

1 **Mechanical Heterogeneity in the Bone Microenvironment as**
2 **Characterised by Atomic Force Microscopy**

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4 **KEYWORDS.** Force map, viscoelasticity, AFM, bone microenvironment.

5 **RUNNING TITLE.** Mechanical heterogeneity of bone.

6

7 ABSTRACT. Bones are structurally heterogeneous organs with diverse functions that
8 undergo mechanical stimuli across multiple length scales. Mechanical characterisation of the
9 bone microenvironment is important for understanding how bones function in health and
10 disease. Here we describe the mechanical architecture of cortical bone, the growth plate,
11 metaphysis and marrow in fresh murine bones, probed using atomic force microscopy in
12 physiological buffer. Both elastic and viscoelastic properties are found to be highly
13 heterogeneous with moduli ranging over 3 to 5 orders of magnitude, both within and across
14 regions. All regions include extremely soft areas, with moduli of a few Pascal and viscosities
15 as low as tens Pa·s. Aging impacts the viscoelasticity of the bone marrow strongly but has
16 limited effect on the other regions studied. Our approach provides the opportunity to explore
17 the mechanical properties of complex tissues at the length scale relevant to cellular processes
18 and how these impact on aging and disease.

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20

21 SIGNIFICANCE. The mechanical properties of biological materials at cellular scale are
22 involved in guiding cell fate. However, there is a critical gap in our knowledge of such
23 properties in complex tissues. The physiochemical environment surrounding the cells in *in-*
24 *vitro* studies differs significantly from that found *in vivo*. Existing mechanical characterisation
25 of real tissues are largely limited to properties at larger scales, structurally simple (*e.g.*
26 epithelial monolayers) or non-intact (*e.g.* through fixation) tissues. In this paper, we address
27 this critical gap and present the micro-mechanical properties of the relatively intact bone
28 microenvironment. The measured Young's moduli and viscosity provide a sound guidance in
29 bioengineering designs. The striking heterogeneity at supracellular scale reveals the potential
30 contribution of the mechanical properties in guiding cell behaviour.

31

32 INTRODUCTION

33 It is increasingly clear that mechanical properties and forces play an essential role in
34 controlling many aspects of cell biology of tissues and organs including growth, migration,
35 differentiation, homeostasis and communication (1-3). How these fundamental cellular
36 processes are influenced by such forces requires an understanding of the mechanical properties
37 at a subcellular scale but in the context of the tissue, organ, or even the whole organism.
38 Obtaining this information is particularly challenging as most traditional methods for tissue
39 processing (*e.g.* sectioning) or fixation radically change the mechanical properties of at least
40 part of the structure (*e.g.* by freezing and thawing). Similarly, obtaining the relevant spatial and
41 temporal resolution with sufficient force sensitivity to systematically determine the relevant
42 mechanical variation at the different cell and tissue scales requires new specialised approaches.
43 Here we present an atomic force microscopy (AFM) based approach for tackling these issues
44 allowing the study of bones, a unique and mechanically complex organ.

45 Bone tissue consists predominantly of two types of bone, cortical and trabecular. The
46 cortical bone (also known as dense bone) is structurally compact and bears the load of the
47 body's weight. The trabecular bone (also known as cancellous bone or spongy bone) has a
48 more loosely organized structure and highly metabolic activity (4). Both types of bone contain
49 inorganic (bone mineral and water) and organic components (bone cells and matrix) that form
50 complex, interconnected structures (5). In addition to being central to mechanical support, bone
51 contains bone marrow which plays a significant role in mammalian physiology through
52 hematopoiesis and by regulating immune and stromal cell trafficking (6). Bone tissues undergo
53 continuous dynamic remodelling in response to changes in mechanical loading and during the
54 homeostatic repair of microdamage occurring through normal 'wear and tear'. The balance
55 between bone deposition and resorption is orchestrated by bone cells, called osteoblasts and
56 osteoclasts, respectively (4, 5). Osteoblasts are derived from mesenchymal stem cells (MSCs)
57 and osteoclasts are derived from hematopoietic stem cells (HSCs), both of which are resident
58 in bone marrow (6). In addition there are a multitude of progenitor cells, immune cells, stromal
59 cells and adipocytes involved in maintaining the homeostasis of bone.

60 The mechanical properties of bone are fundamental to the function of the skeleton (7) and
61 changes in bone loading can influence bone turnover, a process whereby bone formation and
62 absorption is in physiological balance, thereby preventing the net gain or loss of bone tissue
63 (8). Investigations of bone mechanics are necessary to fully understand how the mechanical
64 properties of the bone microenvironment (BMeV) influence the biological functions such as
65 bone turnover, and have been well developed in recent decades. *In-vitro* models mimicking the

66 BMEv are commonly used to study the role of mechanical properties in bone function and
67 related diseases, such as cancer-induced bone metastasis (9, 10). However, these *in vitro*
68 models do not fully recapitulate the complexity of the *in vivo* BMEv and hence lack
69 fundamental components of the underlying biology (10).

70 Numerous studies using a variety of techniques have characterised the mechanical
71 properties of bone at multiple length scales. Elastic and viscoelastic studies on both cortical
72 and trabecular bones generally give Young's/shear moduli in the GPa range, though the results
73 vary due to different experimental conditions (11-22). The Young's/shear modulus of
74 trabecular bone is slightly lower than that of the cortical bone and occasionally as low as 10s -
75 100s MPa (23, 24). The growth plate is more compliant than both cortical and trabecular bone
76 tissues, with the Young's modulus ranging from 300 kPa to 50 MPa (25-28). In contrast, most
77 studies on bone marrow have considered it as a purely viscous tissue (29), reporting viscosity
78 values ranging from below 1 Pa·s to 100 Pa·s (29-31).

79 The structural complexity of bone samples can hinder accurate mechanical monitoring. A
80 number of approaches have been taken to overcome this, through exposing bone to freezing
81 (32, 33), dehydration (34), jet washing (35), polishing (12, 19, 36) and homogenising (37, 38)
82 in addition to sectioning or fracturing. Although differences in the mechanical properties of the
83 BMEvs may be extracted from samples prepared via these methods, such treatments
84 substantially modify the surface structures (35) and consequently cause a shift in the measured
85 mechanical properties (14, 19). However, the ability to quantify the mechanical properties of
86 tissues at different length scales is critical in building models for theoretical simulations or in
87 tissue engineering to replicate key features of such tissues. A recent study on intact bone
88 marrow demonstrated both the feasibility and advantages of mechanical measurements using
89 minimally deconstructed samples (39). In contrast to other studies, this work identified that
90 the bone marrow is predominantly elastic, and suggested that the extracellular matrix
91 contribution to the mechanical properties from fresh samples is essential to explain the
92 observed heterogeneity. The accessible scale in this study was not sufficient to reveal the
93 mechanical heterogeneity of bone, and suggested that techniques suitable for smaller scale
94 characterisation are necessary.

95 Several methods to evaluate the local (*i.e.* cellular or subcellular) mechanical properties of
96 biological tissues have emerged over the last decade, including optical (40, 41) and acoustic
97 (42) techniques. However, the structural and mechanical heterogeneity of the BMEv makes the
98 application of these approaches particularly challenging. AFM enables characterisation at

99 length scales ranging from nm to tens of μm (25, 43-45), which is of particular relevance for
100 understanding the biological mechanisms arising at the molecular and cellular level.

