

1 **High spatial resolution analysis using indentation mapping differentiates biomechanical properties**
2 **of normal vs. degenerated mouse articular cartilage**

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15 **Abstract**

16 Characterizing the biomechanical properties of articular cartilage is crucial to understanding processes
17 of tissue homeostasis vs. degeneration. In mouse models, however, limitations are imposed by their
18 small joint size and thin cartilage surfaces. Here we present a 3D automated surface mapping system
19 and methodology that allows for mechanical characterization of mouse cartilage with high spatial
20 resolution. We performed repeated indentation mappings, followed by cartilage thickness
21 measurement via needle probing, at 31 predefined positions distributed over the medial and lateral
22 femoral condyles of healthy mice. High-resolution 3D x-ray microscopy (XRM) imaging was used to
23 validate tissue thickness measurements. The automated indentation mapping was reproducible, and
24 needle probing yielded cartilage thicknesses comparable to XRM imaging. When comparing healthy
25 vs. degenerated cartilage, topographical variations in biomechanics were identified, with altered
26 thickness and stiffness (instantaneous modulus) across condyles and within anteroposterior sub-
27 regions. This quantitative technique comprehensively characterized cartilage function in mice femoral
28 condyle cartilage. Hence, it has the potential to improve our understanding of tissue structure-function
29 interplay in mouse models of repair and disease.

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39 **Introduction**

40 The articular cartilage in synovial joints has a specialized three-dimensional structure and biochemical
41 composition that provides low-friction, wear-resistance, and load-bearing properties to the tissue.
42 Mechanical loading is essential to tissue homeostasis and influences gene expression, chondrocyte
43 metabolism, extracellular matrix (ECM) maintenance, and associated interstitial fluid permeability ^{1,2}.
44 Therefore, the biomechanical properties of cartilage are unique indicators of tissue homeostasis versus
45 degeneration. Indeed, research into cartilage-related changes during the development of degenerative
46 diseases such as osteoarthritis (OA) has demonstrated that early tissue dysfunction involves loss of
47 proteoglycans and increased water content, resulting in reduced compressive strength and higher
48 tissue permeability ^{3,4}. Disease progression leads to further structural damage and altered mechanics ⁵,
49 ultimately resulting in tissue failure.

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51 Mouse models are commonly used in pre-clinical studies focused on cartilage repair and
52 pathophysiology of OA ⁶. Mice are advantageous because of the well-established development of
53 genetically modified strains ⁷ and the availability of diverse model systems mimicking mechanisms of
54 spontaneous and induced tissue degeneration and regeneration ⁸⁻¹¹. In this regard, mouse models serve
55 as powerful tools for the targeted assessment of cellular and molecular processes and the discovery of
56 novel therapeutics related to cartilage regeneration and OA. Yet, while the structural integrity and
57 biochemical composition of murine cartilage are routinely assessed through histological and
58 molecular approaches, the evaluation of how these features translate into mechanical function is
59 limited. The main challenge in mechanical function assessment stems from their small joint size and
60 thin cartilage found in mice relative to other species ¹². Prior efforts to overcome these challenges
61 include finite element modelling and optimization of small-scale indentation techniques ^{3,13-15}.

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63 Mechanical indentation (including AFM techniques), performed using either creep or stress-relaxation
64 protocols, is widely employed for assessing the biomechanical behavior of cartilage and OA-related
65 changes in many species ¹⁶⁻¹⁸, and considered the gold standard for small animal joints ¹⁹. Unlike
66 confined and unconfined compression tests, no sectioning or subsampling of tissue (i.e., cylindrical

67 explants) is required²⁰. Instead, the cartilage tissue and its subchondral bone interface is kept intact,
68 providing more physiologically relevant data. Indentation is also advantageous in that it is non-
69 destructive and allows for repeated measures *in situ*²¹. However, the natural curvature of joint
70 articular surfaces poses a challenge to testing, as indentation must be conducted perpendicularly to the
71 surface²².

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73 Recently, a novel automated indentation technique has been developed for the mechanical assessment
74 of cartilage²³. This commercially available multi-axial apparatus (Mach-1TM, *Biomomentum Inc*,
75 Laval, QC) is capable of detecting specimen surface orientation at each position of measurement and
76 subsequently indent normal to the surface^{23,24}. As such, it can map entire cartilage surfaces using a
77 single setup with high spatial density, and has been previously shown to discriminate between healthy
78 and diseased human cartilage samples^{23,25}. This apparatus has been recently employed by Woods and
79 colleagues²⁶ to evaluate altered biomechanics in a mouse model of cartilage degeneration. However,
80 the study was conducted in the non-load-bearing region of the mouse knee joint. Considering that the
81 knee range of motion in mice is between 40° to 145° (i.e., unable to fully extend)²⁷, with normal gait
82 range between 90.5° and 120° (extension-flexion)²⁸, the contact regions in the distal femoral condyles
83 are located further posteriorly compared to humans. Careful consideration of species-specific
84 differences in knee joint anatomy and kinematics is imperative for proper translation of pre-clinical
85 models²⁹.

86

87 Woods and colleagues²⁶ were also unable to account for site-specific cartilage thickness variations in
88 their measurements, instead using the mean cartilage thickness obtained via histological analysis in
89 their assessment²⁶. Other studies have employed mean cartilage thickness values retrieved through
90 histology or imaging techniques to characterize and model tissue mechanical parameters in mice^{13,15}.
91 Implicit in this approach is the underlying assumption that cartilage thickness is relatively uniform
92 among medial and lateral compartments (e.g., femoral condyles or tibial plateaus) and along their
93 anteroposterior or mediolateral axis. Yet, regional variations in thickness are recognized within these
94 cartilage surfaces^{12,15,30}, and can impact indentation measurements^{22,31}.

95 Hence, the purpose of this study was to investigate the reliability of automated indentation mapping in
96 the assessment of healthy femoral articular cartilage in mice and characterize site-specific variations
97 in cartilage thickness. We also employed concurrent contrast-enhanced 3D x-ray microscopy (XRM)
98 imaging to validate the cartilage thickness measurements from our optimized needle probing protocol
99 ³². Finally, this approach was used to investigate biomechanical differences in a clinically relevant
100 mouse model of cartilage degeneration ³³. Together, we show that automated indentation is reliable
101 and able to characterize topographic and mechanical variations across condyle cartilage locations in
102 intact cartilage. Moreover, this technique was able to identify regional changes in cartilage thickness
103 and stiffness in degenerated cartilage. A comprehensive and standardized biomechanical evaluation of
104 cartilage in repair and disease can greatly contribute to our understanding of tissue structure-function
105 interplay, thereby enhancing the clinical relevance of mouse models.

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107 **Results**

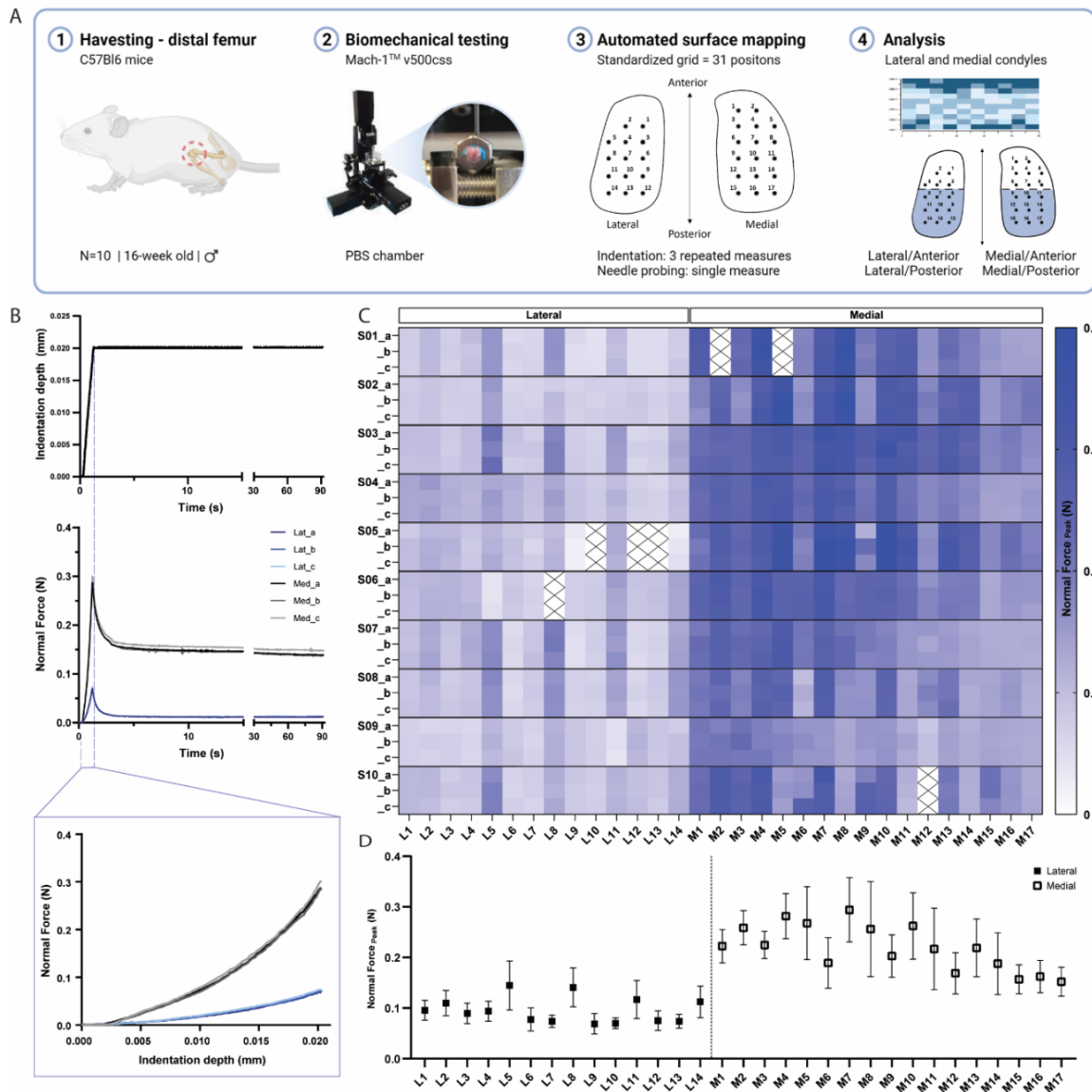
108 *Automated indentation mapping reliability*

