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Compositional and mechanical properties of growing cortical bone tissue: a study of the human fibula

Emmanuelle Lefevre, Delphine Farlay, Yohann Barla, Fabien Subtil, Uwe Wolfram, Sébastien Rizzo, Cécile Baron, Philippe Zysset, Martine Pithioux, Hélène Follet

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1 **Title:**

2 Compositional and mechanical properties of growing cortical bone tissue: a study of the
3 human fibula

4

5 **Short title:**

6 “Tissue analysis of growing bone”

7

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46 **Abstract:**

47 Human cortical bone contains two types of tissue: osteonal and interstitial tissue. Growing
48 bone is not well-known in terms of its intrinsic material properties. To date, distinctions
49 between the mechanical properties of osteonal and interstitial regions have not been
50 investigated in juvenile bone and compared to adult bone in a combined dataset. In this work,
51 cortical bone samples obtained from fibulae of 13 juveniles patients (4 to 18 years old) during
52 corrective surgery and from 17 adult donors (50 to 95 years old) were analyzed.
53 Microindentation was used to assess the mechanical properties of the extracellular matrix,
54 quantitative microradiography was used to measure the degree of bone mineralization (DMB),
55 and Fourier transform infrared microspectroscopy was used to evaluate the physicochemical
56 modifications of bone composition (organic versus mineral matrix). Juvenile and adult
57 osteonal and interstitial regions were analyzed for DMB, crystallinity, mineral to organic
58 matrix ratio, mineral maturity, collagen maturity, carbonation, indentation modulus, indicators
59 of yield strain and tissue ductility using a mixed model. We found that the intrinsic properties
60 of the juvenile bone were not all inferior to those of the adult bone. Mechanical properties
61 were also differently explained in juvenile and adult groups. The study shows that different
62 intrinsic properties should be used in case of juvenile bone investigation.

63

64

65 **Keywords :** Human Cortical bone, Growing bone, Micromechanical properties,
66 Mineralization, Collagen maturity.

67

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69

70 **Introduction**

71 From a clinical point of view, juvenile bone is of interest since various congenital, acquired
72 diseases or trauma influence bone development in childhood and adolescence. Juvenile bone
73 growth is well described ¹ and starts with the formation of primary bone, formed by the early
74 primary Haversian system, which is then remodeled into a more complex secondary
75 Haversian system with new lamellar bone and oriented cylindrical osteons. Bone modeling
76 allows increasing bone size, as resorption and formation occur simultaneously on different
77 surfaces of the bone (periosteal apposition and endocortical resorption). After reaching final
78 size, bone remodeling occurs with the coupling of resorption and formation at the same
79 location. This could lead to different intrinsic properties. Even if this growth process is well
80 described, tissue mechanical properties and their relationships with compositional properties
81 are not well established in comparison to adult bone.

82 Cortical bone contains both osteonal and interstitial tissue. Osteons are comprised of 5 to 30
83 concentric lamellae with different collagen fibril orientations ^{2 3 4 5} that are arranged around
84 Haversian canals, which ensure an adequate blood supply and innervation of bone ⁶.
85 Secondary osteons are the product of bone remodeling in which old bone is replaced with new
86 bone ⁷. Interstitial tissue is found between osteons and is made of osteonal remnants that
87 remain after bone remodeling. Both osteons and interstitial bone represent bone structural
88 units (BSUs). The characterization of juvenile bone *in vivo* using HRpQCT demonstrated that
89 the transient increase in distal forearm fractures during adolescent growth is associated with
90 alterations in cortical bone which include cortical thinning and increasing porosity ⁸. The
91 assessment of mineral metabolism is also complex in pediatrics ⁹. Characterization of juvenile
92 bone on iliac biopsies by histomorphometry ^{10 11} from 58 healthy subjects showed that the
93 ilium growth occurred through simultaneous periosteal and endosteal resorption and
94 apposition in inner and outer cortices leading to an increase of cortical width from 0.52 mm
95 at an age of 2 years to 1.14 mm by the age of 20 years. It also showed that a lateral modeling
96 drift of the inner cortex encroaches on the marrow cavity. According to Bala et al., the pore
97 volume fraction did not significantly differ between children and adults but originates from
98 different microarchitectural patterns ¹².

99 The mechanical properties of bone tissue change during growth. For example, bone becomes
100 stiffer and more resistant to fracture ^{13 14 15}. As bone is a hierarchical bio-composite material
101 made of an organic matrix (a network of type I collagen fibrils) filled and impregnated with a
102 mineral component consisting of apatite crystals that interact with collagen fibrils ¹⁶, mineral
103 content plays a major role in bone strength ^{17 18}. However, there are very few data on

104 characterization of healthy juvenile bone at the tissue level (at the micro meters length-scale)
105 ^{19 20} and none contrasting such data with a conjoined adult set of samples. The
106 micromechanical properties of bone can be assessed by microindentation tests which is not
107 available for juvenile bone. In adult bone, plane strain modulus was in the range of 7 to 35
108 GPa ^{21 22 23 24 25} and cortical bone stiffness is predominantly associated with mineral content
109 and bone density while cortical bone toughness correlates with the quality of the collagen
110 matrix ²⁶. At the BSU level, axial elastic properties and hardness of bone are dependent on the
111 degree of mineralization ^{6 27}. Using quantitative backscattered electron imaging in juvenile
112 iliac crest bone biopsies, Fratzl-Zelman *et al.* showed that at the trabecular level, no variation
113 of bone mineralization with age was present. However, the average values of mineralization
114 density were slightly lower than in the adult reference population. The cortices appeared to be
115 less mineralized than the trabecular bone ^{19 10} with a lower mineralization in the inner
116 compared to the outer cortex between 1.5 year to 14 years. Mechanical properties of cortical
117 bone also depend on the size and distribution of mineral crystals ^{28 29}. Characteristics such as
118 crystallinity, i.e. crystal size and/or lattice perfection, may influence bone mechanical
119 properties ^{30 31}. The role of collagen, however, remains unclear ³² but cortical bone toughness
120 is reduced by dehydration ^{33 34} and crack bridging is a predominant mediator of cortical bone
121 toughening ^{35 36}. The elastic properties of lamellae depend on the orientation of collagen
122 fibers ^{37 38 39} and it has been demonstrated that, at the lamellar level, collagen is involved in
123 plastic properties ^{6 36}. As described by Bala et al, mineral density (degree of mineralization of
124 bone, DMB), mineral quality (crystallinity), and collagen maturity (age of collagen matrix)
125 are the minimum necessary variables required to define intrinsic properties of adult bone
126 tissue. Thus, they are likely to be the predictive indicators of bone mechanical properties at
127 the osteon level ⁶. To date, this is not clear in case of the mechanical properties of juvenile
128 bone, especially when differentiating between osteonal and interstitial regions and a
129 significant gap of knowledge concerning juvenile bone in comparison to adult tissue exists.
130 Therefore, the aim of this study was for the first time to analysis together the composition and
131 indentation properties of osteonal and interstitial tissue from juvenile and adult bone to
132 understand how structure, composition, and mechanics affect each other. For this purpose,
133 micromechanical properties will be assessed at the same location by instrumented
134 microindentation ⁴⁰, the degree of mineralization by digitized microradiography ⁴¹, and
135 mineral and organic characteristics (crystallinity, mineral/matrix ratio, mineral and collagen
136 maturities, carbonate content) by Fourier Transform Infrared Microspectroscopy (FTIRM) ⁴²
137 ^{43 44}. Specifically, we investigated (i) the relation between tissue mechanical as well as

138 compositional properties with donor age, (ii) the relation of these properties with tissue age,
139 and (iii) their interrelation with each other. We hypothesized that intrinsic properties would
140 change with age in juveniles but not in adults.

141

142 **Results**

143 We analyzed the effect of gender using a multiple regression on adult and juvenile bone, and
144 no influence of sex was found.

145

146 *1. Evolution of parameters with chronological age in juveniles and adults (osteonal and* 147 *interstitial tissue properties)*

148 Figure 1 shows the evolution of the fibula with age in transverse DMB sections. Figure 2
149 shows a transverse fibulae section at two ages (Male 7 years old, Male 80 years old) with the
150 specific location of the high magnification seen in Figure 3 and Figure 4. On juvenile bone,
151 drifting osteons⁴⁵ are visible and exhibit a variation in their main direction. In adult bone,
152 those types of osteon are no more observed (Figure 4).