101 Here we focused on measuring the subcellular mechanical properties of the non-load bearing
102 components of untreated bone on the internal surfaces (except for longitudinal splitting, which
103 is necessary to access the tissue). Mechanical measurements were made using colloidal probe
104 AFM, as opposed to nanoindentation (19, 34) or AFM with conventional sharp probes (13, 14,
105 17, 25), due to the extreme softness of non-mineralised components in the BMev. Point
106 measurements were performed randomly over a macro-scale ($> 100 \mu\text{m}$) of bone surfaces. Both
107 the elastic and viscoelastic properties of different regions of the BMev were quantified by
108 fitting data obtained from force-distance and creep (*i.e.* strain relaxation) curves acquired with
109 the AFM with appropriate mechanical models. Bones from young (6-8 weeks) and mature (11-
110 13 weeks) mice were compared to determine any mechanical changes due to age-associated
111 skeletal maturation. Finally, AFM force mapping was used to collect images (max. size $100 \times$
112 $100 \mu\text{m}^2$) that represented the heterogeneity of the BMev morphology and mechanics at a
113 supracellular scale (from $5 \mu\text{m}$ to $100 \mu\text{m}$). The implications of our findings for the potential
114 correlation with biological functions in bones are also discussed.

115 **MATERIALS AND METHODS**

116 **Animals**

117 All experiments involving animals were approved by the University of Sheffield project
118 Applications and Amendments (Ethics) Committee and conducted in accordance with UK
119 Home Office Regulations, under project license number 70/8964 (NJB). Mice were housed in
120 a controlled environment with 12 hours light/dark cycle, at 22°C . Mice had access to food and
121 water *ad libitum*, Teklad Diet 2918 (Envigo, UK). A total of 21 mice were used in these studies.

122 **Bone sample preparation**

123 Femurs from both young (6 to 8 weeks old, 9 bones from 8 mice) and mature (11 to 13
124 weeks old, 24 bones from 13 mice) mice were used in this study unless otherwise stated. The
125 mice were culled by cervical dislocation and the hind limbs detached from the pelvis at the
126 femoral head. Muscle tissue connected to the bone was removed and the femur separated from
127 the tibia at the knee joint. The dissected femurs were placed in phosphate buffered saline (PBS)
128 (Lonza, US) at 4°C and split prior to measurement, which was within 36h of culling. A custom
129 designed tool with a razor blade (Fig. S1a) was used to longitudinally fracture the bone, which
130 was immobilised on a platform using a two component dental impression putty (Provil Novo

131 Light, Kulzer, UK). The split bone was then immersed in PBS and immobilised in a petri-dish
132 (TPP, Switzerland) with the same dental impression material. Care was taken to maintain
133 hydration of the exposed bone surface during the entire process.

134 **Colloidal probe cantilever preparation**

135 Rectangular Si₃N₄ cantilevers with a nominal spring constant of 0.02 N/m (MLCT cantilever
136 B, Bruker, USA) were used for all AFM measurements. A polystyrene microsphere with 25
137 µm diameter (Sigma Aldrich, USA) was attached to the cantilever using a UV-curing adhesive
138 (NOA 81, Norland Inc., USA), using an AFM (Nanowizard III, JPK, Germany) and combined
139 with an inverted optical microscope (Eclipse Ti, Nikon, Japan) as a micro-manipulator. Before
140 attachment of the colloidal probe, the spring constant of each cantilevers was determined in air
141 using the thermal noise method (46). To prevent strong non-specific binding between the probe
142 and sample surface, the cantilever with attached colloidal probe and cantilever holder were
143 passivated by 20 min immersion in 10 mg/mL bovine serum albumin (BSA), (Sigma Aldrich,
144 USA). Excess BSA was removed by washing with 5 × 1 mL PBS.

145 **AFM force spectroscopy and mapping**

146 AFM measurements were all performed on a Nanowizard III system (JPK, Germany) using
147 a heated sample stage (35 to 37 °C). The cantilever deflection sensitivity was calibrated prior
148 to each measurement, from the hard contact regime of force-distance curves (average of 10
149 repeated curves) obtained from a clean petri-dish containing PBS. Prepared bone samples in
150 PBS (Fig. 1a) were then mounted on the sample stage and probed after the temperature of the
151 liquid had stabilised. Force-distance curves (Fig. 1b) were acquired at randomly selected
152 positions from different regions of interest towards the distal end of each femur, as shown in
153 Fig. 1a. For the majority of samples, 5 to 10 positions within each BMEv region were measured
154 from each femur, but on occasion the number varied beyond this range depending on sample
155 quality (*e.g.* surface roughness). The approach speed was 1 µm/s unless otherwise stated.
156 Subsequently, curves with 3 s dwell under constant force (0.5 nN), *i.e.* creep curves, were also
157 acquired from the same position (Fig. 1d). For both force-distance and creep curves, a
158 minimum of 3 measurements were taken at each location.

159 A two-dimensional array of force-distance curves (*i.e.* an AFM map, one force curve per
160 pixel) was then produced at randomly selected areas in the different bone regions, with 30 µm/s
161 approach speed. The faster approach speed for force maps was chosen to minimise data
162 collection time and consequent degradation of the sample. Before obtaining maps for analysis

163 (typically 1-1.6 μm per pixel), low resolution “survey” maps (10 to 15 μm per pixel) were
164 collected to ensure that the major features in the selected region could be mapped without
165 exceeding the 15 μm Z range of the AFM.

166 All AFM measurements (force-distance curves, creep curves and force maps) on a single
167 bone sample could typically be collected in 2 to 6 h. The measured bone surfaces were stable
168 during this time (*i.e.* there was no significant dissociation or significantly different features in
169 force curves observed). Reference force-distance curves were acquired on a petri-dish at the
170 end of the measurements to check for colloidal probe contamination, with no significant
171 contaminants (expected to manifest as strong adhesive features in the retract segment of the
172 curves) found.

173 Raw data were exported as .txt format using JPK Data Processing and imported into
174 customised algorithms in MATLAB (generated in-house by XC) for all subsequent analyses.
175 Data processing scripts are available upon request.

176 **Processing and analysis of force-distance curves**

177 Raw force-distance curves were first converted into force (F) - indentation (δ) curves (Fig.
178 1b) by applying baseline correction, determining the contact point and subtracting the
179 cantilever deflection from the Z displacement in the contact regime (see Supporting Methods
180 and Fig. S1b-e). Curves were fitted to a Hertz-Sneddon model (47) using a custom Matlab
181 routine, with the Young's modulus (E_{H-S}) and a virtual contact point as free fitting parameters
182 (see Supporting Methods). The E_{H-S} at any measured position was determined by the mean
183 value resulting from fitting to all repeated F - δ curves at the same position, unless otherwise
184 specified. The quality of F - δ curves (*i.e.* tilt in baselines) and the fitting quality variation only
185 had a minor impact on the results, as discussed in the Supporting Methods. It is important to
186 note that the analysis described in this section implicitly assumes that the mechanical response
187 of the BMeV is purely elastic.

188 **Processing and analysis of creep curves**

189 Raw force (F) – time (t) curves from the dwell segment of force-distance curves were
190 checked and discarded if the force could not be maintained constant by the feedback loop. The
191 Z displacement value at the start of the dwell segment was subtracted from the displacement-
192 time curve to yield an indentation variation ($\Delta\delta$) - time (t) curve (creep curve). This was fitted
193 to both a Standard Linear Solid (SLS) model (Fig. S1f) and a Kelvin-Voigt (K-V) model (Fig.
194 S1g), based on the theory developed by Cheng *et. al.* (48) (see Supporting Methods). The

195 instantaneous elastic modulus (E_1), delayed elastic modulus (E_2) and viscosity (η) were free
196 fitting parameters using the SLS model, and the Young's modulus (E_{K-V}) and viscosity (η) using
197 the K-V model. Results were obtained from the mean value of repeated measurements at each
198 position and those with low fit quality ($R^2 < 0.9$) were discarded if not specified.