109 While previous studies have employed indentation mapping on mouse cartilage, to our knowledge
110 none have measured its accuracy and precision. Therefore, we performed three repeated mappings per
111 position for all specimens to undertake a reliability analysis of the automated surface indentation
112 technique. Each of the 31 predefined positions distributed over the femoral condyles of ten C57Bl6
113 mice was included in this analysis (Figure 1A). The setup was developed and optimized (Figure S1) to
114 assess the load-bearing regions of the femoral condyles and achieve non-destructive retrieval of
115 specimen post-testing for subsequent 3D XRM imaging analysis. The imposed step deformation (i.e.,
116 indentation depth) on the femoral cartilage yielded typical stress-relaxation behavior, characterized by
117 a sharp increase in force followed by gradual relaxation over time until equilibrium (Figure 1B).
118 Assessment of stress-relaxation and corresponding force-displacement curves (Figure 1B)
119 demonstrated consistency among repeated measurements for single positions and visible differences
120 in peak reaction forces between condyles. These observations were further evidenced by the spatial
121 distribution of peak force values across condylar testing sites (Figure 1C, Table S1). A total of 930
122 indentation measurements were retrieved, out of which only 21 produced invalid curves (seven testing
123 sites at specimen's periphery with higher angles yielded noisy signals, Figure 1C), representing a 2.26
124 % error rate during data acquisition. High reliability and absolute agreement between repeated
125 measures for individual testing sites were observed, with 4.7% intra-assay average coefficient of
126 variation (CV) (Table S1) and intraclass correlation coefficients - ICC (lower 95%, upper 95%) -
127 ranging from 0.974 (0.966, 0.981) for the lateral condyle to 0.971 (0.963, 0.978) for the medial
128 condyle. Mean peak force values illustrate site-specific variations within and between condyles
129 (Figure 1D). The lateral condyle values varied significantly per position ($p < 0.0001$), ranging from
130 0.07 to 0.15 N and showed a trend for higher values at outermost positions, with a slight decrease in
131 force posteriorly. The latter was also seen for the medial condyle, wherein heterogeneities in peak
132 force were also apparent ($p < 0.0001$) and had a wider range – from 0.15 to 0.294 N. Since the
133 analysis per testing site also reflects inherent deviations due to anatomical positioning across

134 specimens, data was pooled for regional (between condyles) and sub-regional (between and within

135 anteroposterior locations) comparisons.

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138 **Figure 1.** (A) Schematic overview of experimental design employed for biomechanical testing of
 139 murine articular cartilage using Mach-1™ v500css mechanical tester (B) Step displacement used for
 140 cartilage indentation (top), with typical force-relaxation response curves obtained for three repeated
 141 measures on representative lateral and medial condyle positions (middle) and corresponding force
 142 increase with indentation depth (bottom) (C) Normal peak force recorded for all three repeated
 143 measures (a-c) considering each of the 31 testing sites, L1-L14 at lateral condyle and M1-M17 at
 144 medial condyle, for each specimen ($n = 10$, S01-S10), demonstrates general agreement for intra-
 145 specimen measurements on both condyles (D) Mean peak force values varied within and between
 146 condyle locations and higher within medial condyle testing sites. Data is presented as mean \pm SD.

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152 As Table 1 shows, the average peak force was significantly higher on the medial condyle and on both
 153 its anterior and posterior sub-regions when compared to lateral counterparts (Lat/Ant vs. Med/Ant and
 154 Lat/Post vs. Med/Post). Interestingly, no significant differences were observed between sub-regions of
 155 the lateral condyle (Lat/Ant vs. Lat/Post). In contrast, the mean peak force yielded at the Med/Post
 156 sub-region was 20% lower than on the Med/Ant ($p < 0.01$). As cartilage thickness variations between
 157 and within condyle locations could affect peak forces measured at same indentation depth³⁴, with
 158 thinner cartilage yielding higher force values, we sought to determine the cartilage thickness
 159 distribution within the same surfaces and validate this approach using XRM imaging.

160

161 **Table 1.** Mean and standard deviation (SD) values for peak force (N) as determined by automated
 162 indentation test performed for $n = 10$ distal femur samples of murine AC. Mean values compared
 163 between condyles (Lateral/Medial; unpaired, Student's t test, $\alpha = 0.05$) and within sub-regions of
 164 condyles (Lat/Ant, Lat/Post, Med/Ant, Med/Post; *one-way* ANOVA, $p < 0.05$).

Peak Force [N]				
Condyle	Mean (SD)	Condyle	Mean (SD)	Lat - Med
Lateral	0.096 (0.011)	Medial	0.219 (0.037)	$p < 0.0001$
<i>Lat/Ant</i>	0.107 (0.017)	<i>Med/Ant</i>	0.250 (0.037)	$p < 0.0001$
<i>Lat/Post</i>	0.090 (0.011)	<i>Med/Post</i>	0.199 (0.037)	$p < 0.0001$
Ant - Post	<i>ns</i>	Ant - Post	$p < 0.01$	-

165 *ns*, no significant difference; -, comparison not applicable

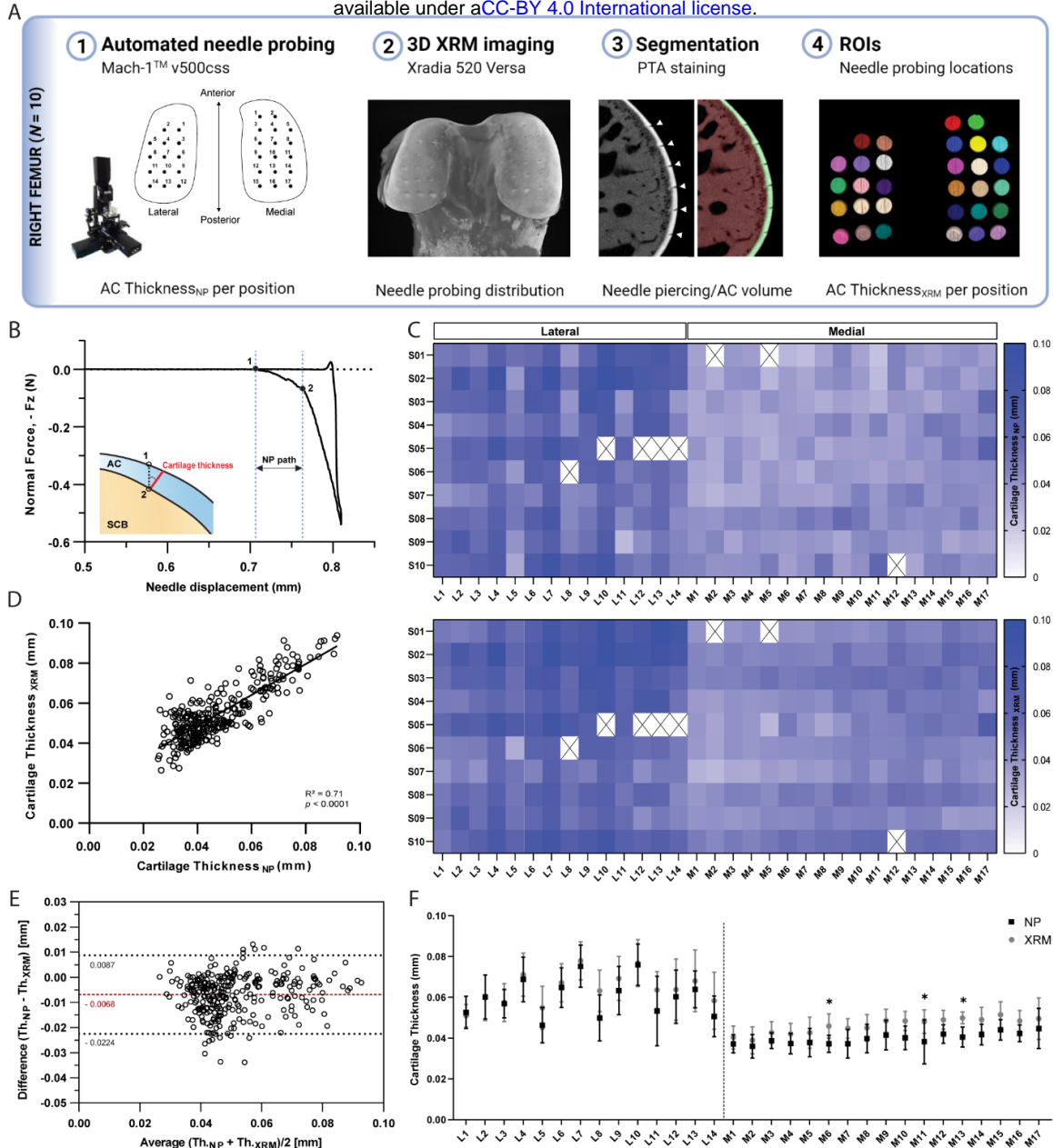
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168 *Cartilage thickness characterization: comparison between needle probing and XRM imaging*