153 Figure 5 shows the evolution of all age-dependent variables in osteonal and interstitial areas.
154 The gap between juveniles and adults prevents from using a linear or non-linear regression
155 curve to evaluate the relationship between age and tissue properties. Table 1 shows the
156 correlations between intrinsic parameters with chronological age within each subgroup
157 (juvenile/osteonal, juvenile/interstitial, adult/osteonal, adult/interstitial). Collagen maturity
158 and plane strain modulus (E^*) were positively linked to age in the juvenile/osteonal subgroup,
159 which was not observed in adults. E^* was also positively linked to age in the
160 juvenile/interstitial subgroup but not in adult (both areas). Mineral maturity was negatively
161 linked to age in adult bone (both osteonal and interstitial). Compositional and indentation
162 variables of adult bone were equivalent or higher than juvenile bone, with the exception of
163 H/E^* in osteonal tissue ($p = 0.02$) (Figure 6 and Table 3).

164

165 *2. Comparison of osteonal and interstitial tissue properties in juveniles and adults (Figure 6.)*

166 - Juvenile vs adult: (Mann-Whitney unpaired test):

167 A significant difference between juveniles and adults was observed in both osteonal and
168 interstitial areas for DMB, crystallinity, carbonation, and E^* (based on the Mann-Whitney
169 unpaired test). No differences in mineral maturity, collagen maturity, and $W_{\text{plast}}/W_{\text{tot}}$ in
170 juveniles and adults were observed. A significant difference in the mineral/matrix ratio was

171 found in the interstitial area, whereas H/E* ratio showed a significant difference in the
172 osteonal area.

173 - Osteonal vs interstitial (Wilcoxon paired test)

174 Significant differences between the osteonal and interstitial areas were observed for all
175 variables, except for the H/E* ratio and $W_{\text{plast}}/W_{\text{tot}}$ in the juvenile group.

176 - Interaction Category/Region: Comparison with a mixed-model (Table 3):

177 A mixed-model was used to evaluate the effect of the area on the interaction between juvenile
178 and adult groups (Table 3). Differences between the juvenile and adult groups in the osteonal
179 and interstitial regions are shown in Table 3. If the interaction is statistically significant, its
180 sign is indicated as +/- . E* and mineral/matrix ratio showed a negative interaction which
181 indicates that the difference between juveniles and adults is higher in the osteonal than in the
182 interstitial area. A positive interaction was observed for collagen maturity, which indicates
183 that the difference between adults and juveniles is higher in the interstitial area than in the
184 osteonal area.

185

186 *3. Correlations between compositional and indentation tissue properties in juveniles and*
187 *adults in all areas (Table 2 and Table 4.)*

188 When all data are pooled, E* correlated with all parameters (DMB, crystallinity, carbonation,
189 mineral/matrix ratio, and collagen maturity) except for mineral maturity, whereas H/E* and
190 $W_{\text{plast}}/W_{\text{tot}}$ correlated only with carbonation. Correlations between other parameters for each
191 technique are shown in Table 2 Part2A.

192 When groups are separated (Table 2. Part 2B & 2C), the link with mechanical parameter is
193 different. Plane strain modulus is correlated with DMB and carbonation, but also with
194 collagen maturity in juvenile bone and with mineral/matrix in adult bone. H/E* is only linked
195 to crystallinity in the adult group. $W_{\text{plast}}/W_{\text{tot}}$ correlated with H/E* in the juvenile group, and
196 with both H/E* and crystallinity in the adult group.

197 Using a multiple regression to investigate the relation between mechanical and compositional
198 parameters (Table 4), only E* was explained by simultaneously DMB, carbonation and
199 mineral/matrix in the juvenile group. In the adult group, plane strain modulus was only
200 explained by DMB whereas no link was found for the juvenile group. H/E* can be explained
201 by DMB, crystallinity, mineral maturity and collagen maturity. $W_{\text{plast}}/W_{\text{tot}}$ is explained in the
202 model by both crystallinity and mineral maturity in the adult group.

203

204

205 Discussion

206 The aim of this study was to challenge our hypothesis that intrinsic properties would change
207 with age in juveniles but not in adults. Different trends were observed, depending on age and
208 the intrinsic material properties of juvenile and adult bone. Contrary to what we hypothesized,
209 not all the intrinsic properties of the juvenile bone changed with age compared to those of the
210 adults. Mechanical properties were also differently explained in juvenile and adult groups.

211

212 1. Chronological age in juveniles and adults in osteonal and interstitial tissue properties

213 Several bone properties correlated with chronological age (except for collagen maturity
214 and $W_{\text{plast}}/W_{\text{tot}}$), including higher bone mineralization, increased crystal size/perfection and
215 carbonation, decreased mineral maturity, and increased mechanical properties (Table 1).
216 Based on these results, the skeleton appears to reach a stable condition in adult bone (Figures
217 2(c-d), 4) compared to juvenile bone with drifting osteons (Figures 2(a-b), 3).

218 E^* positively correlated with age in juveniles but not in adults (Figure 5.) A study by
219 Akkus *et al.* (2004) that analyzed rat femurs of young adult 3 months old, middle-aged 8
220 months old, and aged 24 months old female Sprague-Dawley rats by Raman spectroscopy
221 demonstrated that increased mineralization, increased crystallinity, and increased type-B
222 carbonate substitution significantly correlated with decreased elastic deformation capacity
223 with age⁴⁶. The results of their study demonstrated that the physicochemical status of mineral
224 crystals of bone tissue impacts the mechanical properties of rat femoral cortical bone, as in
225 our study.

226 We found in juvenile bone only, that mineral/matrix ratio, and total carbonation (types A
227 and B and labile carbonates) increased with chronological age without changing crystallinity.
228 This suggests that in juvenile bone mineral content and carbonation increase without change
229 in crystal size/perfection in osteonal or interstitial bone. In adult bone, except for mineral
230 maturity (decreasing with age), no change in mineral/matrix ratio, carbonation or crystallinity
231 were found. Mirzaali *et al.* (2016) showed comparable results with no change in mineral
232 content or crystallinity with donor age in the elderly²⁵ when using Raman spectroscopy.
233 Yerramshetty *et al.* (2006) also used Raman spectroscopy and showed that crystallinity does
234 not change with age in adult human cadaveric bone (femurs, 52-85 year-old) but no
235 distinction between tissue type was made⁴⁷. They found a significant increase of carbonation
236 with donor age (type B carbonates/ $v\text{IPO}_4$). Different results were reported by Follet *et al.* on
237 adult vertebral bone (54-93 year-old) where crystallinity increased with donor age and was
238 associated with decreased carbonation (types A and B and labile carbonates)⁴⁸. These

239 differences may have been due to differences in bone type that were analyzed by the same
240 technique (cortical fibula compared to trabecular vertebrae) or to differences in bone regions
241 that were analyzed (osteonal / interstitial bone in the present study; no distinction in Follet *et*
242 *al.* ⁴⁸). For comparative studies, care must then be taken to use the same type of bone and the
243 same location, but it seems a pattern arise which is that physicochemical status and
244 mechanical properties change with age-related.

245

246 2. Comparison of osteonal and interstitial tissue properties in juveniles and adults (Figure 6)

247 Material composition of bone tissue was studied at the level of osteons and interstitial tissue
248 using microradiography and FTIRM. The relationship between parameters for osteonal and
249 interstitial tissue were the same for juveniles and adults. In juveniles and adults, crystallinity,
250 mineralization/matrix, mineral maturity, collagen maturity, DMB, and E* were lower in
251 osteonal than in interstitial tissue, whereas carbonation and $W_{\text{plast}}/W_{\text{tot}}$ were higher. Those
252 parameters are always higher in adult bone as compared to juvenile bone in both osteonal and
253 interstitial tissue. Our results show that interstitial tissue had higher levels of mineralization
254 than osteonal tissue in adult bone which is consistent with previous studies ^{6 18}. Our results
255 allow to extend this feature to juvenile bone.

256 A study on ewes ⁴⁹ showed that during the first 6-months of mineralization, the degree of
257 mineralization and microhardness significantly increased, followed by a slower increase until
258 reaching maximal values at 30-months. This secondary mineralization progression was
259 associated with an improvement in both maturation and crystal perfection of the mineral part
260 of bone matrix ⁴⁹ which is comparable to our results. Bergot *et al.* (2009) acquired
261 microradiographs of transverse cortical bone sections from 99 female and 94 male donors (N
262 = 193) ranging from 20 years to 90 years of age ⁵⁰. They reported DMB for interstitial and
263 osteonal tissue separately. Among all donors, osteonal and interstitial DMB was 88.79 ± 7.07
264 g/cm^3 and $90.56 \pm 7.14 \text{ g/cm}^3$ with a coefficient of variation (CV) of 7.88% and 7.96%,
265 respectively, and a percent difference ranging from 2-3.5% ($p < 0.0001$). In our study, CV
266 values for osteonal and interstitial ranged from 2.30-5.65% while the mean percent difference
267 between osteonal and interstitial tissue for the juvenile and adult groups were 5.2% and 6.9%,
268 respectively ($p < 0.0057$).