199 **Processing of AFM map data**

200 Three types of maps that graphically represented topography and mechanical properties of
201 the scanned areas were generated from the AFM maps. Firstly, the trigger point map was
202 constructed from a vertical position where the interaction force between the probe and the
203 sample reached the trigger value. This represents the topography of the surface, including the
204 indentation of the probe into the sample. Secondly, the contact point map was constructed from
205 the vertical position where the probe was deemed to have made initial contact with the sample
206 surface, determined from the contact point found for every force curve in the map using the
207 triangle thresholding method, as shown in Fig. S1b. This represents an approximation to the
208 topography at zero load and indentation. Thirdly, the elastic map was constructed from the
209 Young's modulus E_{H-S} extracted from all force curves in the map using the Hertz-Sneddon
210 model with a fitted contact point. For both the contact point map and the mechanical map,
211 pixels with curves that could not be properly fitted due to not having a clear non-contact
212 baseline, contact point and monotonic contact regime were considered as "missing data" and
213 coloured black in the figures.

214 **Statistical analysis**

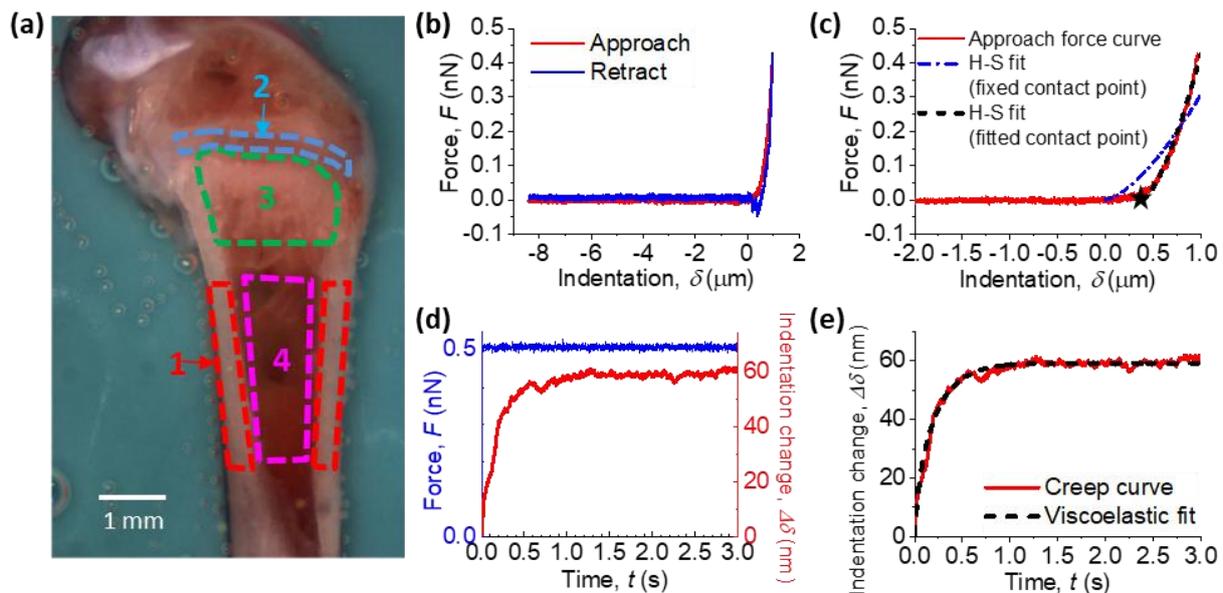
215 Statistical analyses were performed using OriginPro software. A normality test was applied
216 to all distributions prior to any further analysis. Data were then analysed by the Kruskal-Wallis
217 test for comparison between different groups. A statistically significant difference was defined
218 as $p < 0.05$.

219 **RESULTS AND DISCUSSION**

220 Four regions of interest (Fig. 1a) within the BMeV were selected on the surface of split bones
221 for AFM characterisation, due to their diverse composition and function: (i) cortical bone, (ii)
222 growth plate, (iii) metaphysis and (iv) bone marrow in the diaphysis. The four regions of
223 interest are shown by coloured outlines in the bright field image (Fig. 1a). In long bones such
224 as the femur, the growth plate (also known as the epiphyseal plate or physis) is found at each
225 end. This is where longitudinal bone growth takes place and is composed of a large number of
226 chondrocytes and proteinaceous matrix. The narrow portion located just below the growth plate,

227 the metaphysis (4), was also examined. Previous preclinical *in vivo* studies from our group
228 have identified this as the predominant region where circulating metastatic cancer cells home
229 to and colonise in bone (49, 50). In the metaphysis, measurements were made on either the
230 trabecular bone tissue or adjacent soft tissue in the surrounding marrow. In the diaphysis, we
231 measured both the cortical bone, which comprises the outer shaft of the long bone, and the
232 central bone marrow cavity.

233 Only bones with an acceptably smooth surface topography after splitting were used for AFM
234 measurements. However, even on these specimens, the height range of the surface profile could
235 still vary by several hundred micrometres across the whole bone. Tilt or curvature of the split
236 surface occasionally caused fouling of the cantilever, or cantilever holder on parts of the bone
237 that were higher than the region of interest. In these cases it was not possible to measure all
238 regions of interest on the same specimen.



239

240 **Figure 1.** Methods of measuring the mechanical properties on internal bone surfaces (coloured
241 version available online). (a) Bright field image of a prepared bone surface for AFM
242 characterisation. Four regions of interest are indicated by coloured dashed lines: (1) cortical
243 bone (red), (2) the growth plate (blue), (3) the metaphysis (green) and (4) bone marrow in
244 diaphysis (magenta). (b) Representative force (F) vs. indentation (δ) curve obtained during
245 AFM probe approach (red) and retract (blue) taken on the bone surface. The contact position
246 ($\delta = 0$) was determined from the approach curve using triangle thresholding method described
247 in the Supporting Material. (c) Examples of Hertz-Sneddon (H-S) model fits to the approach
248 segment of a force-indentation curve (red) H-S fit either using a fixed contact point (blue dash-
249 dotted line) determined using the triangle thresholding method (described in the Supporting
250 Material), or with the contact point as a free fitting parameter (black dashed line). The star (★)
251 shows the position of the contact point determined by the fit. (d) Representative creep curve
252 ($\Delta\delta$ vs. t) obtained from the dwell segment of a force curve taken on the bone surface. The
253 applied force (blue) was held constant for 3 s while the indentation depth increased (red) due

254 to the material being viscoelastic rather than purely elastic. (e) Example of the viscoelastic
255 model fit (*black dashed line*) on a creep curve (*red line*).

256

257 **Point force-indentation measurements reveal mechanical heterogeneity spanning**
258 **orders of magnitude over each of the different regions of the bone microenvironment,**
259 **with all regions containing very soft (1-10 Pa) areas**

260 To probe the overall mechanical profile for each bone region, we first applied point force
261 (F) -indentation (δ) measurements at randomly selected positions within the specified region.
262 A representative F - δ curve acquired on the surface of a split bone is shown in Fig. 1b (see
263 Supporting Material and Fig. S1b-e for details of conversion of raw deflection-distance curves
264 to F - δ curves). In the majority of curves, features associated with plastic deformation (yield
265 points, plateaus etc.) were not observed, indicating that elastic or viscoelastic analyses were
266 appropriate. For simplicity, the Young's modulus E_{H-S} , together with a virtual contact point,
267 was obtained from Hertz-Sneddon model fits to the F - δ curves (Fig. 1c) assuming a purely
268 elastic response. Data include bones from both young and mature mice.