169 Needle probing (NP) thickness mapping was performed on all ten femoral condyles, which were
 170 subsequently scanned using contrast enhanced XRM imaging. As shown in Figure 2A, reconstructed
 171 3D datasets of murine distal femurs allowed us to validate the spatial distribution of NP testing sites,
 172 thereby the corresponding ROI coordinates for imaging processing could be determined. Additionally,
 173 the 2D slices confirmed that the needle probe pierced the full length of the cartilage, reaching the
 174 subchondral bone (Figure 2A). The cartilage surface and cartilage-subchondral bone interface
 175 positions were identified using the load-displacement curves from NP (Figure 2B) and the cartilage
 176 thickness for each position was then calculated considering the surface angle (*Methods*). There was a

177 2.58% rate of needle probe failure (8 out of 310 measurements, testing sites near the specimen's edge)
178 during data acquisition. NP and contrast enhanced XRM imaging yielded similar cartilage thickness
179 distributions on both condyles (Figure 2C), demonstrating a highly significant correlation between
180 paired values ($R = 0.842$, $n = 302$, $p < 0.0001$) (Figure 2D). Further method-comparison using a
181 Bland-Altman plot (Figure 2E) illustrated XRM measurements were approximately $6.8 \mu\text{m}$ thicker on
182 average than needle probing, representing only a 1.55 voxels difference ($4.39 \mu\text{m}$ resolution - XRM).
183 Moreover, no significant differences were found between pairwise mean thickness values (NP vs.
184 XRM) for individual positions within the lateral condyle (Figure 2F). Similar results were shown for
185 the medial condyle, wherein higher mean thickness values from XRM were only seen on M06 ($\Delta =$
186 $8.68 \mu\text{m}$, $p = 0.028$), M11 ($\Delta = 9.89 \mu\text{m}$, $p < 0.01$), and M13 ($\Delta = 9.34 \mu\text{m}$, $p = 0.012$) testing sites.
187 No obvious reason for these localized differences were found, and dullness of needle was ruled out
188 since subsequent testing sites (M14 to M17) yielded comparable cartilage thickness values between
189 techniques.
190



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192 **Figure 2.** (A) Assessment of agreement between needle probing (NP) and XRM imaging cartilage
 193 thickness per position of measurement within right femoral condyles ($n = 10$) (B) Representative
 194 normal force-displacement curve obtained during NP test depicting articular cartilage, AC, surface (1)
 195 and subchondral bone, SCB, interface (2) positions and cartilage thickness calculated normal to the
 196 surface (red) using the surface angle orientation (C) Mapping distributions of cartilage thickness
 197 values per position as measured by NP and XRM (D) Correlation graph of cartilage thickness
 198 measured by NP vs. XRM, $R = 0.842$, $n = 302$, $p < 0.0001$ and corresponding (E) Bland-Altman plot
 199 showing overall agreement between methods, with average difference of $6.8 \mu\text{m}$ in thickness. Dotted
 200 black lines show upper and lower 95% limit of agreement (F) Pairwise assessment of mean cartilage
 201 thickness NP vs. XRM per position for the lateral and medial condyles ($* p < 0.05$, $** p < 0.01$, two-
 202 way ANOVA). Symbols represent the means and error bars the standard deviation.

203

204 Unlike peak force distributions, spatial heterogeneities in cartilage thickness were apparent among
 205 individual testing sites only on the lateral condyle ($p < 0.0001$), with averaged values ranging from 46
 206 to 76 μm ; whereas thickness distributions within the medial condyle was more uniform ($p = 0.06$),
 207 ranging from 36 to 45 μm . Nevertheless, no significant differences were observed within condyles
 208 when comparing their anteroposterior sub-regions; whereas the medial condyle was significantly
 209 thinner than its lateral counterpart, both in its anterior and posterior sub-regions (Tables 2 and S2).
 210 Together, cartilage thickness appears as a contributing factor but not the sole explanation to
 211 mechanical variations, which is also affected by differences in composition and morphology.

212
 213 **Table 2.** Mean and standard deviation (SD) values for cartilage thickness as determined by needle
 214 probing for distal femur samples of murine articular cartilage ($n = 10$). Mean values compared
 215 between condyles (Lateral/Medial; unpaired, Student's t test) and within sub-regions of condyles
 216 (Lat/Ant, Lat/Post, Med/Ant, Med/Post; *one-way* ANOVA, $\alpha = 0.05$).

Cartilage Thickness [μm]				
Condyle	Mean (SD)	Condyle	Mean (SD)	Lat - Med
Lateral	60.3 (6.3)	Medial	39.8 (2.9)	$p < 0.0001$
<i>Lat/Ant</i>	57.0 (5.9)	<i>Med/Ant</i>	37.5 (3.8)	$p < 0.0001$
<i>Lat/Post</i>	62.3 (7.8)	<i>Med/Post</i>	41.9 (3.0)	$p < 0.0001$
Ant - Post	<i>ns</i>	Ant - Post	<i>ns</i>	-

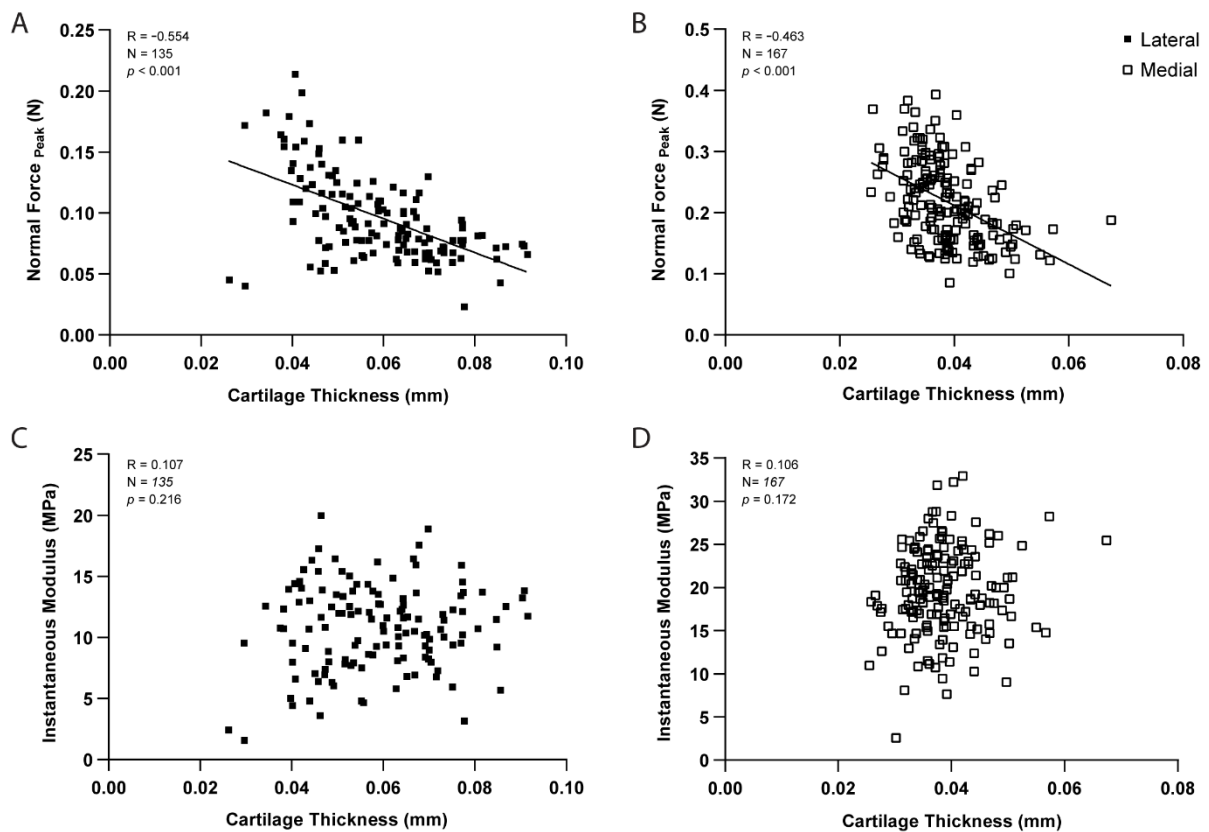
217 *ns*, no significant difference; -, comparison not applicable

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 220 As expected, pairwise comparisons for individual testing sites demonstrated a significant negative
 221 correlation between peak force and thickness measurements within lateral ($R = -0.554$, $n = 135$, $p <$
 222 0.001 , Figure 3A) and medial ($R = -0.463$, $n = 167$, $p < 0.001$, Figure 3B) condyles. Knowledge of
 223 site-specific thickness variations allowed the compressive stiffness to be determined for the same
 224 mechanical strain at each testing site. Instantaneous modulus was calculated using Hayes *et al.* elastic
 225 model³¹ at 20% strain, wherein linear elastic behavior can be assumed and instantaneous response is
 226 considered as flow-independent (*Poisson's* ratio, $\nu = 0.5$ assumed)³⁵. Regardless of femoral condyle,
 227 instantaneous stiffness demonstrated no significant correlation to thickness variations (Figures 3C-D).
 228 Notably, compressive stiffness differed significantly between condyles (Lat vs. Med, $p < 0.001$), but
 229 no longer within a condyle (Lat/Ant vs Lat/Post: $p > 0.99$; Med/Ant vs Med/Post: $p = 0.546$), like

230 seen for peak reaction force on the medial side. Next, we assessed the potential of this indentation

231 testing in identifying microscale biomechanical differences between healthy and degraded cartilage.

232



233

234 **Figure 3.** Correlation graphs per testing site for the lateral ($n = 135$ positions) and medial ($n = 167$)
235 condyles, showing cartilage thickness (needle probing) is significantly correlated to peak indentation
236 force at 20 μm (A-B), but not to instantaneous modulus values as determined by Hayes *et al.* (1972)
237 elastic model at 20% strain. p -values reported.