269 FTIRM was used to study the physicochemical modifications of bone composition
270 regarding the mineral phase as well as the organic matrix ⁴². FTIRM parameters included
271 composition of the crystals and their organization. The results from this study showed that
272 mineral crystallinity values were lower in the juvenile group (crystals were smaller and less

273 perfect). Additionally, significantly lower values in carbonation were observed in the juvenile
274 group, which indicates that lower levels of CO₃ ions were present in the core of the apatite
275 crystal in juvenile bone. However, within each group (juvenile and adult), a different
276 observation was done. In our study, carbonation was lower in both tissue regions in the
277 juvenile group as compared to the adult group. Indeed, carbonation was always lower in
278 interstitial bone than in osteonal bone. We conclude that carbonate-incorporation in bone
279 matrix is different based on chronological age and tissue age (“young” osteon and “old”
280 interstitial). Petra *et al.* (2005) used synchrotron infrared micro-spectroscopy to analyze
281 femoral biopsies taken from an 11-year-old patient (femoral osteotomy), which showed that
282 the carbonate to phosphate ratio in several osteons consistently increased (from 1%) but at a
283 much lower intensity with increasing distance from the center of the osteon⁵¹. The authors
284 concluded that their work confirmed the presence of immature forms of bone mineral (in the
285 $\nu_1\nu_3\text{PO}_4$ area phosphate groups region) in some pediatric cortical bone osteons. Unfortunately,
286 the authors did not perform the same work with interstitial bone⁵¹. In our study, the variation
287 in carbonation within the osteons was <0.1% in both juvenile and adult groups.

288 A study of iliac crest bone from adults without apparent signs of metabolic bone disease
289 found a significant, but weak, correlation between mineral maturity and crystallinity⁵². The
290 crystallinity of this previous study and our study were in the same range (interstitial bone:
291 0.0406 versus 0.0387 (our study); osteonal bone: 0.0397 versus 0.0379 (our study)). The same
292 pattern was observed for mineral maturity (interstitial bone: 2.046 versus 1.813 (our study);
293 osteonal bone: 1.738 versus 1.717 (our study)). Additionally, crystallinity and mineral
294 maturity showed a significant correlation ($p < 0.0001$).

295 Boskey showed in an animal study that the younger animal with mechanically stronger
296 bones has a mixture of small (recently formed) crystals and larger, more mature crystals.
297 Indeed, the mixture of small and large crystals may represent the optimal situation for
298 effective resistance to loads. In aging bone, the average crystal size is larger. When there are
299 only large crystals or only small crystals present, the mechanical properties of the bone
300 composite are considered to be weakened. Boskey’s review also points out the discrepancy
301 between studies (e.g., Table 1 of Boskey, 2003). In a previous study of vertebrae and aging⁴⁸,
302 we showed an increase of both size and perfection of the crystals. Based on the previously
303 referenced review article²⁹, bone containing a greater number of large crystals becomes more
304 brittle and tends to fracture more easily. We showed a significant correlation between
305 crystallinity and E* but not with H/E* or $W_{\text{plast}}/W_{\text{tot}}$, which suggests that larger/more perfect
306 crystals may not be associated with brittle bone. However, deviation from the ideal

307 composition might be considered to be associated with the deterioration of mechanical
308 properties at the microstructural level ⁵³. Indeed, stiffness of cortical bone is predominantly
309 associated with mineral content and bone density, whereas toughness of cortical bone is
310 strongly associated with the quality of the collagen matrix ²⁶. Bone's crystalline structure (i.e.,
311 amount of crystals) provides compressive strength and brittleness; collagen fibrils provide
312 tensile strength and toughness. Remodeling induces regional variability of collagen fiber
313 orientation, leading to changes in bone mechanical properties. It was previously shown that
314 the collagen network loses up to 50% of its capability to absorb energy during aging, likely
315 because of an increase in the percentage of denatured collagen ⁵⁴, but without taking into
316 account the porosity of bone which can account to 76% of the reduction in strength ⁵⁵.

317

318 *3. Correlations between compositional and indentation tissue properties in juveniles and* 319 *adults within all areas (Tables 2)*

320 Results were different when groups were separated or pooled (only Table 2. Part.2A, see §
321 limitations).

322 Yerramshetty et al. (2008) studied the association between mineral crystallinity and the
323 mechanical properties of human cortical bone ³⁰. Using Raman scans and mechanical tests
324 (monotonic and fatigue; n = 64) on 16 human cadaveric femurs (52–85 years old) revealed
325 that crystallinity accounted for only 6.7% to 48.3% of the variation in monotonic mechanical
326 properties, with a significant positive relationship between crystallinity and modulus in
327 tension. Results of this study indicated that increased tissue-level strength and stiffness
328 positively correlated with increased crystallinity while ductility was reduced. The authors
329 concluded that crystallinity could be used as a complementary diagnostic marker for the
330 prediction of bone strength. As Yerramshetty et al. (2008), we also found this relationship
331 between plane strain modulus and crystallinity in juvenile or adult groups when we pool all
332 groups, but this link disappears when groups are separated (Table 2. Part 2A, 2B, 2C).
333 However, crystallinity was associated with H/E^* and $W_{\text{plast}}/W_{\text{tot}}$ in the adults group (Table 2.
334 Part 2C).

335 E^* showed different correlations with compositional properties depending on the group
336 studied (Table 2), but never correlate with H/E^* or $W_{\text{plast}}/W_{\text{tot}}$. This suggests that mechanical
337 competencies of bone are mainly acquired during childhood and growing. However, it has
338 been shown that deterioration in bone macro-mechanical competence occurs during the aging
339 process, mainly because of an increase in porosity. Nevertheless, porosity is not detected by
340 microindentation and this alteration should be considered for studies on a length scale above

341 the one used in this study^{36 25 55}. Although energy absorption, fracture toughness, and
342 ultimate tensile strain show age-related changes of approximately 5–10% per decade, elastic
343 moduli in tension or compression degrade by only approximately 2% per decade^{53 56}.
344 However, these macroscale studies are not being representative for microscales, as toughness
345 tests are influenced by the crack deflecting porosity. At the macroscale, changes in the
346 mechanical competence of bone can be explained by functional adaptation of bone structure,
347 i.e. an age-related increase in porosity and microcracks^{55 57}. Each osteonal remodeling event
348 that fails to replace all of the bone previously removed results in an increase in cortical bone
349 Haversian porosity. Lacuno-canalicular porosity may not be detected by the indenter. The
350 ratio of highly mineralized to new, less mineralized bone tissue is higher when bone
351 remodeling is suppressed, which results in an increase in the homogeneity of cortical bone
352 tissue⁵⁸. More homogenous tissue allows cracks to grow more easily, which reduces the
353 macroscopic toughness of the composite material⁵³. However, the size of bone microcracks is
354 an order of magnitude greater than the indentation depth and therefore will not be detected
355 here^{59 60 61 48}. Modifications of intrinsic mechanical properties involved in this deterioration
356 are also an important point. Indeed, we found a positive linear correlation between age and
357 mineral/matrix ratio and carbonation in the juvenile group but disappear in the adult group.
358 These results reveal that those parameters were rapidly increasing during growth, but remain
359 constant in elderly bone. DMB was highly correlated with the mechanical parameter (E^*) but
360 did not correlate with H/E^* and $W_{\text{plast}}/W_{\text{tot}}$ ratio in juvenile and adult groups. Based on all
361 data, DMB is still highly correlated to E^* .

362 Osteonal and interstitial tissue display different mechanical behaviors in juvenile bone, but
363 these differences are not as pronounced in adult bone. In osteonal bone, we can find primary
364 and secondary osteons⁶². This distinction between primary (totally new) and secondary
365 (replacement) bone is important because it is likely that control of primary bone apposition to
366 periosteal or endosteal bone surfaces is different from that replacing preexisting bone by
367 secondary bone⁶². Primary osteons are quite different (developmentally, morphologically and
368 mechanically) than secondary osteons. Martin and Burr (1989) hypothesized that primary
369 osteonal cortical bone may be mechanically stronger than secondary osteonal cortical bone⁶².
370 Morphologically, the main distinction is that primary osteons do not have cement lines
371 (reversal lines) because they are not the product of bone remodeling. Primary and secondary
372 osteons are considered to be young tissue since they are the result of a remodeling cycle while
373 interstitial tissue is considered to be old tissue. Plane strain modulus strain modulus of old
374 tissue is higher than that of young tissue^{22 40}. The first type of bone tissue that appears in

375 embryonic development and in fracture repair (woven bone) have been recently studied in
376 fetal/infantile bone femurs²⁰. Woven bone can be distinguished from lamellar bone using a
377 polarized light and staining (if needed)⁶³. However, microindentation measurements could
378 not distinguish between primary and secondary osteons. By the nature and definition of
379 primary osteonal bone, we assumed that the primary osteonal bone rate is higher in juveniles
380 and diminishes with age in adults

381

382 4. Discussion of the mixed model and multiple model (Tables 3&4)

383 E* and mineral/matrix ratio showed a negative interaction, indicating that the difference
384 between adults and juveniles was higher in interstitial tissue as compared to osteonal tissue.
385 Interstitial tissue is considered mature bone tissue as compared to osteonal tissue and it is
386 apparent that mechanical properties are higher in older and more mineralized tissue.
387 Interaction of the H/E* and $W_{\text{plast}}/W_{\text{tot}}$ ratio was identical within osteon and interstitial bone in
388 adults and juveniles. The interstitial tissue is considered to be “old bone” and it is known that
389 the plane strain modulus of “old bone” is higher than that of “young bone”^{22 40}.