269 Histograms of E_{H-S} for each bone region are shown in Fig. 2 (note the logarithmic scale of
270 the horizontal axes), and follow neither normal nor log-normal distributions. The median E_{H-S}
271 is (i) cortical bone: 0.29 kPa, (ii) growth plate: 91 Pa, (iii) metaphysis: 17 Pa and (iv) bone
272 marrow: 6.7 Pa. The mean E_{H-S} is (i) cortical bone: 2.7 kPa, (ii) growth plate: 0.19 kPa, (iii)
273 metaphysis: 0.42 kPa and (iv) bone marrow: 0.14 kPa. These data indicate that the BMEv is
274 very soft.

275 The distributions of E_{H-S} are very broad in all regions of interest, covering several orders of
276 magnitude (see Fig. S2 for data on a linear scale), revealing the extreme mechanical
277 heterogeneity within each region, which is consistent with the known heterogeneous structure
278 of bone (12, 26, 33).

279 The mechanical distributions vary significantly between different bone regions ($p < 10^{-4}$
280 under Kruskal-Wallis test). Histograms of E_{H-S} measured for cortical bone show a wide
281 distribution that lacks a sharp peak with a large portion of data at relatively high E_{H-S} (*i.e.* E_{H-S}
282 $> 10^3$ Pa), compared to the mechanical distribution of the other bone regions measured. This
283 is in agreement with the high level of mineralisation in cortical bone (5). The mechanical
284 distribution of both the growth plate and bone marrow are narrower, reflecting the more
285 homogeneous composition and/or structure compared to other bone regions (51). The measured
286 modulus of the metaphysis covers the widest range, due to the presence of both bone tissue and

287 bone marrow in this region, enhancing the structural and consequently mechanical
288 heterogeneity.

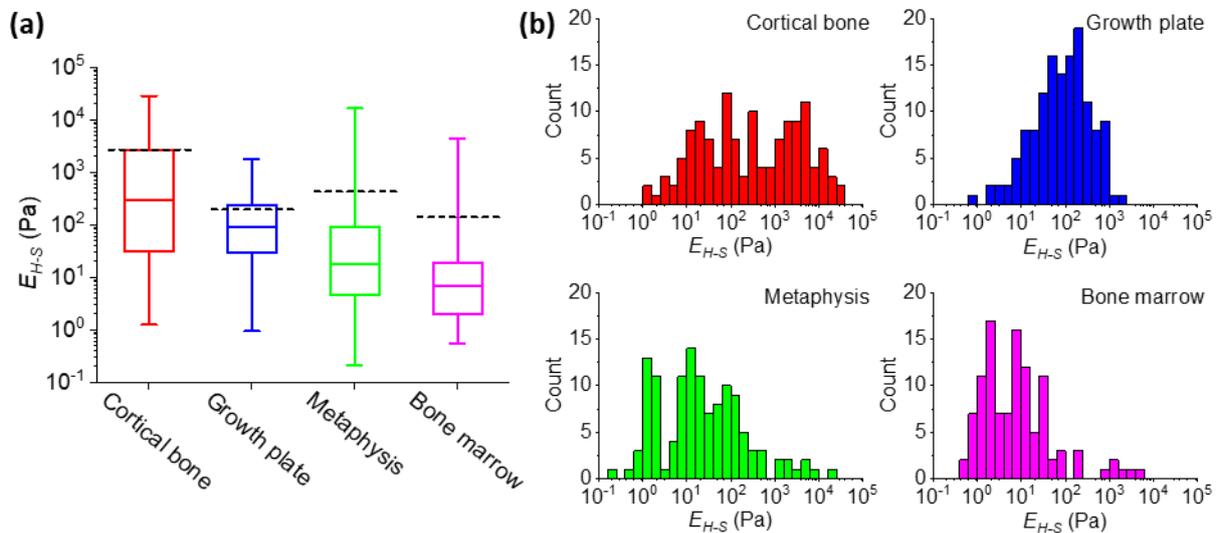
289 Our approach has limits in acquiring data beyond the range shown in the histograms. At the
290 low stiffness end of the distribution (Pa), errors are dominated by inaccuracies in determining
291 the contact point, and tend to lead to an overestimate of the extracted moduli, so the
292 distributions may extend to still lower moduli. At the high stiffness end (100s kPa) there is
293 insufficient indentation at the maximum (trigger) forces used which may lead to an
294 underestimate in the extracted moduli. As a control, the E_{H-S} extracted from hard wall $F-\delta$
295 curves, from the polystyrene petri-dish (expected $E_{H-S} \sim 3$ GPa) under the same experimental
296 conditions, does not exceed 10s to 100s kPa, which is the upper limit of E_{H-S} using the current
297 AFM setup (that is focused on measuring soft components). The experimental setup is not
298 sensitive to the GPa moduli of calcified cortical and trabecular bone previously described (11-
299 15), as this is beyond the scope of this study.

300 However, our approach has the important advantage of being able to measure the subcellular
301 properties of soft tissues, i.e. the cellular microenvironment. The Young's moduli found in the
302 current study are orders of magnitude lower than the values reported in the majority of previous
303 studies (11-28, 34). Direct comparison is difficult, but the ability to measure at the length scale
304 of the cellular components is likely to contribute to the difference. Previous experiments
305 performed with non-AFM indentation approaches (19, 34) normally average over large areas
306 (hundreds μm^2) and thus are unable to determine the properties of the smaller scale. Meanwhile,
307 the low spring constant cantilever and relatively large contact radius probe were selected for
308 use in the current study after extensive preliminary experiments, and provide sufficient force
309 sensitivity to assess subcellular scale mechanics in soft tissue. This is in contrast to using
310 conventional sharp AFM probes and enables accurate measurements of the soft components in
311 the BMev, rather than simply being able to obtain measurements from the calcified regions
312 (11-15).

313 In previous studies mechanical characterisation of the bone marrow is reported to be distinct
314 from other regions within the BMev. The marrow region is traditionally considered as a viscous
315 fluid (29) and there are only a few studies describing the elastic properties. . However, a recent
316 study suggested that the bone marrow is predominantly elastic, and reported an effective
317 Young's modulus ranging from 0.1 to 10.9 kPa measured at physiological temperature (39).
318 This value obtained from porcine bone is similar to that obtained in the current study from
319 murine bone, the difference in the resultant moduli most likely due to the use of rheological
320 measurements instead of indentation. It is important to note that the bone marrow used both in

321 the previous (36) and the current study, is essentially intact with no processing (such as fixation)
322 prior to measurement of the mechanical properties.

323 The H-S model with fitted contact point does not fit the entire F - δ curve perfectly (Fig. 1c).
324 This is likely because the H-S model assumes that the probed material is homogeneous and
325 linearly elastic, whereas the BMev is known to be structurally heterogeneous (5) (Additional
326 errors in the model are discussed in the Supporting Materials). Elastic models describing
327 discontinuous materials (*e.g.* cell-polymer brush model (52)) may be helpful for improved
328 determination of the elasticity of the BMev. Also, the BMev is suggested to be a viscoelastic
329 material in many studies (18, 21, 34, 53). To explore this further, we have characterised the
330 viscoelastic properties by indentation creep measurements.



331
332 **Figure 2.** Elastic properties of different bone regions (coloured version available online). (a)
333 The Young's modulus (E_{H-S}) of different regions in the BMev calculated using the Hertz-
334 Sneddon model with fitted contact point. The central box spans the lower quartile to the upper
335 quartile of the data. The solid line inside the box shows the median and whiskers present the
336 lower and upper extremes. The mean values are indicated by black dashed lines. Data were
337 obtained at randomly selected positions within regions of interest on bones from both young
338 and mature mice. The Young's modulus reported at each position is the mean value of
339 individual fits to all force-indentation curves taken at that location. Curves with strongly tilted
340 baselines (*i.e.* $d_b > d_c/4$, as in Fig. S1c) were discarded. Results from low quality fittings (*i.e.*
341 $R^2 < 0.9$) were also discarded. (b) Histograms of the E_{H-S} of different bone regions, as in (a).
342 The corresponding histograms in linear scale are presented in Fig. S2.