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241 *Altered biomechanical properties in degenerated murine articular cartilage*

242 To assess the changes in mechanical response within the context of cartilage degeneration, we

243 employed the same testing protocol on age-matched Proteoglycan 4 (PRG4) knockout mice ($n = 6$)

244 and compared the outcomes to the C57Bl/6 controls. PRG4 is a mucin-like glycoprotein highly

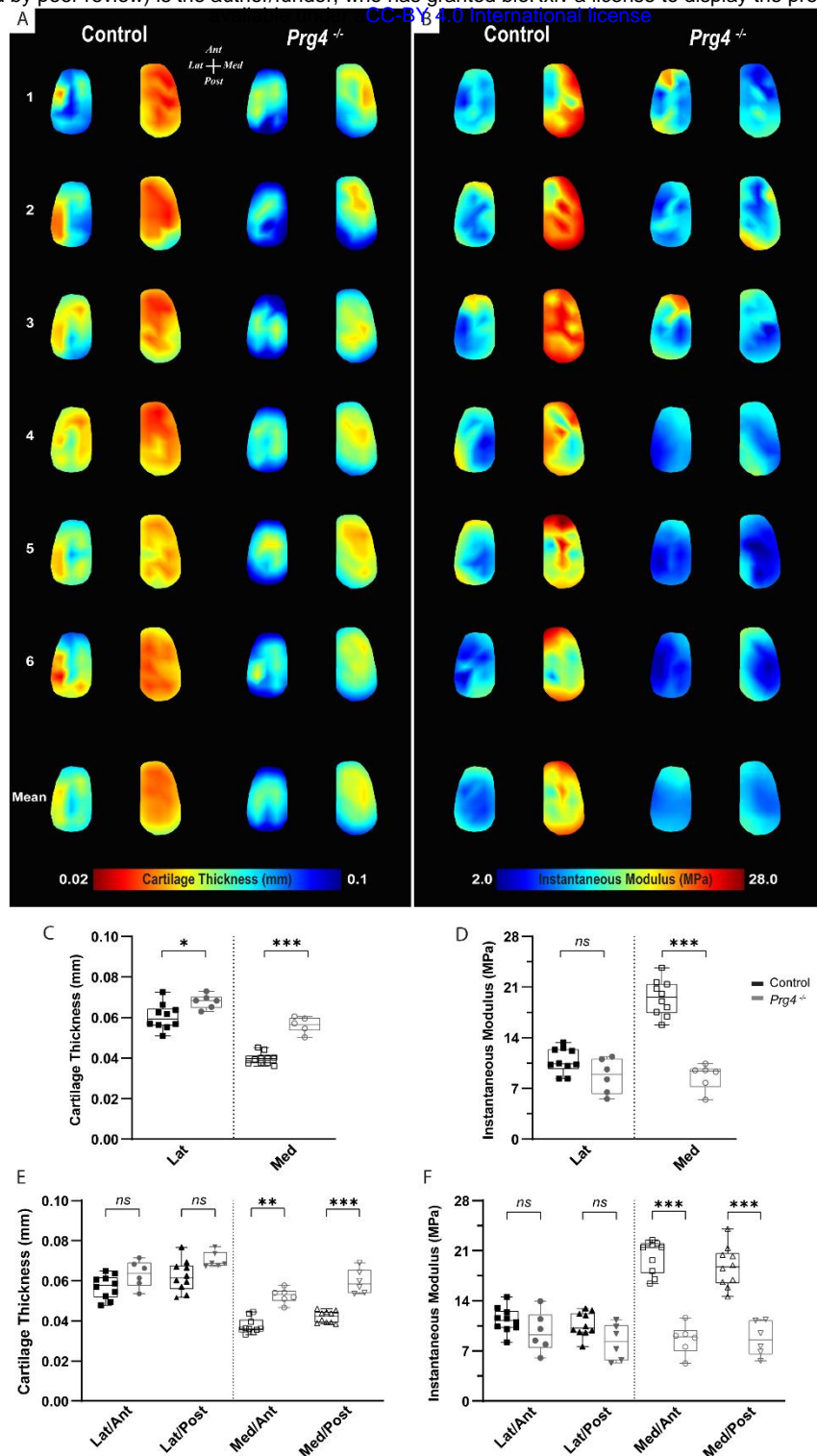
245 conserved across species^{36,37} and functionally relevant in joint homeostasis and lubrication^{33,38,39}.

246 PRG4 loss of function, as seen in knockout mice ($Prg4^{-/-}$), leads to degenerative joint changes

247 recapitulating the phenotype of human camptodactyly-arthropathy-coxavara-pericarditis (CACP)

248 syndrome^{33,40}. Histological alterations of articular cartilage have been comprehensively described,

249 and include surface roughness, tissue thickening, and loss of collagen parallel orientation at the
250 superficial layer, progressing to irreversible tissue damage with age^{33,41}. Yet, the microscale
251 assessment of site-specific mechanical variations, aside from friction, has not been described so far
252 for *Prg4*^{-/-} knee cartilage surfaces. Mapping of biomechanical parameters allowed site-specific
253 differences in *Prg4*^{-/-} cartilage to be visualized, and outcomes were largely reproducible across
254 femoral specimens (Figure 4A-B). These findings were supported by the combined quantitative
255 assessment between genotypes for the different condyle regions and subregions (Figure 4C-F). *Prg4*^{-/-}
256 mean cartilage thickness on both lateral ($67.9 \pm 3.5 \mu\text{m}$, $p = 0.032$) and medial ($56.3 \pm 3.7 \mu\text{m}$, $p <$
257 0.001) sides of the knee was higher compared to controls (Figure 4C). Differences in cartilage
258 thickness between genotypes on anteroposterior sub-regions, however, were only detected on the
259 medial condyle (Figure 4E). The site-specific thickness measurements enabled us to determine the
260 instantaneous modulus at each testing site for the same mechanical strain. The utilized Hayes *et al.*
261 elastic model could fit the response of mouse cartilage with great accuracy (RMSE equal to $0.017 \pm$
262 0.007 MPa for controls and 0.010 ± 0.003 MPa for *Prg4*^{-/-}). Cartilage compressive stiffness was
263 significantly lower on the medial condyle of *Prg4*^{-/-} mice compared to controls (8.67 ± 1.79 MPa vs.
264 19.52 ± 2.44 MPa, respectively, $p < 0.001$). Similar patterns were seen when considering the
265 anteroposterior subregions of the medial condyle (Figures 4F). No significant differences in lateral
266 condyle cartilage stiffness were observed (10.85 ± 1.73 MPa - control vs. 8.73 ± 2.39 MPa - *Prg4*^{-/-}, p
267 = 0.187).



268

269 **Figure 3.** (A-B) Heat maps and corresponding boxplots comparing (C-D) regions - lateral and medial
 270 - and their (E-F) sub-regions - Lat/Ant, Lat/Post, Med/Ant, Med/Post - highlight spatial differences in
 271 biomechanical parameters on femoral cartilage surface between controls (C57Bl/6, $n = 10$) and PRG4
 272 knockout mice (*Prg4*^{-/-}, $n = 6$). Maps shown for six representative samples per genotype, as well as the
 273 corresponding averaged map of all samples. Parameters illustrated are thickness measured by NP (A,
 274 C, E) and instantaneous modulus as determined by Hayes *et al.* (1972) elastic model (B, D, F).
 275 Pairwise comparison between genotypes for mean values on the lateral and medial condyles and

276 anteroposterior sub-regions (Mann-Whitney *U* with Bonferroni-Dunn correction, * $p < 0.05$, ** $p < 0.01$,
277 *** $p < 0.001$).

278 Discussion

279 Using a novel microscale instrumented apparatus, we were able to reliably detect and quantify spatial
280 variations in biomechanical parameters across murine cartilage surfaces, both within healthy and
281 degenerated femoral condyles. Compared to recent mouse studies using this commercially available
282 apparatus^{26,42}, the optimization of sample mounting and needle probing protocols allowed for
283 unprecedented quantitative mapping of the mechanical behavior and associated cartilage thickness on
284 load-bearing regions of distal femurs with high spatial density.

285

286 For healthy, 4-month old C57Bl6 mice, micro-indentation measurements were reproducible at any
287 given testing site, also indicating that the instantaneous deformation employed and subsequently
288 sustained compression protocol did not compromise the mechanical behavior of the freshly harvested
289 cartilage tissue. In addition, heterogeneity in peak forces were identified across anatomical locations.
290 Notably, the medial condyle surface yielded approximately two times higher peak forces on average
291 than its lateral counterpart. Moreover, both condyles displayed a shift towards lower values at
292 posterior regions. Previous studies have shown significant variation in mechanical properties within
293 different cartilage surfaces in healthy joints^{43,44}, or even over a single surface^{18,45}. However, the
294 effect of site-specific cartilage geometry, such as thickness, when characterizing the mechanical
295 properties of cartilage in indentation is often overlooked^{23,46}.

296

297 Studies in mice commonly rely on mean thickness values from histology, requiring longer turnaround
298 and lacking spatial specificity. The optimized needle probing protocol used here, was able to resolve
299 spatial thickness variations across condyle surfaces, and for the first-time closely localize cartilage
300 thickness measures to the footprint of indentation on mouse distal femurs. Moreover, three-
301 dimensional visualization of the femoral cartilage surfaces after XRM imaging validated the
302 positioning and distribution of the testing grid used, allowed us to pinpoint individual testing sites and
303 compute their thickness. A highly significant correlation between pairwise cartilage thickness values

304 from NP and XRM, per measurement site, was observed. Small variations seen between averaged
305 thickness values could be explained by differences in instrument resolution capabilities (Mach-1 z-
306 axis: 0.5 μm vs XRM: 4.39 μm voxel size), partial volume effects in XRM segmentation, and/or
307 compromise of cartilage integrity by needle probing, which might have led to cartilage swelling and
308 overestimation on subsequent XRM thickness measures. Nevertheless, mean thickness values were in
309 line with earlier reports for mouse cartilage^{29,47,48}, and marked greater cartilage thicknesses were
310 measured on the lateral condyle compared to the medial counterpart¹².