390 Collagen maturity showed a higher difference in osteonal tissue as compared to the
391 interstitial tissue between adults and juveniles. The organic matrix constitutes the principal
392 toughening mechanism in bone and plays a substantial role in determining properties of
393 energy absorption and toughness⁶⁴. Collagen maturity cannot be attributed to a single
394 phenomenon, since the peak of amide I is sensitive to the secondary structure of collagen,
395 which itself can change based on mineral maturity, degree of mineralization, dehydration of
396 collagen fibers, maturation of collagen fibers. We previously demonstrated that this ratio was
397 not correlated to enzymatic crosslinking of collagen⁶⁵. Increase in collagen maturity in
398 interstitial bone is then the result of a combination of different processes (cited above).

399 Other parameters (e.g., crystallinity, carbonation, mineral maturity, DMB) showed a null
400 interaction, meaning that the difference between juvenile and adult were the same for osteonal
401 and interstitial bone.

402 However, using a multiple regression model, we can point out a different relationship
403 between mechanical parameters and intrinsic properties. Whereas for the adult group all
404 mechanical properties are linked with several intrinsic properties, in the juvenile group only
405 the plane strain modulus is explained by simultaneously DMB, carbonation and
406 mineral/matrix. After growth, plane strain modulus is only explained by DMB. That’s
407 different trend is a novelty in the study of juvenile bone field.

408

409 5. *Limitations*

410 In some results, we stated that no difference was observed between juvenile and adult
411 groups. Due to the small sample size, this means that this could be due either to a lack of
412 statistical power or to an insignificant difference. Statistical differences between osteonal and
413 interstitial tissue of both juvenile and adult groups prevented us from pooling the tissue
414 samples together. Indeed, when we conducted the measurements, we did not express the
415 osteonal or interstitial region as a percentage of total bone, but instead used a fixed number of
416 measurements per tissue type. This means that we assigned equal “weight” to those tissue
417 types, which may not be true, especially in the case of a growing skeleton. In a recent study,
418 Gauthier *et al.* separated those compartments and found in adult a ratio of 41% for osteonal
419 tissue and 54.5% for interstitial tissue compare to total bone, the remain was due to osteocyte
420 lacunae morphometric parameters, with a significant differences in shape and morphometric
421 parameters in lacunae density, lacunae main length, and anisotropy, higher in interstitial tissue
422 compared to osteonal one.^{66 67}

423 In juveniles, it was difficult without polarized light to distinguish drifting osteons.
424 Schnitzler *et al.* (2013) used histomorphometry to analyze osteons and their canals for age-
425 related changes in numbers, size, and shape in 87 iliac crest bone samples of subjects aged 0-
426 25 years⁷. The authors identified three types of secondary osteons: drifting, eccentric, and
427 concentric, as previously described by Jones *et al.*⁶⁸. The authors concluded that these
428 structures of osteons and canals varied during growth. Large asymmetrical drifting osteons
429 with giant active canals (remodeling space) were predominant until the mid-teens and
430 accounted for > 70% of juvenile cortical porosity. In our study, we noticed these different
431 types of structures only in the juvenile group (as shown of Figure 2.).

432 Another limitation was preparation and PMMA embedding (also called infiltration) of
433 the samples. Based on Raman spectroscopy analysis of three elderly human femurs, Nyman *et*
434 *al.* found that compositional properties were still detectable in samples embedded in PMMA
435⁶⁹. However, bound water is a primary contributor to the mechanical behavior of bone in that
436 it is responsible for giving collagen the ability to confer ductility or plasticity to bone, but
437 little is known about why bound water decreases with age in hydrated human bone, which
438 may have had, or not, an influence on our results^{70 25}. In our study, microindentation tests
439 were conducted on PMMA infiltrated bone samples. Microindentation is particularly sensitive
440 to sample preparation methodologies, since hydration, alcohol fixation, and inclusion and
441 roughness of bone samples can influence test results^{71 72 73 24 40}. According to Rodriguez
442 Florez *et al.*, who compared the effects of different preparation protocols (inclusion, i.e.:

443 coating, resin surrounding the material or infiltration, i.e. embedding) on nanoindentation
444 results, the E^* and model viscosity values were similar for included or embedded samples ⁷⁴.
445 However, hardness was higher for samples included in the PMMA. This seems to come from
446 the infiltration of the resin into the pores. In this study, samples from both groups were
447 prepared using the same protocol. Therefore, preparation methodology is not critical for
448 making comparisons between different groups.

449 Another limitation concerns the samples themselves. Although our study tried to quantify
450 parameters of growing bone, we were unable to obtain a healthy bone sample to serve as a
451 control for the study. Our patients are the closest to a healthy patient that we could get ⁽⁷⁵⁾.
452 Indeed, all samples were obtained after surgery, which meant that juvenile patients were not
453 considered healthy at the time of sample acquisition. The ideal control bone sample would
454 have to be obtained from a healthy individual after accidental death, for example. However,
455 this is ethically impossible in the authors' countries. Thus, our results should be interpreted
456 with caution.

457

458 6. Conclusions

459 Juvenile osteonal or interstitial bone is less mineralized, contains smaller/less-perfect
460 apatite crystals, and is less carbonated, as compared to adult bone. Mineral and collagen
461 maturity was not significantly different. Dominated by the mineral phase, indentation
462 modulus and hardness were also lower in juvenile bone.

463 The differences between osteonal and interstitial properties are distinct in juvenile and
464 adult bone (significant interaction term). Crystallinity, mineralization index, mineral maturity,
465 collagen maturity, DMB, and E^* were always lower in osteonal tissue than in interstitial
466 tissue, whereas carbonation and $W_{\text{plast}}/W_{\text{tot}}$ were higher. There were no consistent trends
467 suggesting that indicators of tissue ductility (H/E^* and $W_{\text{plast}}/W_{\text{tot}}$) were different between
468 juvenile and adult bone.

469 It has been clearly established that child bone has a different mechanical behavior from
470 adult bone but, despite some recent studies on the subject, the characterization of juvenile
471 bone remains poorly documented. The issue of the present work is to gain insight into the
472 compositional and mechanical properties of growing cortical bone tissue and compare them to
473 adult bone as reference material. The challenge is to improve the knowledge on juvenile bone
474 in order to develop adapted clinical devices, based in particular on ultrasound measurements,
475 particularly suitable for new born or children in whom anesthesia should be avoided ^{14 75}.

476

477 **Material and Methods**

478 *1. Specimens*

479 Bone samples were collected at the same location from the distal third of the fibula of 13
480 children (10 male and 3 female) 4 to 18 years old (mean age of 9.9 years \pm 4.0 years) during
481 corrective surgery for a growth plate fracture, clubfeet, or for chondrodystrophy, hypoplasia,
482 epiphyseal dysplasia. Surgeries were performed at the Timone Hospital (Marseille, France).
483 All children were ambulatory prior to surgery and none received medications known to affect
484 bone remodeling. In accordance with the French Code of Public Health and after approbation
485 of the study by the Committee for the Protection of Persons, informed consent was obtained
486 from a legal guardian of each child. Adult bone samples were harvested from the distal third
487 of the fibula from 17 donors (7 male and 10 female) 50 to 95 years old (mean age of 76.4
488 years \pm 13.9 years). Autopsies were performed to build a bone sample bank (French body
489 donation to science program, declaration number: DC-2015-2357; Laboratory of Anatomy,
490 Faculty of Medicine Lyon Est, University of Lyon, France)

491 All bone samples were fixed in 70% alcohol, dehydrated in absolute alcohol, and infiltrated in
492 methyl methacrylate (MMA), which resulted in a block of infiltrated bone samples. For
493 quantitative microradiography, 150 μ m-thick sections of the block were cut in a plane
494 perpendicular to the Haversian canals with a precision diamond wire saw (Well, Escil,
495 Chassieu, France). Sections were progressively ground to a thickness of $100 \pm 1 \mu$ m with
496 silicon carbide and polished with a diamond suspension (0.25μ m)⁽⁷⁶⁾

497 For FTIRM, sections of 2 to 5 μ m were cut from the infiltrated bone samples using a
498 Polycut E microtome (Leica, Wetzlar, Germany). The residual block of the infiltrated bone
499 samples was used for nanoindentation testing.