343

344 **Point indentation-creep measurements demonstrate the bone microenvironment can**
345 **be described as a heterogeneous viscoelastic Kelvin-Voigt solid.**

346 To extract the viscoelastic properties of the BMev, an equation based on a three elements
347 Standard Linear Solid (SLS) model (Fig. S1f) developed by Cheng *et. al.* (48) was first used to
348 fit the indentation change ($\Delta\delta$) vs. creep time (t) curves (Fig. 1d-e). The $\Delta\delta$ - t curves from 90%
349 of the total 779 measured positions in all BMev regions of bones from both young and mature
350 mice could be fitted well by this simple viscoelastic model ($R^2 > 0.9$). This supports the premise
351 that the BMev is viscoelastic rather than purely elastic.

352 The moduli presenting both the instantaneous elastic behaviour (E_1) and the delayed elastic
353 behaviour (E_2) could be obtained from the SLS model (Fig. S3). It is notable that in the majority
354 of cases E_1 was significantly higher than E_2 . For all positions that were well fitted by the SLS
355 model, 93% have $E_1 > 10E_2$ and 84% have $E_1 > 100E_2$ (Fig. S3d). This indicated any strain
356 caused by the instantaneous elasticity was negligible. Thus, the complete SLS model is largely
357 unnecessary and can be further simplified to a two element Kelvin-Voigt (K-V) model (Fig.
358 S1g). The $\Delta\delta$ vs. t curves were then fitted to the simplified equation (for development and
359 validation see the Supporting Material) based on the K-V model. Compared to using the SLS
360 model, a similar number of measured positions could be fitted well ($R^2 > 0.9$). This suggests
361 the K-V model is sufficient to describe the viscoelasticity and the majority of BMev acts like
362 a Kelvin-Voigt solid in nature. A K-V type solid is predominantly a viscous liquid at short time
363 scales and predominantly an elastic solid at long time scales. This is biologically meaningful
364 in the context of the BMev. Being a K-V type solid, ensures fast energy damping through the
365 BMev in reaction to any abrupt mechanical loading, as well as maintaining a stable shape in
366 the long term as part of the body scaffold.

367 The Young's modulus determined by the K-V model E_{K-V} and viscosity η for the different
368 bone regions are shown in Fig. 3. The median E_{K-V} for the different regions of interest in the
369 BMev, represented in Fig 3a, are (i) cortical bone: 0.86 kPa, (ii) growth plate: 1.5 kPa, (iii)
370 metaphysis: 0.40 kPa and (iv) bone marrow: 0.14 kPa. The mean E_{K-V} for the different regions
371 of interest in the BMev are (i) cortical bone: 4.2 kPa, (ii) growth plate: 2.9 kPa, (iii) metaphysis:
372 1.7 kPa and (iv) bone marrow: 0.52 kPa. These values are significantly higher than the E_{H-S}
373 values obtained from the pure elastic fit (as in Fig. 2a, cortical bone: $p < 0.01$, growth plate: p
374 $< 10^{-33}$, metaphysis: $p < 10^{-18}$, marrow: $p < 10^{-22}$), which is not surprising since the latter does
375 not take the presence of the viscous effect into account. The E_{K-V} distributions of all BMev
376 regions (Fig. 3c) cover several orders of magnitude, with significant differences ($p < 0.05$)
377 found between the bone regions.

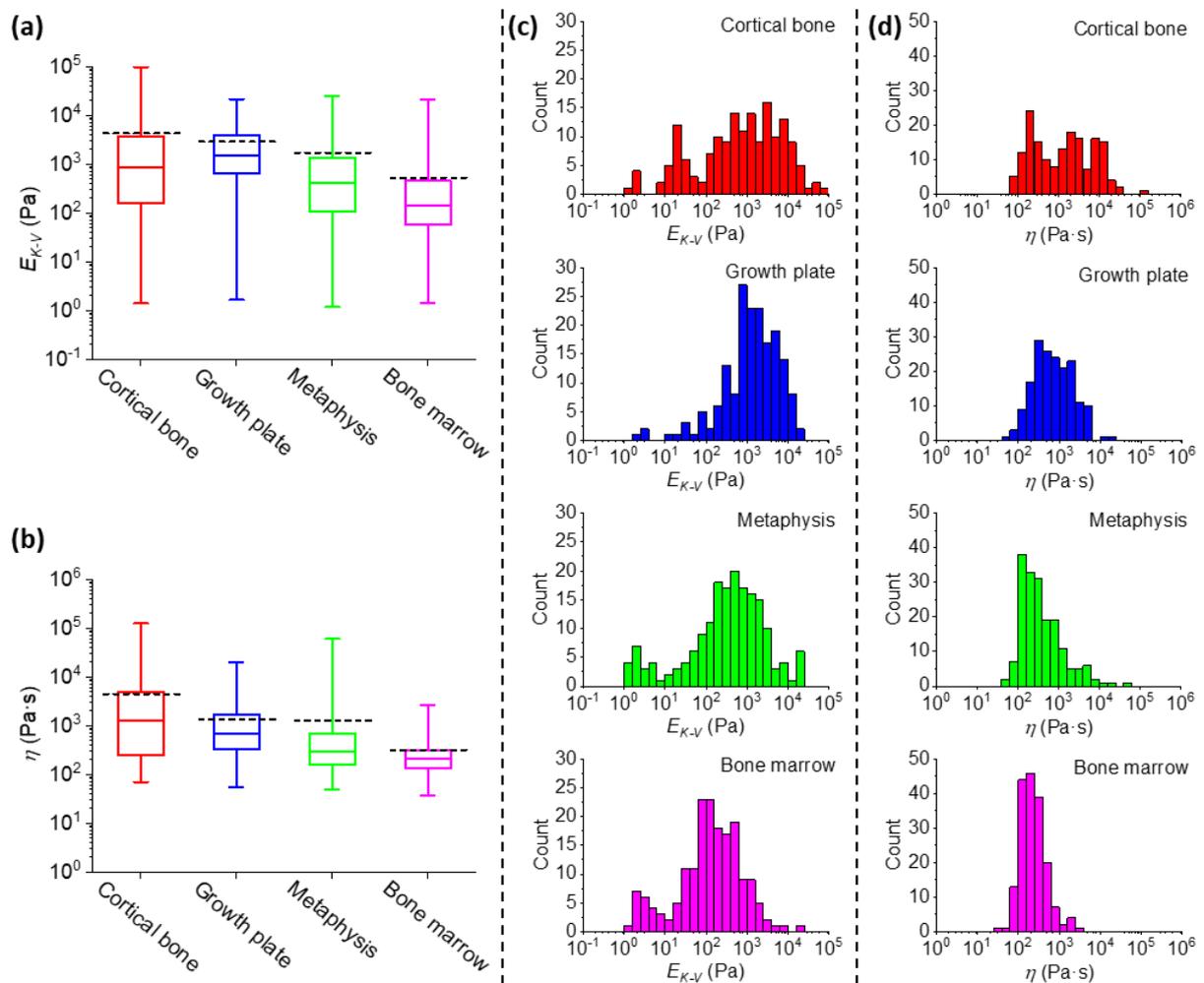
378 The median resultant viscosity η of different BMeV regions are represented in Fig. 3b, as (i)
379 cortical bone: 1.3 kPa·s, (ii) growth plate: 0.68 kPa·s, (iii) metaphysis: 0.29 kPa·s and (iv) bone
380 marrow: 0.21 kPa·s. The mean resultant viscosity η of different BMeV regions are (i) cortical
381 bone: 4.4 kPa·s, (ii) growth plate: 1.3 kPa·s, (iii) metaphysis: 1.3 kPa·s and (iv) bone marrow:
382 0.31 kPa·s. The width of η distribution (Fig. 3d) is smaller than that of E_{K-V} , but still spans
383 several orders of magnitude. Significant differences between different bone regions ($p < 0.01$)
384 are also found as for the elasticity data. The creep time τ , defined as the time for the strain to
385 decay to $1/e$ of its total change, can be calculated from the values of E_{K-V} and η of each measured
386 position by $\tau = 3\eta/E_{K-V}$. The median creep time τ of different BMeV regions are (i) cortical
387 bone: 2.2 s, (ii) growth plate: 1.3 s, (iii) metaphysis: 2.1 s and (iv) bone marrow: 4.1 s. The
388 mean creep time τ of different BMeV regions are (i) cortical bone: 140 s, (ii) growth plate: 16
389 s, (iii) metaphysis: 34 s and (iv) bone marrow: 36 s. The median creep time for all regions is
390 similar to the indentation time in the elastic measurements, so creep relaxation and indentation
391 are most likely occurring simultaneously in force-distance experiments. As such, the viscous
392 drag force is likely to have a strong effect on the F - δ curve, especially in softer areas and around
393 the contact point, where the loading rate is highest. This is most likely the main reason for the
394 discrepancy between the Hertz-Sneddon fit and the modulus obtained from the creep data.