311
312 In general, higher peak reaction forces in intact cartilage were associated with lower thickness values
313 at the same indentation depth, and likely amplified by contribution of the underlying subchondral
314 bone on thinner areas⁴⁹. Localized cartilage thickness measurements enabled us to account for the
315 effect of cartilage geometry when calculating compressive stiffness distributions. Quantitative
316 analysis was adjusted to 20% strain (considered as physiological loading)⁵⁰, and corresponding
317 instantaneous modulus could be derived from Hayes's analytical formulation. According to the
318 present results, there was no significant variation in mean thickness of healthy cartilage over a
319 condyle surface, therefore biomechanical parameters were compared between matched regions and
320 sub-regions when assessing degeneration.

321
322 Compared to C57Bl6 control, the loss of *Prg4* function resulted in an increase in thickness and
323 decrease in stiffness of the knee cartilage in 16-weeks-old mice. This is consistent with previous
324 observations on hip articular cartilage of *Prg4*^{-/-} mice (same age), using nanoscale atomic force
325 microscopy (AFM) indentation³⁹. The cartilage thickness values determined by needle probing in our
326 study are in a similar range to those reported by Karamchedu *et al.*⁴¹. In their microCT imaging
327 study, *Prg4*^{-/-} mice displayed cartilage thickness averaging $61.0 \pm 4.3 \mu\text{m}$ and $42.4 \pm 2.6 \mu\text{m}$ for the
328 load-bearing regions of the lateral and medial condyle, respectively. Furthermore, *Prg4*^{-/-} cartilage
329 was significantly thicker than in control littermates, even though mice were younger (10-weeks-old)
330 than in our study.

331

332 Reduction in the instantaneous compressive modulus in our study was confined to the medial
333 compartment. We attribute that to differences in thickening in *Prg4*^{-/-} femoral cartilage compared to
334 controls, 12.6% versus 41% on lateral and medial condyles respectively, as well as *Prg4*^{-/-}
335 compromised structural integrity^{33,38,51} having a bigger functional impact on the medial compartment,
336 recognized as the main load bearing region in most mammalian joints. Microscale indentation
337 characterizes the overall tissue resistance to deformation⁵⁰. Due to the rapid compression rate
338 employed in the present protocol, normal cartilage tissue is expected to deform with minimal change
339 in volume³⁵, as its low permeability restrains fluid flow; thereby localizing the strain near the surface
340 as fluid pressure pushes against the collagen meshwork. Thus, under instantaneous (impact) loading
341 the ability of cartilage to resist compression is known to be affected by the collagen fibril meshwork
342^{49,52}, particularly tangentially oriented collagen fibrils on the superficial tissue layer⁴³. In *Prg4*^{-/-} the
343 normal parallel organization of collagen fibrils adjacent to the surface is known to be disrupted³⁸,
344 likely affecting tissue permeability as well, helping explain the lower instantaneous modulus and
345 slightly faster dissipation of stress.

346
347 Limitations of this study include evaluation of the anatomy of the distal murine femoral condyles and
348 not the opposing articulation (i.e., proximal tibia). Also, we focused on a single timepoint analysis,
349 however, investigations related to aging and progressive degeneration are of interest, as they are
350 known to affect the biochemical composition and structural integrity of cartilage^{33,53,54}, thereby
351 influencing its mechanical properties. Finally, due to the biphasic/poroviscoelastic nature of cartilage,
352 future studies could consider the use of more complex analytical models^{55,56} able to capture the
353 viscoelastic behavior of cartilage in mice.

354
355 In this study, we have gained insights into the patterns of varying surface geometry, and mechanics
356 present within murine articular cartilage at the microscale. Three-dimensional indentation mapping
357 was able to resolve site-specific differences in thickness and mechanical properties across knee
358 cartilage surfaces in healthy mice. Moreover, it identified functional changes on the *Prg4*^{-/-} mouse
359 model. This technique could also prove helpful for the study of other mouse models mimicking

360 different mechanisms of, or therapies focused on, repair and degeneration of articular cartilage, as
361 microscale indentation with high spatial density can provide a more comprehensive characterization
362 of cartilage's mechanical properties.

363

364 **Materials and methods**

365 *Ethics statement*

366 All mouse experiments were carried out following the Canadian Council on Animal Care Guidelines
367 recommendations and approved by the University of Calgary Animal Care Committee (protocols
368 AC16-0043 and AC20-0042).

369 *Animals*

370 Male C57Bl6 mice were purchased from *Jackson Laboratories* (Bar Harbor, ME). We based on
371 sample on a recent paper that used indentation (but not needle probing) in mice²⁶, however, because
372 of the addition of needle probing we decided to increase the sample size. Animals were housed under
373 a standard light cycle and had free access to feed (standard diet) and water. Ten mice were euthanized
374 at 16-weeks of age, and hind limbs ($n = 10$ right) were harvested for biomechanical testing and 3D X-
375 ray microscopy (XRM) imaging. Age-matched PRG4 knockout mice (*Prg4*^{-/-}, $n = 6$) were generated
376 and maintained on a C57BL/6 genetic background, as previously described⁵⁷. Limbs were
377 disarticulated at the hip, followed by transection of the ligaments and careful isolation of distal femurs
378 from tibiae and menisci with the help of a dissection microscope (*Leica*). Femurs were preserved
379 gently wrapped in Kimwipe soaked in phosphate-buffered saline (PBS, pH 7.4) until the time of
380 assessment. All samples were mechanically tested no longer than 3h after dissection to prevent tissue
381 degradation.

382 *Automated Indentation Mapping*

383 The shafts of isolated femurs were glued into a 0.1-10 μ L pipette tip (VWR) using cyanoacrylate
384 adhesive, fixed into a stainless-steel hex nut (*Paulin*, Model 848-216) and secured to the sample
385 holder (Figure S1). This customized setup allowed for simple and proper positioning of the sample
386 exposing the load-bearing region of the condyles²⁷ for data acquisition, as well as non-destructive

387 retrieval of samples after testing, such that subsequent XRM imaging could be carried out. A
388 standardized mapping grid ($n = 31$ positions) was superimposed on an image of the cartilage surface,
389 consisting of 14 and 17 measurement sites at the lateral and medial condyles, respectively (Figure
390 1A). The testing chamber was filled with PBS solution at room temperature, and the tissue was
391 allowed to equilibrate before testing. Automated indentation mapping under stress-relaxation was then
392 performed using the Mach-1TM v500css (Biomomentum Inc., Laval, QC) device, equipped with a
393 calibrated multiple-axis load cell (± 17 N, 3.5 mN force resolution) and associated software. With each
394 site, the contact coordinates (and contact coordinates at four adjacent places in a 0.075 mm scanning
395 grid) were measured using a contact criteria of 0.035 N. Surface orientation was then calculated using
396 the surrounding contact coordinates. Indentation was achieved by moving the indenter perpendicular
397 to the surface while moving the Mach-1 stages concurrently in all 3-axis. The spherical indenter (0.3
398 mm in diameter) was driven into the cartilage to a depth of 20 μ m over one second and held constant
399 for 90 seconds. For C57Bl6, a total of 3 indentation tests were performed per sample, approximately
400 45 minutes apart. Data reported consist of peak force and instantaneous modulus, as determined by
401 fitting the Hayes et al. (1972)³¹ elastic model to the load-displacement curves at 20% strain.
402 Assessment of how well the model fit the resulting curve per test site was done using root mean
403 square error (RSME). Since the analysis per position across specimens and genotypes reflect
404 deviations due to anatomical positioning, calculated parameters were compared between lateral (Lat)
405 and medial (Med) femoral condyles, as well as on four condylar sub-regions (Lateral/Anterior -
406 Lat/Ant, Lateral/Posterior – Lat/Post, Medial/Anterior - Med/Ant, Medial/Posterior - Med/Post), each
407 containing at least 5 positions of measurement (Figure 1A).

408 *Needle probing – thickness measurement*

409 After indentation mapping, the spherical indenter was replaced by a 30G x 1.4” hypodermic needle
410 (TSK Laboratory, Japan) adapted to the 1 mm spherical indenter (*Biomomentum Inc.*, Laval, Canada)
411 (Figure S2). Automated thickness mapping (Figure 2A) was done on the knee cartilage surface using
412 the needle probing technique³², and previous mapping grid distribution slightly shifted as appropriate
413 to account for the off-axis position of the needle’s bevel tip. The needle was driven vertically into the

414 cartilage surface at a constant speed until a 0.5 N stop criteria was reached in the subchondral bone.
415 The cartilage surface and cartilage/subchondral bone interface positions were identified in the load-
416 displacement curves (Figure 2B) generated at each measurement site using the automatic mode of
417 analysis²⁴. A 0.25 N/s loading limit was defined to identify the interface position. Manual correction
418 was employed when the algorithm failed to identify the inflection point²⁴. The cartilage thickness
419 reported corresponds to the vertical needle displacement from cartilage surface to subchondral bone
420 multiplied by the cosine of the surface orientation angle (Figure 2B) as determined during automated
421 indentation for each position. After testing, samples were preserved in 10% NBF solution for 24h and
422 stored in 70% ethanol for 24h to 48h before imaging.