500 All measurements detailed the biological tissue age from each region (“young” osteonal and
501 “old” interstitial tissue) and the chronological donor age of each group (juvenile and adult).

502

503 *2. Quantitative microradiography*

504 Quantitative microradiography of 100 μ m-thick bone sections was performed using an X-ray
505 diffraction unit (L9421-02, Microfocus, Hamamatsu Photonics, Japan) to assess the degree of
506 mineralization of bone (DMB). X-ray images of bone samples and an aluminum standard
507 were acquired with a CCD camera with the following settings: active area of 36×24 mm
508 (4008×2671 pixels) with a 12-bit (4096 values) digital image, scintillator Gd₂O₂S: Tb, 12
509 μ m aluminum filter (FDI VHR 11 M, Photonic science, Robertsbridge, UK). Each acquisition
510 was an average of five images with an exposure time of 7-seconds per image. Due to the high

511 magnification, multiple areas were needed to rebuild an entire sample. After calibration of
512 gray level using the aluminum standard ⁷⁶, the mean gray level of BSUs was converted into
513 degree of mineralization values (in g/cm³). For each bone sample, 20 BSUs (10 osteons and
514 10 interstitial areas) were individually selected by drawing a region-of-interest (ROI) around
515 the BSU and analyzed to obtain DMB values for these areas by a unique operator. The entire
516 bone section was also analyzed to obtain an average DMB value ⁴¹.

517

518 3. *Fourier-transform infrared microspectroscopy (FTIRM)*

519 A GXII Auto-image microscope (Perkin-Elmer, Norwalk, CT, USA) equipped with a
520 wideband detector (mercury–cadmium–telluride) (7800-400 cm⁻¹) was used to perform
521 FTIRM in transmission mode on 2 μm-thick sections. A Cassegrain objective (numerical
522 aperture of 0.6) with a spatial resolution of 10 μm at typical mid-infrared wavelengths (4000-
523 400 cm⁻¹) was used for measurements. Osteonal and interstitial bone were clearly identified
524 under the device's microscope. Twenty measurements per sample (10 measurements in
525 osteonal and interstitial regions) with a spatial resolution of 40 × 40 μm were performed. Each
526 spectrum was collected at a resolution of 4 cm⁻¹ and 50 scans per spectrum. Contributions to
527 the spectrum of air and MMA were subtracted from the original spectrum. Following
528 automatic baseline correction (Spectrum Software) and curve fitting of every individual
529 spectrum, bone characteristics were quantified using GRAMS/AI software (Thermo Galactic,
530 Salem, NH, USA) ^{77 78}.

531 In a bone spectrum (Figure 1), six distinct regions were identified based on the
532 vibrational response of different constituents:

- 533 - The amide (I, II, III) areas [1300, 1700] cm⁻¹, corresponding to the signal of the
534 organic matrix in the bone tissue.
- 535 - The ν₁ν₃PO₄ area [900, 1,200] cm⁻¹, corresponding to symmetric and
536 antisymmetric stretching of the phosphates.
- 537 - The ν₂CO₃ area [800, 900] cm⁻¹, corresponding to symmetrical stretching of the
538 carbonates.
- 539 - The ν₄PO₄ area [500, 650] cm⁻¹, corresponding to antisymmetric deformation of the
540 phosphates.

541 The following five variables within these regions were determined to characterize each
542 sample:

543 **Crystallinity**, which is calculated as the inverse of the full-width at half-maximum
544 (1/FWHM) parameter of the 604 cm⁻¹ peak (apatitic phosphate environment) that
545 corresponds to both crystal size and perfection ⁷⁹.

546 **Ratio of mineral to organic matrix**, (mineral/matrix) which is the area ratio of the [910-
547 1184] cm⁻¹/[1592-1730] cm⁻¹ bands describing mineral over organic matrix ratio ⁸⁰;

548 **Mineral maturity**, which is the area ratio of the apatitic phosphate over non-apatitic
549 phosphate (1030/1110 cm⁻¹ area ratio) that reflects the age of mineral ⁷⁹.

550 **Collagen maturity**, which is the ratio of organic matrix bands (1660/1690 cm⁻¹ area
551 ratio) ⁸¹ that reflects the change in secondary structure of collagen in relation to the
552 mineralization process ⁸⁰.

553 **Carbonation**, which is the ratio of the v₂CO₃ area [862, 894] cm⁻¹ to the v₁v₃PO₄ area
554 [910, 1184] cm⁻¹ that reflects the incorporation of CO₃ ions into the crystal.

555

556 4. Microindentation tests

557 Flat and parallel surfaces on the residual blocks of infiltrated samples were produced
558 with an ultra-miller (Polycut E, Reichert-Jung, Germany). Indentations were performed under
559 dry conditions with an Ultra Nano Hardness Tester (UNHT, CSM Instruments, Switzerland)
560 equipped with a long-shaft reference tip and a Berkovich indenter. Five indentations were
561 made on 10 osteonal and 10 interstitial regions in a plane perpendicular to the bone axis.

562 To minimize the effect of creep on the measurements, a trapezoidal protocol in load
563 control up to a maximum depth of 1 μm with a loading rate of 100 mN/min, a holding time at
564 maximum force of 30 seconds ³³, and an unloading rate of 400 mN/min was used ²⁵. Plane
565 strain modulus (E*, GPa), indentation hardness (H, GPa), elastic work (W_{elast}), and total work
566 (W_{tot}) were extracted ²⁵. Plane strain modulus was recovered from the experimentally
567 measured reduced modulus E_r ⁸² for known isotropic constants E_i and ν_i of the diamond
568 indenter tip using equation (1). Parameters were measured on the unload curves.

$$E = \left(\frac{1}{E_r} - \frac{1 - \nu_i^2}{E_i} \right)^{-1} \quad (1)$$

569 Indentation hardness is given by the ratio of maximum load (P_{max}) to contact area at
570 maximum depth A_c

$$H = \frac{P_{max}}{A_c} \quad (2)$$

571 The total, elastic, and plastic works are defined as:

$$W_{tot} = \int_0^{h_m} Pdh, W_{elast} = \int_{h_m}^{h_p} Pdh, W_{plast} = W_{tot} - W_{elast} \quad (3)$$

572 with load P, depth h, maximum depth h_m , and residual depth h_p . For analyses, the ratios
 573 W_{plast}/W_{tot} and H/E^* were used since they represent surrogate measurements of ductility and
 574 yield strain, respectively²⁵.

575

576 5. Statistical analysis

577 Statistical analysis was performed in SPSS 20.0 (IBM, Amonk, NY, USA) using a
 578 significance level of 5%. Depending on the main parameters (E^* , DMB, carbonation and
 579 crystallinity), the power analysis range from 68% to 99% in osteonal tissue and from 95% to
 580 99% in interstitial tissue. All tests were two-tailed. Results are reported as scattergram and
 581 boxplots. Distribution of variables was tested with the Shapiro–Wilk procedure. Non-
 582 parametric tests were used to evaluate variables that were not normally distributed. The
 583 influence of the microstructure on mechanical behavior was studied using bivariate
 584 correlations that were tested by the Spearman’s rank correlation test. The Mann-Whitney
 585 unpaired test was used to test for differences between juvenile and adult groups (category).
 586 The Wilcoxon paired test was used to test for differences between osteonal and interstitial
 587 tissue (region) for each category.

588 Due to the study design, a measurement of heterogeneity based on standard deviation of
 589 the parameters was used. Those results show that the heterogeneity is the same between
 590 juvenile and adult groups in osteonal and interstitial bone for all parameters obtained with the
 591 FTIRM and microradiography technics. In indentation, heterogeneity is different between
 592 juvenile and adult groups in osteonal and interstitial bone for W_{plast}/W_{Tot} and only in
 593 interstitial bone for H/E^* .

594 A mixed model is used in cases of fixed and random effects. Our custom mixed-model
 595 evaluates the interaction between category and region, using R statistics package. The
 596 function used in this analysis, lme, is a generic function that fits a linear mixed-effects model
 597 in the formulation described by Laird and Ware (1982) but allows for nested random effects
 598⁸³. Within-group errors are allowed to be correlated and/or have unequal variances.
 599 Significance (p) is also obtained for each region. The sign of the interaction (+/-) is indicated
 600 when the model is significant (based on p-values). The interaction is positive when the
 601 difference between adults and juveniles is higher in the osteonal area than in the interstitial
 602 area. A negative interaction indicates that the difference between adults and juveniles is
 603 higher in the interstitial area than in the osteonal area. A non-significant interaction indicates

604 that the equivalent differences are observed between adults and juveniles within the two tissue
605 areas.