395 It is difficult to make comparisons with the previous published studies quantifying the
396 viscoelastic properties of the hard regions of the bone, as these studies have measured the
397 potential load bearing mechanics (i.e. mineralised regions) (18-20) while the current study
398 focused on obtaining measurements from the cellular microenvironment. Studies of the
399 metaphysis have measured trabecular bone (23, 34), whereas our measurements are dominated
400 by a more marrow-like component which accounts for most of the surface area in the region
401 quantified in our samples.

402 In contrast to the hard bone regions, the composition of bone marrow in previous studies are
403 more comparable to the values reported here. The Young's modulus from both the elastic (E_H -
404 s) and viscoelastic (E_{K-V}) characterisation in our study, is in good agreement with the effective
405 Young's modulus obtained in (39). The viscosity η from rheological measurements (29-31)
406 ranges from far below 1 Pa·s to approximately 100 Pa·s. However, the resultant median and
407 mean value of the bone marrow viscosity is significantly greater (0.2 k Pa·s and 0.3 k Pa·s) in
408 our study. This can be explained by both sample preparation and instrument limits. The
409 preparation and post-processing of the sample have been shown to affect the bone marrow
410 viscosity. For instance, the viscosity of samples undergoing a freeze-thaw cycle prior to testing
411 was lower by an order of magnitude compared to the fresh samples (31). Also, in our studies,

412 the bone marrow was not extracted from the medullary cavity. Thus, connections of the marrow
 413 and bone tissues, and subsequently the rigidity of bone marrow structure, were maintained and
 414 relatively intact.

415 It is notable that viscosity is often a function of velocity and consequently varies with
 416 different measurement frequencies used in different studies. The viscosity of bone marrow has
 417 been shown to decrease as the shear rate increases according to a power law (31, 39). In contrast
 418 to dynamic measurements, the quasi-static method used in our study does not cover a broad
 419 range of frequencies. This quasi-static method requires the creep time τ of the sample to be
 420 significantly larger than the time required to overcome system inertia during the transition from
 421 indentation to dwelling (approximately 0.05 s). As τ is correlated with both E_{K-V} and η , the
 422 lower limit in detectable τ indicates that the resultant viscosity η using such a quasi-static
 423 method cannot be found to be much lower than the lowest values we measured (or E_{K-V} much
 424 higher than current highest values we measured).



425

426 **Figure 3.** Viscoelastic properties of different bone regions (coloured version available online).
 427 (a) The Young's modulus E_{K-V} and (b) viscosity η of different bone regions calculated from fits

428 to creep curves using the viscoelastic Kelvin-Voigt model. The central box spans the lower
429 quartile to the upper quartile of the data. The solid line inside the box shows the median and
430 whiskers represent the lower and upper extremes. The mean values are indicated by black
431 dashed lines. Data were obtained at the same positions as elastic measurements (Fig. 2) from
432 all mice bones. The results are from the mean value from all repeated measurements at each
433 position. Results from low quality fittings (*i.e.* $R^2 < 0.9$) were discarded. Histograms of (c) E_{K-V}
434 ν and (d) η of different bone regions are also shown.

435

436 **Aging impacts on the subcellular mechanical properties at the macro-scale of bone** 437 **marrow but only minor effects on other regions of the bone microenvironment**

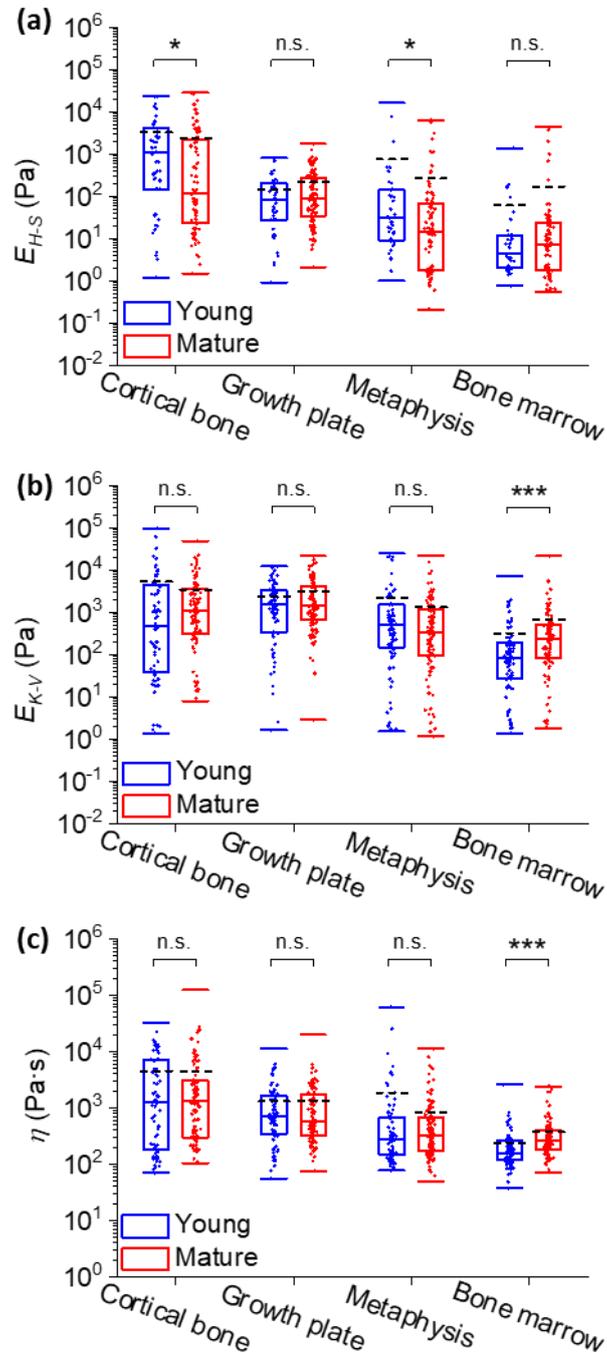
438 Bone turnover in health and disease such as osteoporosis and cancer-induced bone loss show
439 age dependent features (26, 49, 54-57). Therefore, comparing the mechanical properties of the
440 BMeV from mice at different ages will elucidate changes occurring in the mechanics of the
441 BMeV during skeletal maturation. The Young's modulus from the elastic model, E_{H-S} , together
442 with the viscoelastic parameters E_{K-V} and η are classified into two groups: data obtained from
443 young mice (age 6 to 8 weeks old, *blue*) and mature mice (age 11 to 13 weeks, *red*) (Fig. 4).
444 Two groups of data in each region were compared by the Kruskal Wallis (KW) method.