423 *3D X-ray Microscopy (XRM) imaging*

424 3D XRM imaging was used for non-destructive assessment of cartilage morphology after
425 biomechanical testing. Fixed femurs were incubated for 16-18h in 1% phosphotungstic acid (PTA)
426 solution at room temperature for cartilage contrast enhancement before imaging¹⁵. Samples were
427 enclosed onto a custom specimen chamber, with 1% PTA in 70% ethanol added to the chamber's
428 bottom to minimize tissue dehydration. Zeiss Xradia 520 versa (*Carl Zeiss X-Ray Microscopy*,
429 Pleasanton, CA) scans of each distal femur were obtained following previously described protocol
430^{58,59}. In brief, high-resolution scans of 2001 axial slices were acquired at a 4.39 μm voxel size, with
431 low-energy (40 kVp voltage, 3 W power) x-rays.

432 *Imaging processing*

433 The contrast-enhanced cartilage surface was segmented by determining a threshold intensity, thereby
434 delineating the femur scan into cartilage and subchondral bone voxels. Needle probing left a physical
435 deformity in the articular cartilage of the right femurs visible on XRM imaging (Figure 2A), allowing
436 for all 31 regions of interest (ROIs) corresponding to needle probing to be manually landmarked.
437 Landmarks were placed manually using the two-dimensional axial, sagittal, and coronal planes
438 centered along the cartilage thickness and within each needle probing site. Cartilage segmentations
439 were corrected manually to ensure the cartilage mask encompassed resulting volume gaps at needle
440 probing positions. Then, the segmented cartilage was masked by a sphere of radius 75 μm placed on

441 each landmark, leaving a thin disk of cartilage. The thickness transform was computed for each disk⁶⁰
442 and the mean thickness values, taken as a statistic of the thickness distribution, were used to minimize
443 variability due to morphological changes in the cartilage caused by the mechanical testing. Image
444 processing was performed in SimpleITK⁶¹ (Insight Software Consortium, v1.2.4), and morphometry
445 was performed in Image Processing Language (IPL v5.42, SCANCO Medical AG, Brüttisellen,
446 Switzerland).

447 *Statistical analysis*

448 Analyses were performed in GraphPad Prism software (version 9), $\alpha = 0.05$ was considered statically
449 significant. Continuous parameters are reported as mean values and corresponding standard deviations
450 (SDs). Normality was assessed by Shapiro-Wilk normality test. C57Bl6 peak load and thickness data
451 was analysed by Student *t* test or ANOVA, with Bonferroni *post-hoc* comparisons. To assess
452 differences in biomechanical properties between genotypes non-parametric Mann-Whitney U test
453 with *Bonferroni-Dunn's* correction was used. To test reliability and absolute agreement between
454 repeated measurements, SPSS 27 (IBM, Chicago, IL) was used to obtain single-measurement, two-
455 way mixed effect intraclass correlation coefficient estimates and respective 95% limits of agreement
456^{62,63}. When assessing cartilage thickness measurements between needle probing and XRM imaging
457 techniques, *Pearson* correlation coefficient (R) was used for method-comparison and Bland-Altman
458 analysis to assess bias between methods.

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481

482 **Author's contribution**

483 A.O.M. was responsible for the study design, data collection, data analysis, data interpretation and
484 draft of the manuscript. B.B. carried out imaging processing and data analysis. W.B.E. contributed to
485 the study design. R.K. contributed to the study design and data interpretation. B.B., W.B.E. and R.K.
486 provided critical review, commentary and revisions to the manuscript.

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488 **References**

- 489 1. Vincent, T. L. & Wann, A. K. T. Mechanoadaptation: articular cartilage through thick and
490 thin. *J. Physiol.* **597**, 1271–1281 (2019).
- 491 2. Grodzinsky, A. J., Levenston, M. E., Jin, M. & Frank, E. H. Cartilage Tissue Remodeling in
492 Response to Mechanical Forces. *Annu. Rev. Biomed. Eng.* **2**, 691–713 (2000).
- 493 3. Stolz, M. *et al.* Early detection of aging cartilage and osteoarthritis in mice and patient samples
494 using atomic force microscopy. *Nat. Nanotechnol.* **4**, 186–192 (2009).
- 495 4. Setton, L. A., Mow, V. C., Müller, F. J., Pita, J. C. & Howell, D. S. Mechanical Properties of
496 Canine Articular Cartilage Are Significantly Altered Following Transection of the Anterior
497 Cruciate Ligament. *J. Orthop. Res.* **12**, 451–463 (1994).
- 498 5. Kleeman, R. U., Krockner, D., Cedrano, A., Tuischer, J. & Duda, G. N. Altered cartilage
499 mechanics and histology in knee osteoarthritis: Relation to clinical assessment (ICRS Grade).
500 *Osteoarthr. Cartil.* **13**, 958–963 (2005).
- 501 6. McCoy, A. M. Animal Models of Osteoarthritis. *Vet. Pathol.* **52**, 803–818 (2015).
- 502 7. Helminen, H. J., Säämänen, A. M., Salminen, H. & Hyttinen, M. M. Transgenic mouse models
503 for studying the role of cartilage macromolecules in osteoarthritis. *Rheumatology* **41**, 848–856
504 (2002).
- 505 8. Bedelbaeva, K. *et al.* Lack of p21 expression links cell cycle control and appendage
506 regeneration in mice. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 5845–50 (2010).
- 507 9. Kyostio-Moore, S. *et al.* STR/ort mice, a model for spontaneous osteoarthritis, exhibit elevated
508 levels of both local and systemic inflammatory markers. *Comp. Med.* **61**, 346–355 (2011).
- 509 10. Murphy, M. P. *et al.* Articular cartilage regeneration by activated skeletal stem cells. *Nat. Med.*
510 **26**, 1583–1592 (2020).
- 511 11. Christiansen, B. A. *et al.* Non-invasive mouse models of post-traumatic osteoarthritis.
512 *Osteoarthr. Cartil.* **23**, 1627–1638 (2015).
- 513 12. Malda, J. *et al.* Of Mice, Men and Elephants: The Relation between Articular Cartilage
514 Thickness and Body Mass. *PLoS One* **8**, 1–8 (2013).
- 515 13. Cao, L., Youn, I., Guilak, F. & Setton, L. A. Compressive properties of mouse articular

- 516 cartilage determined in a novel micro-indentation test method and biphasic finite element
517 model. *J. Biomech. Eng.* **128**, 766–771 (2006).
- 518 14. Berteau, J. P., Oyen, M. & Shefelbine, S. J. Permeability and shear modulus of articular
519 cartilage in growing mice. *Biomech. Model. Mechanobiol.* **15**, 205–212 (2016).
- 520 15. Das Neves Borges, P., Forte, A. E., Vincent, T. L., Dini, D. & Marenzana, M. Rapid,
521 automated imaging of mouse articular cartilage by microCT for early detection of
522 osteoarthritis and finite element modelling of joint mechanics. *Osteoarthr. Cartil.* **22**, 1419–
523 1428 (2014).
- 524 16. Athanasiou, K. A., Rosenwasser, M. P., Buckwalter, J. A., Malinin, T. I. & Mow, V. C.
525 Interspecies comparisons of in situ intrinsic mechanical properties of distal femoral cartilage.
526 *J. Orthop. Res.* **9**, 330–340 (1991).
- 527 17. Risch, M. *et al.* Mechanical, biochemical, and morphological topography of ovine knee
528 cartilage. *J. Orthop. Res.* **39**, 780–787 (2021).
- 529 18. Hoch, D. H., Grodzinsky, A. J., Koob, T. J., Albert, M. L. & Eyre, D. R. Early changes in
530 material properties of rabbit articular cartilage after meniscectomy. *J. Orthop. Res.* **1**, 4–12
531 (1983).
- 532 19. Lakin, B. A., Snyder, B. D. & Grinstaff, M. W. Assessing Cartilage Biomechanical Properties:
533 Techniques for Evaluating the Functional Performance of Cartilage in Health and Disease.
534 *Annu. Rev. Biomed. Eng.* **19**, 27–55 (2017).
- 535 20. Mansour, J. M. Biomechanics of cartilage. *Kinesiol. Mech. Pathomechanics Hum. Mov.*
536 *Second Ed.* 69–83 (2013).
- 537 21. Lu, X. L. & Mow, V. C. Biomechanics of Articular Cartilage and Determination of Material
538 Properties. *Med. Sci. Sport. Exerc.* **40**, 193–199 (2008).
- 539 22. Swann, A. C. & Seedhom, B. B. Improved Techniques for Measuring the Indentation and
540 Thickness of Articular Cartilage. *Proc. Inst. Mech. Eng. Part H J. Eng. Med.* **203**, 143–150
541 (1989).
- 542 23. Sim, S. *et al.* Electromechanical Probe and Automated Indentation Maps Are Sensitive
543 Techniques in Assessing Early Degenerated Human Articular Cartilage. *J. Orthop. Res.* 858–