606 Within each group, a multiple regression was used to explain the variations of the
607 dependent variables (mechanical properties) by the variations of the independent ones
608 (compositional properties). Adjusted regression coefficients R^2 are indicated in bold if
609 significance was found.

610

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616
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618 All authors state that they have no conflict of interest.

619
620 **Authors' roles:**

621 Study design: EL, MP, and HF. Study conduct: EL, CB, HF, Data collection: EL, YB, SR,
622 UW, Data analysis: EL, DF, FS, HF, Data interpretation: EL, DF, UW, PZ, HF, Drafting
623 manuscript: EL, DF, UW, HF, Revising manuscript content: DF, CB, UW, PZ, HF,
624 Approving final version of manuscript: EL, DF, UW, CB, PZ, MP, HF.

625 HF takes responsibility for the integrity of the data analysis.

626
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835 **Tables and Figures legends**

836

837 **Figure 1:** Top left to bottom right: Evolution of transverse section of fibula with age (age and
838 sex are indicated above each section), taken at the same location. Illustrations come from
839 DMB measurement by X-rays. Note the evolution of the cortex and trabeculae. Scale bars
840 represent 1.5 mm. The right bottom graph shows the DMB distribution in juveniles (Solid
841 line) and adults (Dot line) in osteonal and interstitial bone.

842

843 **Figure 2:** Transverse fibulae section at low magnification at two ages (x1). a)-c) Quantitative
844 digitized microradiography was used to measure the degree of mineralization of bone (DMB,
845 g/cm^3), and b)-d) identical sections observed in polarized light. Rectangles show the location
846 of the high magnification illustrations in Figures 3 & 4. Illustrations will be available at high
847 resolution.

848

849 **Figure 3:** At high magnification (x2.5) from left Figure2, top image shows the drifting
850 osteons present in juvenile bone. Note the heterogeneity of the mineralization, with dark (low
851 mineralization) and white osteons (higher mineralization). Red arrow and blue lines point of
852 drifted osteons as shown in Robling et al. 1999. Middle image shows the corresponding
853 section seen in polarized light. Drifting osteons exhibit a variation in the direction of
854 transverse drift along their longitudinal axes, intermitent regions of concentric morphology
855 and change in drift direction over time. Bottom image illustrates at $8\mu\text{m}$ thin-section of May-
856 Grünwald Giemsa staining, from endosteal (E) to periosteal (P) area, at high magnification
857 (x10). Bone modeling is characterized by a formation (F) and resorption (R) at different
858 location. Formation is mostly oriented throught the perioste and resorption in endoste,
859 allowing the bone growth. Illustrations will be available at high resolution

860

861 **Figure 4.** At high magnification (x2.5) from right Figure2, Top image shows the circular
862 osteons present in adult bone. Note the heterogeneity of the mineralization, with dark (low
863 mineralization) and white osteons (higher mineralization). Red arrows and blue lines point of
864 the circular and smaller osteons. Middle image shows the corresponding section seen in
865 polarized light. Bottom image illustrates at $8\mu\text{m}$ thin-section of May-Grünwald Giemsa
866 staining, from endosteal (E) to periosteal (P) area, at high magnification (x10). Black arrows
867 shows a limited bone cellular activity, with cavity filled with giant adipocytes. Illustrations
868 will be available at high resolution

869 **Figure 5:** Evolution of the parameters with age for both osteonal and interstitial bone regions.
870 Juvenile data in triangle and adult data in circle, sharp symbol for osteonal region and plane
871 symbol for interstitial region. Solid lines represent significant interactions with age in juvenile
872 or adult group, using the pooled osteonal and interstitial regions.

873

874 **Figure 6.** Box Plot of Juvenile data in light grey, Adults data in dark grey, for osteonal and
875 interstitial regions. P-value comparing groups are indicated. The difference (p) between
876 Osteonal and Interstitial is shown using a Wilcoxon paired test and the difference between
877 Juvenile/Adult is shown using a Mann-Whitney unpaired test.

878

879 **Table 1.** Spearman correlation coefficients (r') obtained between chronological age and the
880 different parameters of the FTIRM tests and the mechanical parameters obtained by
881 indentation for the four subgroups (* $p < 0.05$, ** $p < 0.01$).

882

883 **Table 2.** Spearman correlation coefficients (r') obtained between the different variables of
884 microradiography, FTIRM and indentation in: Part 2.A: All samples (juvenile and adult bone)
885 with pooled osteonal, interstitial, (* $p < 0.05$, ** $p < 0.01$); Part 2B: Juvenile bone with pooled
886 osteonal and interstitial, (* $p < 0.05$, ** $p < 0.01$, BL: borderline $0.05 < p < 0.07$); Part 2C: Adult
887 bone with pooled osteonal and interstitial, (* $p < 0.05$, ** $p < 0.01$)

888

889 **Table 3.** Mean and (SD, coefficient of variation %) for each parameter (osteonal and
890 interstitial separated). Difference Adult/Juvenile is indicated for each region, using a mixed
891 model. The significance is also obtained (p) for each region, using a Mann-Whitney unpaired
892 test. Interaction between Category and Region is obtained using a mixed model. The sense of
893 the interaction (+/-) is indicated when the model is significantly relevant (p-values for
894 interaction)

895

896 **Table 4.** Multiple regression analysis describing micromechanical variables (elastic modulus
897 (E^*), ratio (H/E^*) and $W_{\text{plast}}/W_{\text{tot}}$) as a function of the degree of mineralization of bone
898 (DMB), crystallinity, carbonation, mineral/matrix, mineral maturity and collagen maturity.
899 Multiple regressions are done separately for each category (Juvenile or Adult) with osteonal
900 and interstitial bone pooled. Bold values indicate significant results.

901 **Table 1.**

902

Age	n	DMB	Crystallinity	Carbonation	Mineral/Matrix	Mineral Maturity	Collagen maturity	E*	H/E*	W_{plast}/W_{tot}
Juvenile/Osteonal	13	0.077	0.420	0.279	0.340	0.293	0.577*	0.561*	-0.185	-0.011
Juvenile/Interstitial	13	0.287	0.359	0.530	0.547	0.169	0.334	0.622*	0.072	-0.191
Adult/Osteonal	17	-0.128	-0.196	0.292	-0.299	-0.493*	-0.285	0.109	-0.026	0.075
Adult/Interstitial	17	-0.227	-0.137	0.144	-0.447	-0.492*	-0.039	0.213	0.019	0.007

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Table 2.

Part 2A

ALL (n=30)	DMB	Crystallinity	Carbonation	Mineral/Matrix	Mineral Maturity	Collagen maturity	E*	H/E*
Crystallinity	0.375**	-						
Carbonation	0.063	0.016	-					
Mineral/Matrix	0.594**	0.499**	-0.272*	-				
Mineral Maturity	0.153	0.557**	-0.476**	0.467**	-			
Collagen maturity	0.522**	0.218	-0.052	0.555**	0.383**	-		
E*	0.710**	0.321*	0.374**	0.479**	-0.040	0.348**	-	
H/E*	-0.127	-0.057	-0.402**	0.160	0.035	-0.013	-0.257	-
W _{plast} /W _{tot}	0.009	-0.021	0.289*	-0.242	-0.019	-0.048	0.121	-0.893**

Part 2B

Juveniles (n=13)	DMB	Crystallinity	Carbonation	Mineral/Matrix	Mineral Maturity	Collagen maturity	E*	H/E*
Crystallinity	0.326	-						
Carbonation	-0.004	-0.439*	-					
Mineral/Matrix	0.482*	0.506**	-0.185	-				
Mineral Maturity	0.471*	0.747***	-0.362	0.404*	-			
Collagen maturity	0.677***	0.343	0.067	0.644***	0.515**	-		
E*	0.573**	-0.032	0.554**	0.372 ^{BL}	0.092	0.486*	-	
H/E*	-0.220	-0.103	-0.261	0.051	-0.273	-0.266	-0.290	
W _{plast} /W _{tot}	0.082	0.123	0.177	-0.192	0.316	0.136	0.154	-0.861**

Part 2C

Adults (n=17)	DMB	Crystallinity	Carbonation	Mineral/Matrix	Mineral Maturity	Collagen maturity	E*	H/E*
Crystallinity	0.237							
Carbonation	-0.308	-0.664***						
Mineral/Matrix	0.561**	0.580***	-0.720***					
Mineral Maturity	0.044	0.778***	-0.620***	0.558**				
Collagen maturity	0.357*	0.221	-0.166	0.483**	0.297			
E*	0.671***	0.280	-0.420*	0.549***	0.133	0.30	-	
H/E*	0.100	0.386*	-0.298	0.326	0.171	0.283	0.087	-
W _{plast} /W _{tot}	-0.036	-0.434*	0.214	-0.316	-0.191	-0.235	-0.079	-0.923**