445 The KW test comparing E_{H-S} (Fig. 4a) shows a minor yet statistically significant difference
446 ($0.05 > p > 0.03$) in the cortical bone and the metaphysis. Whereas the E_{H-S} of growth plate and
447 the bone marrow did not vary with age. For E_{K-V} and η (Fig. 4b-c), the KW test demonstrates a
448 high level of significance ($p < 0.001$) in bone marrow but not for any other BMeV region. There
449 are differences in the statistical comparison of pure elastic fit results (*i.e.* E_{H-S}) and viscoelastic
450 fit results (*i.e.* E_{K-V} and η). The main reason is most likely that the Hertz-Sneddon model does
451 not adequately describe the BMeV properties due to not considering the viscous force,
452 especially in soft areas such as bone marrow.

453 Very few studies have been performed quantifying the mechanical properties of the BMeV
454 in relation to age. Tensile tests on whole bones reported in 1976, showed only moderate
455 differences between bones from humans at different ages (54). Recently, micro-/nano-
456 indentation tests revealed that the mechanical properties of human cortical (55) and trabecular
457 (56) bones were remarkably constant as a function of age. The elastic modulus of murine
458 growth plate are reported to be significantly different between the embryonic stage and
459 postnatal stage, age 2 week, but did not vary significantly through the next growth stages until
460 adulthood (up to age 4 months) (25). These results are consistent with our findings. Dynamic
461 stiffness of bone marrow stromal cells were significantly higher in adult horses compared to

462 foals (58), which is in agreement with the age dependent trend of bone marrow mechanics
463 observed in our study.

464 Such statistical comparison reflects the impact of aging on the subcellular mechanical
465 properties of different regions of the BMeV only at macro-scales. It would be helpful to
466 integrate the impacts of aging and different length scales in future studies.



467
468 **Figure 4.** Comparison of the mechanical properties of bones from young (*blue*) and mature
469 (*red*) mice (coloured version available online). (a) The Young's modulus E_{H-S} obtained from
470 the H-S model. Data are identical to those in Fig. 2. (b) The Young's modulus E_{K-V} and (c)
471 viscosity η obtained from the K-V model. Data are identical to those in Fig. 3. The central box

472 spans the lower quartile to the upper quartile of the data. The solid line inside the box shows
473 the median and caps below and above the box represent the lower and upper extremes. The
474 mean values are indicated by black dashed lines. Dots represent individual data points overlaid
475 on top of the boxplots. Data include results from both young and mature mice. The significance
476 of statistical comparisons using the Kruskal Wallis method have been indicated above each
477 group of boxplots (n.s.: not significant; *: $p < 0.05$; ***: $p < 0.001$).

478

479 **Force mapping at the supracellular scale reveals high local mechanical heterogeneity**
480 **within the different regions of the bone microenvironment.**

481 The data discussed in the previous sections were from point measurements, which does not
482 inform on the spatial distribution of mechanical heterogeneity at the cellular scale. To explore
483 this further, we carried out AFM force mapping. The maximum area for AFM force mapping
484 in our setup is $100 \times 100 \mu\text{m}^2$ with each pixel in the map down to $1 \mu\text{m}^2$. This ensures AFM
485 mapping is sufficient to resolve the subcellular mechanical profile at a supracellular scale,
486 considering the dimensions of most cells in bone (mean diameter ranging from 0.5 to
487 approximately $100 \mu\text{m}$) (59-61).

488 Fig 5a-c show representative maps obtained from cortical bone. The trigger point map (Fig.
489 5a) reveals the surface topography of the BMeV at the trigger force. Tightly packed structures
490 are visible (as indicated by the dashed circle), the size and shape of which are consistent with
491 those expected for individual cells. An approximation to the surface topography of the BMeV
492 at zero load is given by the contact point map (Fig. 5b). This appears significantly smoother
493 than the trigger point map, due to the minimal indentation at the contact point and indicates
494 that the bone surface after splitting is relatively smooth on a supracellular scale. The lateral
495 resolution of both maps is limited by the colloidal probe and its convolution with the surface
496 topography. Black pixels in Fig. 5b represent missing data, due to the force distance curves at
497 these pixels being unsuitable for fitting (see Methods). These include the large black regions
498 indicated by arrows where the topography exceeds the lower and upper limits of the AFM's
499 vertical scan range. The corresponding map of E_{H-S} is shown in Fig. 5c. The R^2 is greater than
500 0.9 for 54% and 0.5 for 95% of all curves in this map, and independent of E_{H-S} values.
501 Interestingly, the mechanical heterogeneity is correlated with the topographic structures in Figs
502 5a and 5b, and thus helps to distinguish cellular and acellular components (the former is
503 normally stiffer due to the nuclear, cytoskeletal and membrane components) and mineralisation
504 status (mineralised components are expected to be much stiffer). These maps clearly show the
505 mechanical complexity of the BMeV at the supracellular scale, which is likely to be important

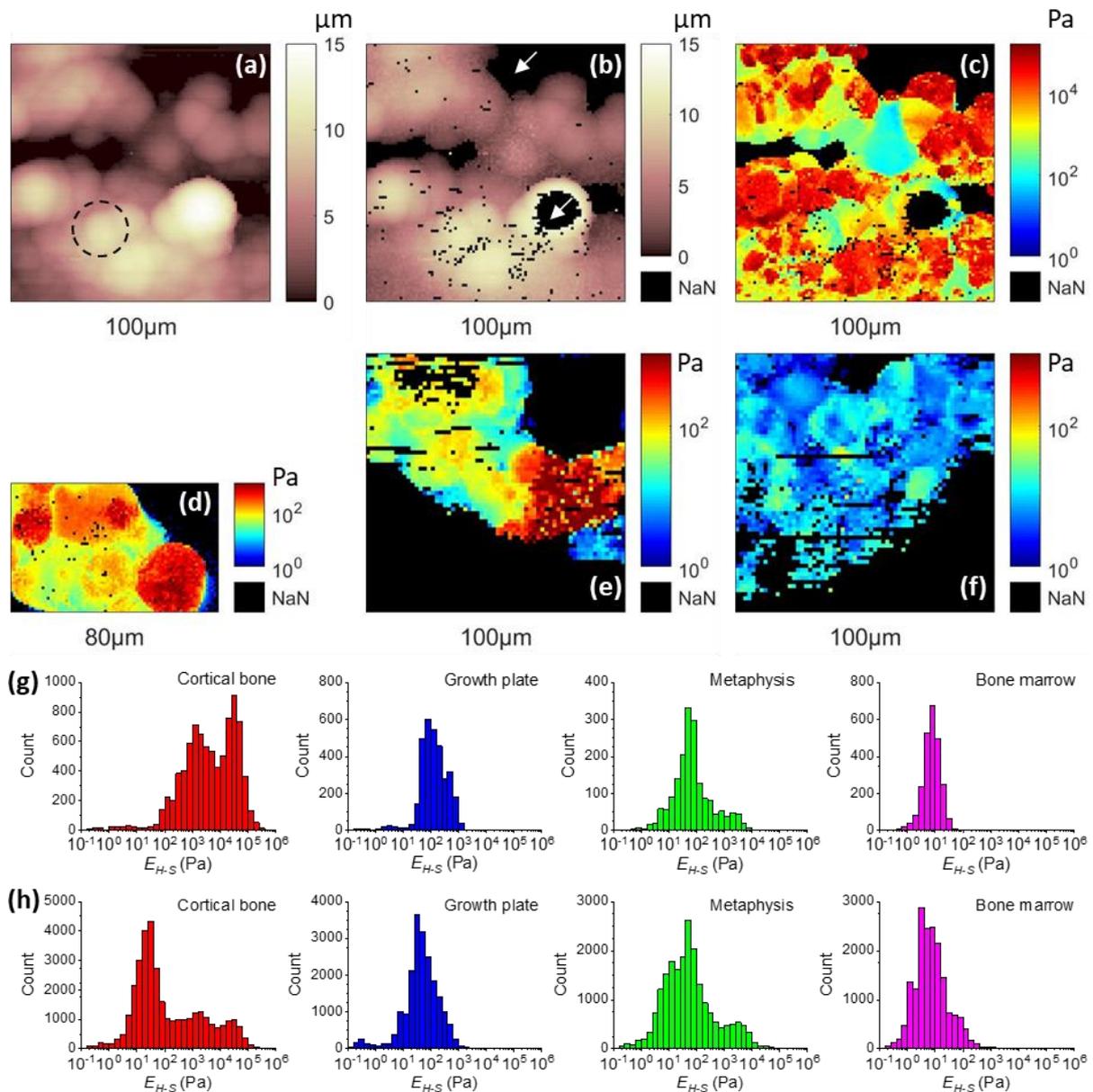
506 for processes such as cell migration and proliferation that are mediated by the mechanical
507 properties of both cells and surrounding extracellular matrix (2, 62).