- 544 867 (2017) doi:10.1002/jor.23330.
- 545 24. Biomomentum. *Mach-1 Analysis User Manual v6.4*. (2020).
- 546 25. Seidenstuecker, M. *et al.* Viscoelasticity and histology of the human cartilage in healthy and
547 degenerated conditions of the knee. *J. Orthop. Surg. Res.* **14**, 256 (2019).
- 548 26. Woods, P. S. *et al.* Automated Indentation Demonstrates Structural Stiffness of Femoral
549 Articular Cartilage and Temporomandibular Joint Mandibular Condylar Cartilage Is Altered in
550 FgF2KO Mice. *Cartilage* 194760352096256 (2020) doi:10.1177/1947603520962565.
- 551 27. Jia, H. *et al.* Loading-Induced Reduction in Sclerostin as a Mechanism of Subchondral Bone
552 Plate Sclerosis in Mouse Knee Joints During Late-Stage Osteoarthritis. **70**, 230–241 (2018).
- 553 28. Hu, X., Charles, J. P., Akay, T., Hutchinson, J. R. & Blemker, S. S. Are mice good models for
554 human neuromuscular disease? Comparing muscle excursions in walking between mice and
555 humans. *Skelet. Muscle* **7**, 1–15 (2017).
- 556 29. Oláh, T., Michaelis, J. C., Cai, X., Cucchiarini, M. & Madry, H. Comparative anatomy and
557 morphology of the knee in translational models for articular cartilage disorders. Part II: Small
558 animals. *Ann. Anat. - Anat. Anzeiger* **234**, (2021).
- 559 30. Li, G. *et al.* The cartilage thickness distribution in the tibiofemoral joint and its correlation
560 with cartilage-to-cartilage contact. *Clin. Biomech.* **20**, 736–744 (2005).
- 561 31. Hayes, W. C., Keer, L. M., Herrmann, G. & Mockros, L. F. A mathematical analysis for
562 indentation of articular cartilage. *J. Biomech.* **5**, 541–551 (1972).
- 563 32. Jurvelin, J. S., Räsänen, T., Kolmonens, P. & Lyyra, T. Comparison of optical, needle probe
564 and ultrasonic techniques for the measurement of articular cartilage thickness. *J. Biomech.* **28**,
565 231–235 (1995).
- 566 33. Rhee, D. K. *et al.* The secreted glycoprotein lubricin protects cartilage surfaces and inhibits
567 synovial cell overgrowth. *J. Clin. Invest.* **115**, 622–31 (2005).
- 568 34. Michalak, G. J., Walker, R. & Boyd, S. K. Concurrent Assessment of Cartilage Morphology
569 and Bone Microarchitecture in the Human Knee Using Contrast-Enhanced HR-pQCT
570 Imaging. *J. Clin. Densitom.* **22**, 74–85 (2019).
- 571 35. Armstrong, C. G. An analysis of the stresses in a thin layer of articular cartilage in a synovial

- 572 joint. *Eng. Med.* 55–61 (1986).
- 573 36. Askary, A. *et al.* Ancient origin of lubricated joints in bony vertebrates. *Elife* **5**, 1–13 (2016).
- 574 37. Ikegawa, S., Sano, M., Koshizuka, Y. & Nakamura, Y. Isolation, characterization and mapping
575 of the mouse and human PRG4 (proteoglycan 4) genes. *Cytogenet. Cell Genet.* **90**, 291–7
576 (2000).
- 577 38. Jay, G. D. *et al.* Association between friction and wear in diarthrodial joints lacking lubricin.
578 *Arthritis Rheum.* **56**, 3662–3669 (2007).
- 579 39. Coles, J. M. *et al.* Loss of cartilage structure, stiffness, and frictional properties in mice lacking
580 PRG4. *Arthritis Rheum.* **62**, 1666–1674 (2010).
- 581 40. Marcelino, J. *et al.* CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-
582 arthropathy-coxa vara-pericarditis syndrome. *Nat. Genet.* **23**, 319–22 (1999).
- 583 41. Karamchedu, N. P. *et al.* Superficial zone cellularity is deficient in mice lacking lubricin: a
584 stereoscopic analysis. *Arthritis Res. Ther.* **18**, 64 (2016).
- 585 42. Lavoie, J. *et al.* Mapping Articular Cartilage Biomechanical Properties of Normal and
586 Osteoarthritis Mice Using In. in *Osteoarthritis Cartilage* 23:A524 (2015).
587 doi:10.1016/j.joca.2015.02.463.
- 588 43. Korhonen, R. . *et al.* Importance of the superficial tissue layer for the indentation stiffness of
589 articular cartilage. *Med. Eng. Phys.* **24**, 99–108 (2002).
- 590 44. Froimson, M. I., Ratcliffe, A., Gardner, T. R. & Mow, V. C. Differences in patellofemoral
591 joint cartilage material properties and their significance to the etiology of cartilage surface
592 fibrillation. *Osteoarthr. Cartil.* **5**, 377–386 (1997).
- 593 45. Samosky, J. T. *et al.* Spatially-localized correlation of dGEMRIC-measured GAG distribution
594 and mechanical stiffness in the human tibial plateau. *J. Orthop. Res.* **23**, 93–101 (2005).
- 595 46. Moshtagh, P. R., Pouran, B., Korthagen, N. M., Zadpoor, A. A. & Weinans, H. Guidelines for
596 an optimized indentation protocol for measurement of cartilage stiffness : The effects of spatial
597 variation and indentation parameters. *J. Biomech.* **49**, 3602–3607 (2016).
- 598 47. Poulet, B., Westerhof, T. A. T., Hamilton, R. W., Shefelbine, S. J. & Pitsillides, A. A.
599 Spontaneous osteoarthritis in Str/ort mice is unlikely due to greater vulnerability to mechanical

- 600 trauma. *Osteoarthr. Cartil.* **21**, 756–765 (2013).
- 601 48. Kotwal, N., Li, J., Sandy, J., Plaas, A. & Sumner, D. R. Initial application of EPIC- μ CT to
602 assess mouse articular cartilage morphology and composition: Effects of aging and treadmill
603 running. *Osteoarthr. Cartil.* **20**, 887–895 (2012).
- 604 49. Julkunen, P., Korhonen, R. K., Herzog, W. & Jurvelin, J. S. Uncertainties in indentation
605 testing of articular cartilage: A fibril-reinforced poroviscoelastic study. *Med. Eng. Phys.* **30**,
606 506–515 (2008).
- 607 50. Simha, N. K., Jin, H., Hall, M. L., Chiravambath, S. & Lewis, J. L. Effect of indenter size on
608 elastic modulus of cartilage measured by indentation. *J. Biomech. Eng.* **129**, 767–775 (2007).
- 609 51. Drewniak, E. I. *et al.* Cyclic loading increases friction and changes cartilage surface integrity
610 in lubricin-mutant mouse knees. *Arthritis Rheum.* **64**, 465–473 (2012).
- 611 52. Laasanen, M. S. *et al.* Biomechanical properties of knee articular cartilage. *Biorheology* **40**,
612 133–40 (2003).
- 613 53. Rahmati, M., Nalesso, G., Mobasher, A. & Mozafari, M. Aging and osteoarthritis: Central
614 role of the extracellular matrix. *Ageing Res. Rev.* **40**, 20–30 (2017).
- 615 54. Julkunen, P. *et al.* Biomechanical, biochemical and structural correlations in immature and
616 mature rabbit articular cartilage. *Osteoarthr. Cartil.* **17**, 1628–1638 (2009).
- 617 55. Mow, V. C., Kuei, S. C., Lai, W. M. & Armstrong, C. G. Biphasic creep and stress relaxation
618 of articular cartilage in compression: Theory and experiments. *J. Biomech. Eng.* **102**, 73–84
619 (1980).
- 620 56. Julkunen, P., Harjula, T., Marjanen, J., Helminen, H. J. & Jurvelin, J. S. Comparison of single-
621 phase isotropic elastic and fibril-reinforced poroelastic models for indentation of rabbit
622 articular cartilage. *J. Biomech.* **42**, 652–656 (2009).
- 623 57. Abubacker, S. *et al.* Absence of Proteoglycan 4 (Prg4) Leads to Increased Subchondral Bone
624 Porosity Which Can Be Mitigated Through Intra - Articular Injection of PRG4. *J. Orthop. Res.*
625 **37**, (2019).
- 626 58. Jablonski, C. L., Besler, B. A., Ali, J. & Krawetz, R. J. p21^{-/-} Mice Exhibit Spontaneous
627 Articular Cartilage Regeneration Post-Injury. *Cartilage* 1947603519876348 (2019)

628

629 59. Richard, D. *et al.* Evolutionary Selection and Constraint on Human Knee Chondrocyte

630 Regulation Impacts Osteoarthritis Risk. *Cell* **181**, 362-381.e28 (2020).

631 60. Hildebrand, T. & Ruegsegger, P. A new method for the model-independent assessment of

632 thickness in three-dimensional images. *J. Microsc.* **185**, 67–75 (1997).

633 61. Lowekamp, B. C., Chen, D. T., Ibáñez, L. & Blezek, D. The design of simpleITK. *Front.*

634 *Neuroinform.* **7**, 1–14 (2013).

635 62. Koo, T. K. & Li, M. Y. A Guideline of Selecting and Reporting Intraclass Correlation

636 Coefficients for Reliability Research. *J. Chiropr. Med.* (2015) doi:10.1016/j.jcm.2016.02.012.

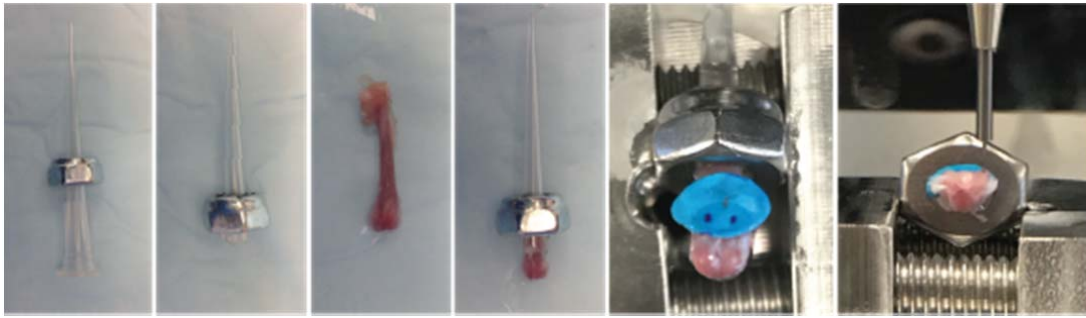
637 63. Müller, R. & Büttner, P. A critical discussion of intraclass correlation coefficients. *Stat. Med.*

638 **13**, 2465–2476 (1994).

