different zones of cartilage in a 53 week old**
357 **BALB/c AKU mouse** (a) TEM micrograph of a flattened chondrocyte in the superficial zone of the HAC.

358 Individual collagen fibres, located in the pericellular matrix (PCM), lie parallel to the articular surface (arrowed)
359 (x26,500). Inset: Location of the chondrocyte in HAC (x8250). **(b)** TEM micrograph of a chondrocyte in the deep
360 zone of the HAC. Specific structures within the cell have been labelled (x9900). **(c)** TEM micrograph of
361 hypertrophic chondrons in the ACC. Both sets of chondrocytes appeared chondroptotic with chromatin
362 condensation, cellular disintegration and the final stage of chondroptosis, empty lacunae, all present. Concentric
363 lamellae were also visible surrounding the cells (dashed lines). Inset: Location of the chondrocyte in the HAC
364 (arrowed) (x2500). Inset: Location of the cells in the ACC (x2500). Tissue fixed in glutaraldehyde. Scale = (a)
365 0.5µm, (b) 2µm, (c) 5µm.

366

367 **Fig. 4 The appearance of concentric lamellae around chondrocytes in the ACC of aged BALB/c AKU mice**

368 **(a)** A chondrocyte partially surrounded by concentric lamella, yet not completely enclosed in the ACC (x6000).
369 Inset: Location of chondrocyte in the ACC, showing apparent 'opening' of the tidemark (arrowed) resulting in the
370 cell becoming engulfed by the ACC (x2500). **(b)** A chondrocyte almost completely surrounded by lamellae,
371 progressing deeper into the ACC (x6000). Inset: Location of chondrocyte in the ACC, showing apparent 'closing'
372 of the tidemark (arrowed) resulting in the cell becoming completely embedded in the ACC (x2500). **(c)** A
373 chondrocyte surrounded by numerous concentric lamellae (arrowed) in a periodic-like manner (x8200). **(d)**
374 Concentric lamellae surrounding a chondrocyte deep in the ACC, in a periodic manner (arrowed) identical to what
375 was seen in (c) (x8200). Tissues fixed in (a,b) PBFS, (c,d) glutaraldehyde. Ages = (a,b) 60 wks, (c,d) 54.4 wks.
376 Scale = (a,b) 5µm, (c,d) 2µm.

377

378 **Fig. 5 Measurements of concentric lamellae in BALB/c AKU and WT mice**

379 **(a)** Quantification of the lamella
380 in a 7.8 week old AKU mouse showed a general increase in width as they progressed further away from the
381 chondrocyte (x16,500). **(b,e)** The number of lamellae surrounding chondrocytes in aged AKU mice (53 + 61
382 weeks old respectively) increased in comparison to young AKU mice (a), however the widths of the lamellae were
383 significantly narrower (x26,500). **(d)** Quantification of the lamellae in an aged WT mouse (69 weeks) revealed
384 the number of lamellae was comparable to that seen in young AKU mice (a), whilst the width was comparable to
385 that seen in aged AKU mice (b,c) (x4200). Tissues fixed in (a,b) glutaraldehyde (c,d) PBFS. Scale = (a,b,c) 1µm,
386 (d) 5µm.

386

387 **Fig. 6 Identification of collagen fibres in aged BALB/c AKU mice (a)** Collagen fibres were identified in the
388 lamellae of a 56 week old AKU mouse (arrowed). Periodic banding can be seen along the fibres which is
389 distinctive of collagen (x60,000). Inset: Low power image highlighting the location of the collagen fibres in the
390 lamellae (x16,500). Tissue fixed in glutaraldehyde. **(b)** Collagen fibres were identified in the lamella of a chondron
391 deep in the ACC of a 60 week old AKU mouse. Again, periodic banding can be seen along the fibres which is
392 distinctive of collagen (x87,000). Inset: Low power image highlighting the location of the collagen fibres in the
393 lamella (x43,000). Tissue fixed in PBFS. Scale = (a) 0.5 μ m, (b) 0.2 μ m.