508 Structure-correlated mechanical heterogeneity is found in all regions of interest in this study
509 (Fig. 5d-f & S4) with E_{H-S} values covering several orders of magnitude. The structures and
510 mechanical properties reflected in the AFM maps vary from region to region, and are difficult
511 to accurately quantify as some areas are more difficult to map due to their large topography.
512 Semi-quantitative comparison of the mechanical properties of different bone regions is feasible,
513 by comparing the histograms of E_{H-S} calculated from the maps.

514 The histograms of E_{H-S} , corresponding to the Young's modulus maps in Fig. 5c-f, are shown
515 in Fig. 5g. The histograms representing E_{H-S} obtained from all maps of each bone region
516 (minimum 5 maps per region) are shown in Fig. 5h. These histograms include data from all F -
517 δ curves with no selection criteria for curve or fit quality. As discussed in the Supporting
518 Material, removal of poor curves has little effect on the distributions, so map data can be
519 compared directly with individual curves from the point measurements (Fig. 2b). For all
520 regions except the cortical bone, the shape of the distribution of E_{H-S} obtained from a single
521 map (Fig. 5g), all maps (Fig. 5h) and individual curves covering the entire region (Fig. 2b) are
522 comparable. The main peak in each histogram shifts by less than one order of magnitude
523 between the different histograms for each bone region. This shows that the degree of elastic
524 heterogeneity at the supracellular scale is comparable to the heterogeneity at a macroscopic
525 scale. In contrast, the histograms from cortical bones are significantly different from each other.
526 Compared to the profile of the entire cortical bone region (Fig. 2b), the peak of E_{H-S} distribution
527 of the selected force map shifts by more than one order of magnitude. Also, the shape of the
528 histogram for all maps obtained on the cortical bone are highly distinct from that of the
529 histograms of either the selected map or the individual curves over the entire cortical bone
530 region. This indicates that the overall heterogeneity of the subcellular mechanical properties in
531 cortical bone results not only from the heterogeneity at the supracellular scale, but also
532 variations at a macroscopic scale over the region.

533 It is notable that the approach speed used in force mapping was significantly increased (30
534 $\mu\text{m/s}$, compared to 1 $\mu\text{m/s}$ in point measurements). Correspondingly, the indentation time of
535 the majority of F - δ curves in the maps is generally not greater than 0.3 s. This is significantly
536 shorter than the mean/median creep time τ of each of the BMeV regions, as described in the
537 viscoelastic section. Within such short time scales, the BMeV will be predominantly viscous as
538 a K-V type solid (see Supporting Material). We should therefore regard the modulus values

539 obtained from indentation as an effective modulus, specific to strain rate, and encompassing
 540 both elastic and viscous contributions.



541

542 **Figure 5.** Mechanical heterogeneity of different bone regions at supracellular scale (coloured
 543 version available online). (a-c) Example of AFM maps obtained from a randomly selected
 544 position on the cortical bone. (a) Topographic map showing the height at which the trigger
 545 force was reached. Dashed circle highlights an observed spherical structure. (b) Topographic
 546 map showing the height at which the probe first makes contact with the surface. The position
 547 of the contact point was determined by the triangle thresholding method as in Fig. S1b. (c) Map
 548 of the measured Young's modulus E_{H-S} from all force curves in the map. Curves with strongly
 549 tilted baselines (*i.e.* $d_b > d_c/4$, as in Fig. S1c) were discarded. (d-f) Examples of E_{H-S} maps
 550 obtained from (d) growth plate, (e) metaphysis and (f) bone marrow. The data include values
 551 from curves with strongly tilted baselines (*i.e.* $d_b > d_c$). For all maps in (a-f), missing data (*i.e.*
 552 no proper force-indentation curves could be fitted) are indicated by black pixels (by white
 553 arrows). The size scale of the maps has been indicated at the bottom of each map. (g)

554 Histograms of the E_{H-S} distribution compiled from the maps in (c-f). (h) Histograms of the E_{H-}
555 S distribution for different bone regions compiled from all AFM maps. For each region, at least
556 5 maps were recorded and each map was collected from a different mouse.

557

558 CONCLUSION

559 In this work, we have used minimally processed murine bone samples to quantify the micro-
560 mechanical properties of four distinct regions of interest using AFM. The mechanical profile
561 of each bone region at macro-scale, revealed by point force-indentation or creep measurements
562 using pure elastic or viscoelastic models, was found to be highly heterogeneous. The
563 mechanical properties of different BMev regions have also shown significant differences.
564 Moreover, aging was found to strongly impact the viscoelastic properties of the region
565 comprising the bone marrow while having no or minimal effects in other regions of interest.

566 All bone regions contained extremely soft areas (*i.e.* the Young's moduli from both elastic
567 and viscoelastic models was as low as a few Pascal), and the overall moduli were much lower
568 than the values reported from previous studies performed on individual cells *in vitro*, whole
569 tissues *ex vivo* or highly processed bone samples. Indeed, the viscoelastic model described the
570 mechanical response better than the elastic model. The majority of the BMev within all regions
571 of interest acted as a Kelvin-Voigt type solid.

572 This study demonstrates the feasibility of obtaining high-resolution AFM data and maps
573 reflecting both 3D morphology and mechanical properties from complex bone tissues. AFM
574 maps revealed that the mechanical properties of all bone regions are highly heterogeneous at
575 the cellular/supracellular scales, ranging over 3 to 5 orders of magnitude. Such unique
576 mechanical architecture may impact on a substantial number of active biological processes,
577 such as bone remodelling, vascularisation, hematopoiesis and on cancer-induced bone disease.
578 With further improvements, such as combining with post-AFM optical images, this AFM based
579 system will be a powerful tool for further characterisation of bones in the presence of stimuli
580 (*e.g.* hormones, cancer cells and therapeutics) or addressing mechanically relevant questions
581 on other complex biological structures.

582 SUPPORTING INFORMATION.

583 The Supporting Material contains supplementary methods and figures S1–7.

584 **AUTHOR CONTRIBUTIONS**

585 XC designed the study, performed the experiments, analysed and interpreted the data, and
586 wrote the manuscript. RH prepared the bone samples, interpreted the data and wrote the
587 manuscript. NM provided support for the experiments, interpreted the data and wrote the
588 manuscript. RJH improved the theoretical model, interpreted the data and wrote the manuscript.
589 IH, NJB and JKH designed the study, interpreted the data, wrote the manuscript and directed
590 the project.

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595 **COMPETING INTERESTS**

596 We declare no competing interests relevant to this work.

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