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1 Supplementary Material



2

3 **Figure S1.** Specimen preparation and assemble to sample holder for automated indentation mapping
4 using custom setup, allowing for repositioning of the sample and non-destructive retrieval for 3D
5 XRM imaging.

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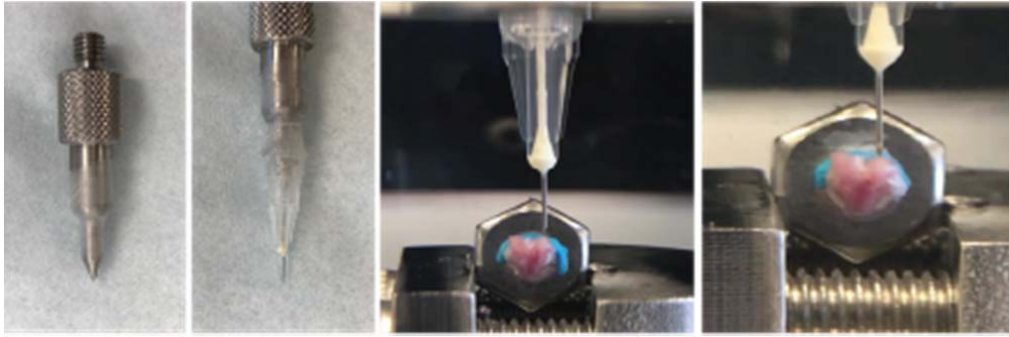
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1 **Table S1.** Mean peak force (\bar{X} : N) and coefficient of variation (CV: %) from triplicate measurements for each of the 31 positions (L1-M17) of
 2 assessment over mice femoral condyles.

	S01		S02		S03		S04		S05		S06		S07		S08		S09		S10	
	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV	\bar{X}	CV
L1	0.08	2.9	0.08	3.0	0.10	4.1	0.13	1.8	0.09	12.1	0.10	2.7	0.10	4.9	0.09	3.3	0.07	1.5	0.11	0.2
L2	0.11	1.3	0.09	2.5	0.10	2.3	0.14	6.1	0.12	8.2	0.11	4.5	0.13	4.9	0.11	6.1	0.07	13.7	0.11	5.7
L3	0.07	4.9	0.08	3.6	0.08	5.0	0.12	2.5	0.11	4.8	0.12	1.5	0.09	3.8	0.08	4.9	0.07	8.5	0.08	8.0
L4	0.10	7.2	0.07	2.8	0.10	1.3	0.11	1.6	0.08	3.6	0.11	4.7	0.13	2.2	0.09	3.0	0.06	4.8	0.09	5.5
L5	0.16	0.7	0.15	5.0	0.21	9.0	0.14	7.7	0.12	7.8	0.04	10.0	0.17	2.4	0.17	4.2	0.11	6.0	0.19	6.3
L6	0.06	9.3	0.07	7.4	0.08	13.4	0.12	4.1	0.09	5.7	0.08	2.9	0.06	9.0	0.06	6.9	0.08	6.0	0.07	5.8
L7	0.06	4.7	0.07	6.2	0.08	6.9	0.10	3.1	0.07	12.3	0.07	3.8	0.09	8.0	0.08	4.9	0.07	3.8	0.07	7.5
L8	0.16	2.2	0.09	15.9	0.19	3.2	0.14	4.9	0.11	7.9	-	-	0.20	5.8	0.17	3.1	0.12	3.5	0.12	6.5
L9	0.05	10.3	0.07	5.4	0.08	2.4	0.09	1.5	0.03	20.2	0.07	0.3	0.05	6.4	0.07	2.9	0.09	0.9	0.08	2.5
L10	0.04	4.2	0.07	6.8	0.07	3.1	0.08	4.5	-	-	0.08	3.3	0.07	3.9	0.07	5.5	0.07	1.1	0.08	3.2
L11	0.10	5.7	0.08	6.1	0.15	2.4	0.11	9.1	0.13	5.1	0.14	6.8	0.16	5.9	0.14	3.7	0.04	9.0	0.11	1.3
L12	0.05	0.9	0.08	4.6	0.07	9.4	0.07	3.6	-	-	0.09	3.1	0.06	6.2	0.06	8.8	0.11	3.6	0.10	3.3
L13	0.05	3.0	0.07	3.6	0.07	6.4	0.07	5.8	-	-	0.10	2.3	0.07	2.9	0.08	4.9	0.09	0.9	0.09	6.0
L14	0.08	5.0	0.08	5.7	0.13	4.9	0.10	4.9	0.04	19.0	0.14	1.1	0.14	3.7	0.16	1.7	0.09	12.0	0.12	1.4
M1	0.29	3.7	0.18	6.9	0.23	4.1	0.23	1.9	0.21	5.5	0.26	1.4	0.22	6.7	0.20	2.2	0.20	1.7	0.22	1.9
M2	-	-	0.25	4.6	0.25	3.2	0.26	1.0	0.28	2.3	0.29	2.1	0.27	1.5	0.24	6.3	0.20	5.4	0.29	0.9
M3	0.23	3.1	0.17	3.8	0.24	0.7	0.24	1.4	0.24	2.8	0.24	1.8	0.23	3.2	0.19	2.7	0.22	6.7	0.25	3.4
M4	0.36	5.0	0.30	6.5	0.28	0.6	0.30	2.6	0.29	3.1	0.31	2.7	0.25	5.8	0.26	3.1	0.19	4.5	0.30	2.7
M5	-	-	0.38	10.0	0.30	3.9	0.30	1.9	0.36	1.1	0.26	0.7	0.30	2.4	0.25	5.5	0.18	4.3	0.15	5.1
M6	0.19	1.7	0.17	6.3	0.25	3.4	0.23	3.8	0.19	5.6	0.26	2.3	0.21	0.7	0.11	10.9	0.16	3.9	0.20	15.1
M7	0.30	3.3	0.30	4.2	0.34	5.9	0.30	1.9	0.37	7.0	0.31	1.7	0.26	2.2	0.28	1.1	0.16	1.4	0.30	6.9
M8	0.37	3.3	0.36	6.2	0.33	2.0	0.28	5.1	0.35	5.6	0.23	2.0	0.23	6.0	0.17	3.6	0.13	1.7	0.14	4.2
M9	0.20	3.8	0.16	2.5	0.27	5.6	0.21	2.8	0.18	19.6	0.25	1.2	0.20	4.2	0.16	0.9	0.15	3.4	0.24	18.6
M10	0.26	2.8	0.30	2.0	0.33	7.1	0.28	1.2	0.39	2.2	0.25	2.4	0.19	2.5	0.25	2.0	0.16	2.4	0.24	2.7
M11	0.28	2.0	0.30	2.8	0.30	6.0	0.21	1.6	0.31	3.8	0.22	2.0	0.16	1.1	0.14	0.7	0.13	7.8	0.11	7.1
M12	0.16	1.6	0.19	5.9	0.25	1.6	0.18	0.6	0.17	2.3	0.18	6.4	0.15	6.9	0.18	5.7	0.09	7.7	-	-
M13	0.22	4.3	0.26	4.6	0.29	3.0	0.20	2.3	0.32	4.0	0.20	2.6	0.15	1.2	0.21	3.6	0.14	0.1	0.20	2.9
M14	0.19	1.6	0.27	1.2	0.25	4.2	0.17	2.6	0.27	1.2	0.20	0.8	0.14	2.9	0.13	4.1	0.12	3.2	0.12	1.8
M15	0.16	3.7	0.16	0.5	0.18	1.6	0.14	1.6	0.18	0.7	0.13	1.9	0.13	1.7	0.14	1.1	0.14	1.6	0.21	9.2
M16	0.14	1.3	0.19	1.3	0.20	0.9	0.14	2.1	0.22	1.4	0.16	2.7	0.12	0.8	0.15	1.2	0.13	1.4	0.16	1.7
M17	0.14	2.2	0.21	1.1	0.17	3.4	0.15	2.6	0.19	3.1	0.15	5.5	0.13	2.1	0.12	3.0	0.13	1.4	0.13	2.2

1



2

3 **Figure S2.** Setup for needle probing thickness measurement, using a 30G x 1.4" hypodermic needle
4 (TSK Laboratory, Japan) adapted to the 1 mm spherical indenter (*Biomomentum Inc.*, Laval, QC).

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1 **Table S2.** Mean and standard deviation (SD) values for cartilage thickness as determined by XRM
2 imaging for distal femur samples of murine articular cartilage ($n = 10$). Mean values compared between
3 condyles (Lateral/Medial; unpaired, Student's t test) and within sub-regions of condyles (Lat/Ant,
4 Lat/Post, Med/Ant, Med/Post; *one-way* ANOVA). p -value reported.

5

Cartilage Thickness [N]				
Condyle	Mean (SD)	Condyle	Mean (SD)	Lat - Med
Lateral	64.4 (8.4)	Medial	46.5 (4.4)	$p < 0.0001$
<i>Lat/Ant</i>	59.0 (8.2)	<i>Med/Ant</i>	42.9 (5.1)	$p < 0.0001$
<i>Lat/Post</i>	67.4 (9.7)	<i>Med/Post</i>	49.5 (4.3)	$p < 0.0001$
Ant - Post	<i>ns</i>	Ant - Post	<i>ns</i>	-

6 *ns*, no significant difference; -, comparison not applicable

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