907

908 **Table 3.**
909

	Osteonal			Interstitial			Interaction Category/Region	
	Juvenile	Adult	p-values	Juvenile	Adult	p-values	Sense of interaction (Adult – Juvenile) (+/-)	p-values
E*(GPa)	24.40 (1.93; 7.9%)	26.09 (1.83; 7.0%)	0.036	25.80 (1.93; 7.5%)	29.63 (1.11; 3.7%)	<0.0001	–	<0.001
H/E*	0.0426 (0.0021; 4.9%)	0.0409 (0.0023; 5.6%)	0.0201	0.0429 (0.0019; 4.4%)	0.0418 (0.0026; 6.2%)	ns		0.087 (ns)
W _{plast} / W _{tot}	0.769 (0.007; 0.91%)	0.774 (0.009; 1.2%)	ns	0.767 (0.008; 1.0%)	0.767 (0.007; 0.9%)	ns		0.105 (ns)
Crystallinity (cm)	0.0360 (0.0024; 6.7%)	0.0379 (0.0011; 2.9%)	0.0144	0.0370 (0.0016; 4.3%)	0.0388 (0.0011; 2.8%)	0.003		0.619 (ns)
Carbonation	0.0066 (0.0008; 12.1%)	0.0078 (0.0005; 6.4%)	0.0002	0.0063 (0.0005; 7.9%)	0.0073 (0.0003; 4.1%)	<0.0001		0.51 (ns)
Mineral/ Matrix	4.31 (0.44; 10.2%)	4.313 (0.345; 8.0%)	ns	4.819 (0.36; 7.5%)	5.141 (0.284; 5.5%)	0.018	–	0.019
Mineral maturity	1.773 (0.235; 13.3%)	1.717 (0.271; 15.8%)	ns	1.954 (0.195; 10.0%)	1.813 (0.276; 15.2%)	ns		0.214 (ns)
Collagen maturity	3.769 (0.422; 11.2%)	3.984 (0.391; 9.8%)	ns	4.516 (0.483; 10.7%)	4.305 (0.330; 7.7%)	ns	+	0.016
DMB (g/cm ³)	1.027 (0.058; 5.6%)	1.079 (0.037; 3.4%)	0.0057	1.191 (0.047; 3.9%)	1.260 (0.029; 2.3%)	<0.0001		0.145 (ns)

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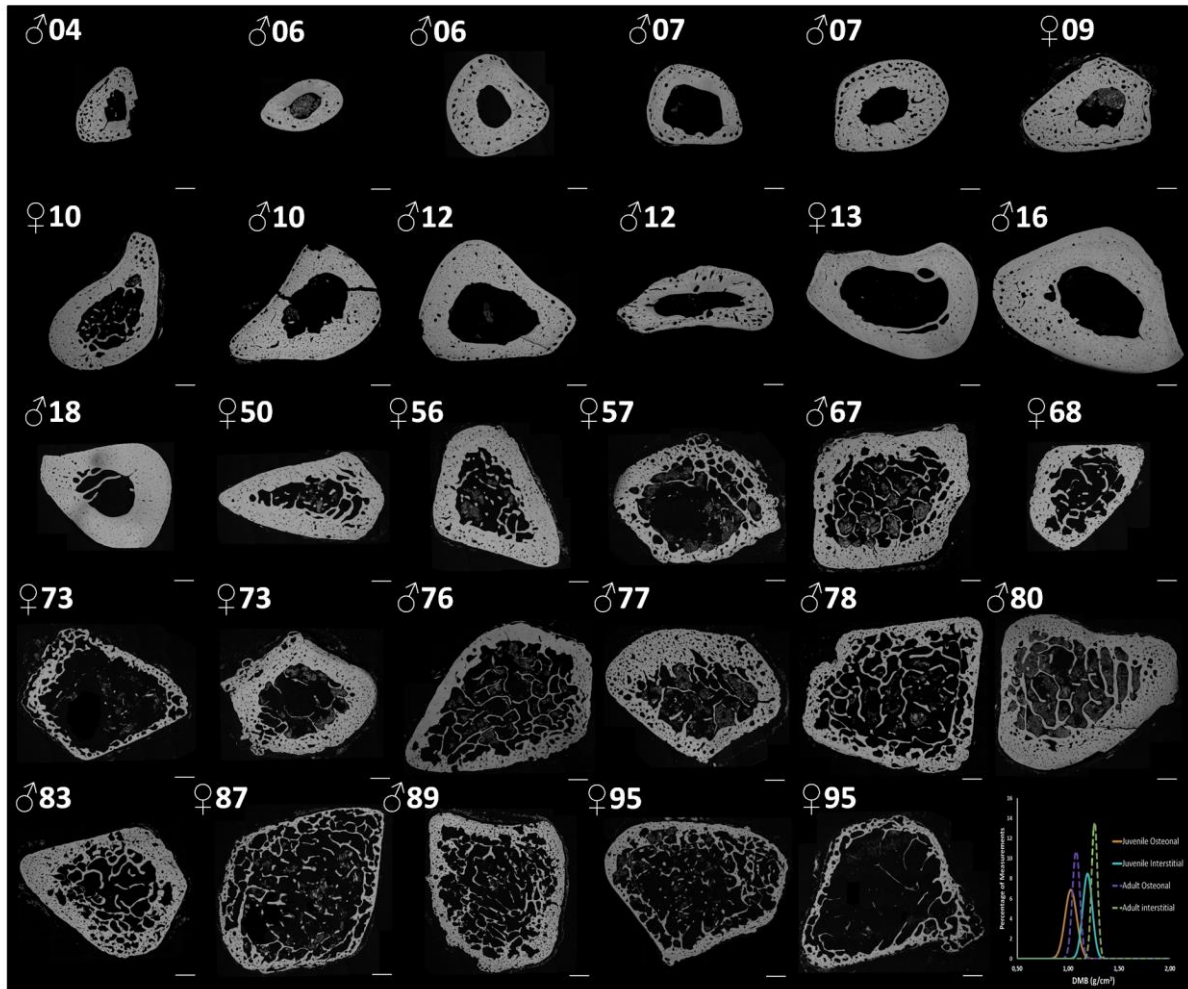
914 **Table 4.**
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Variables		Final adjusted R ²	Part correlation (β)	p-value
Dependent	Independent			
JUVENILES GROUP				
E*	DMB	0.676	0.493	0.006
	Crystallinity		-0.141	0.474
	Carbonation		0.687	0.000
	Mineral/ Matrix		0.549	0.014
	Mineral maturity		0.129	0.515
	Collagen maturity		-0.224	0.322
H/E*	DMB	0.154	0.086	0.744
	Crystallinity		-0.027	0.932
	Carbonation		-0.471	0.054
	Mineral/ Matrix		0.189	0.569
	Mineral maturity		-0.447	0.172
	Collagen maturity		-0.314	0.390
W _{plast} / W _{tot}	DMB	0.256	-0.103	0.678
	Crystallinity		0.143	0.631
	Carbonation		0.386	0.088
	Mineral/ Matrix		-0.580	0.073
	Mineral maturity		0.514	0.098
	Collagen maturity		0.382	0.267
ADULTS GROUP				
E*	DMB	0.479	0.736	0.004
	Crystallinity		-0.002	0.994
	Carbonation		-0.144	0.566
	Mineral/ Matrix		-0.064	0.838
	Mineral maturity		0.010	0.972
	Collagen maturity		0.036	0.829
H/E*	DMB	0.268	-0.620	0.031
	Crystallinity		0.706	0.016
	Carbonation		-0.361	0.229
	Mineral/ Matrix		0.285	0.441
	Mineral maturity		-0.905	0.009
	Collagen maturity		0.433	0.039
W _{plast} / W _{tot}	DMB	0.330	0.482	0.075
	Crystallinity		-0.903	0.002
	Carbonation		0.196	0.490
	Mineral/ Matrix		-0.262	0.459
	Mineral maturity		0.884	0.008
	Collagen maturity		-0.377	0.058

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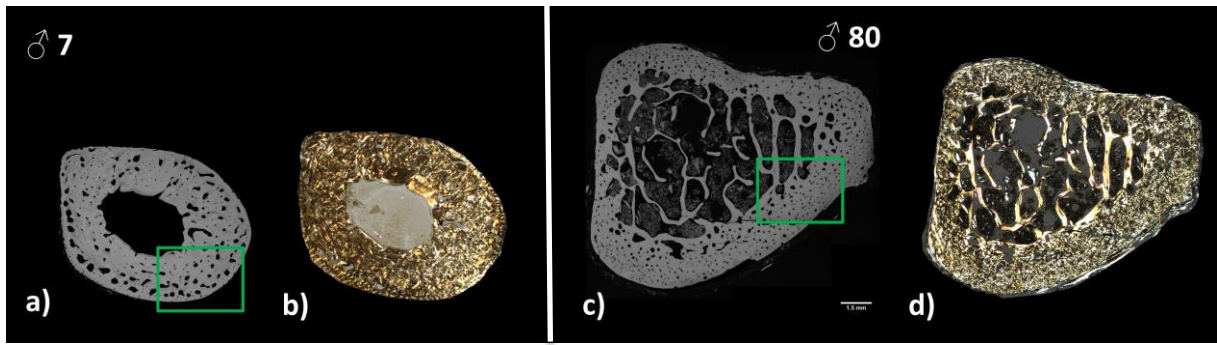
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Figure 1.



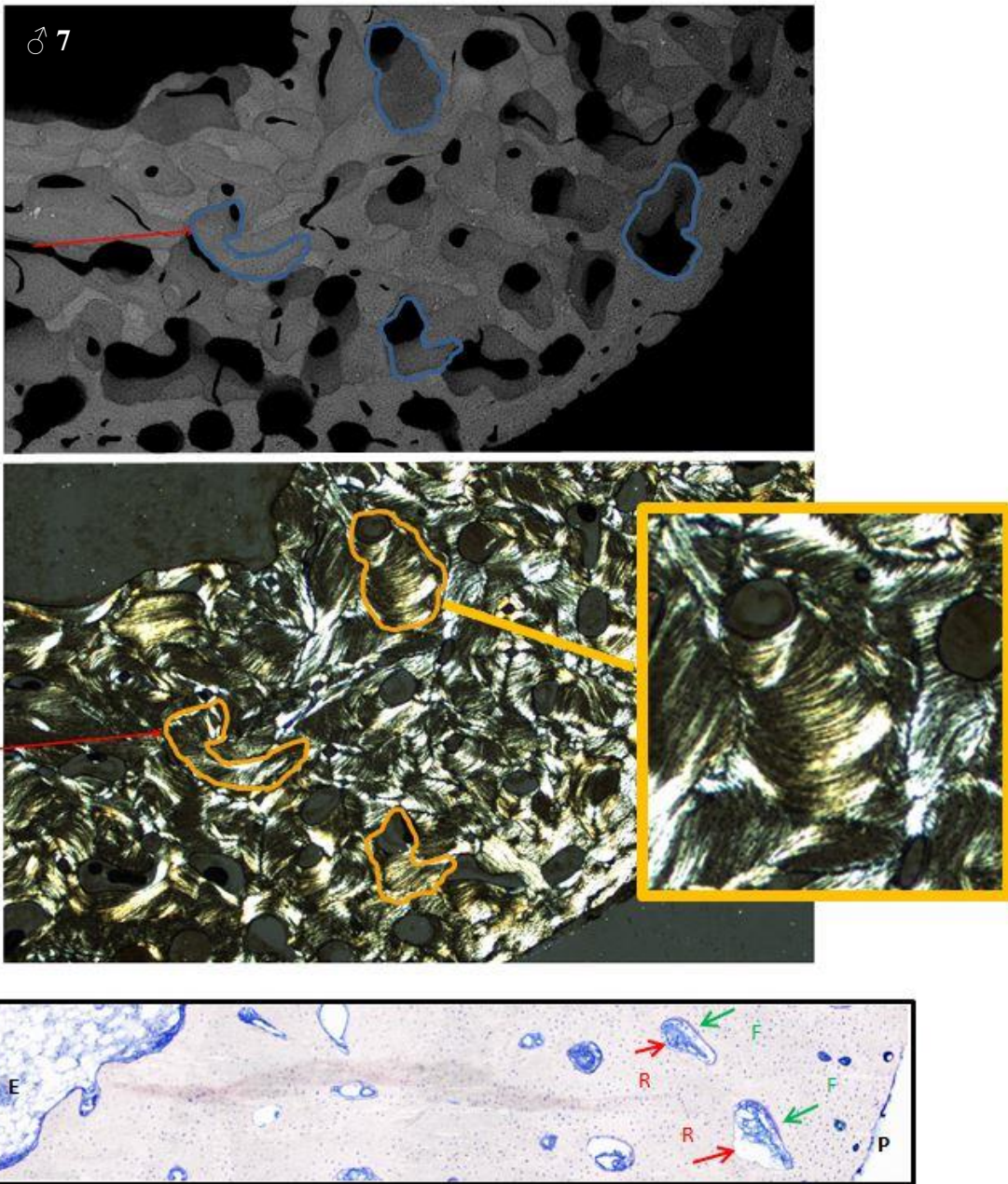
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925 **Figure 2.**
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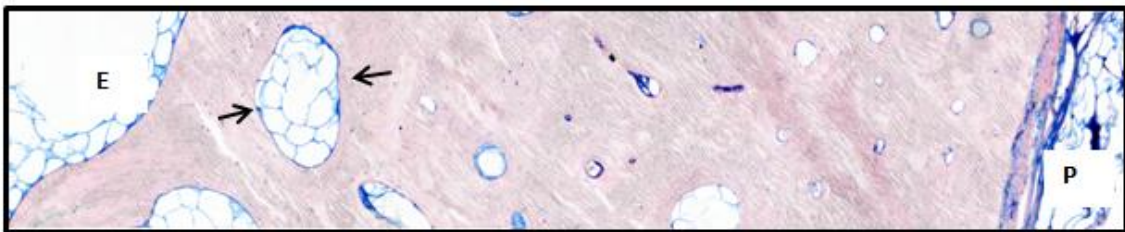
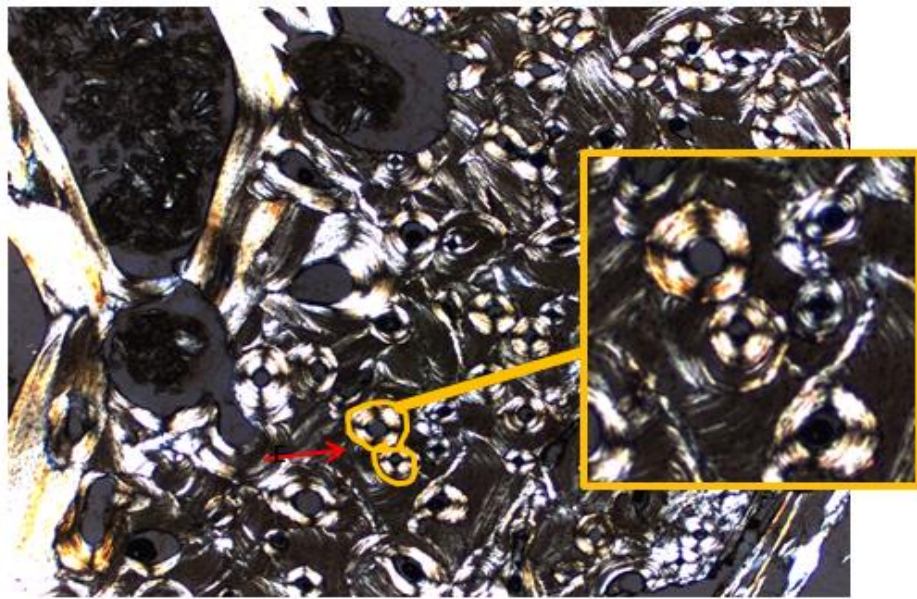
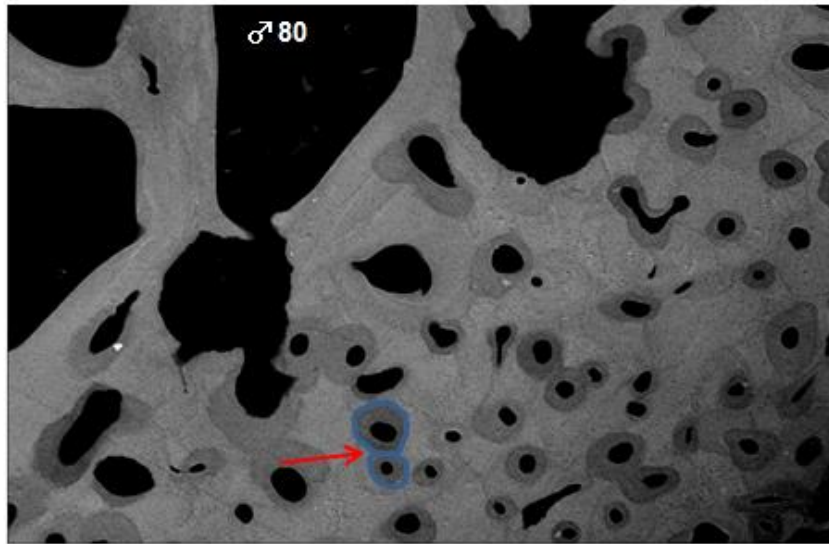
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930 **Figure 3.**
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934 **Figure 4.**
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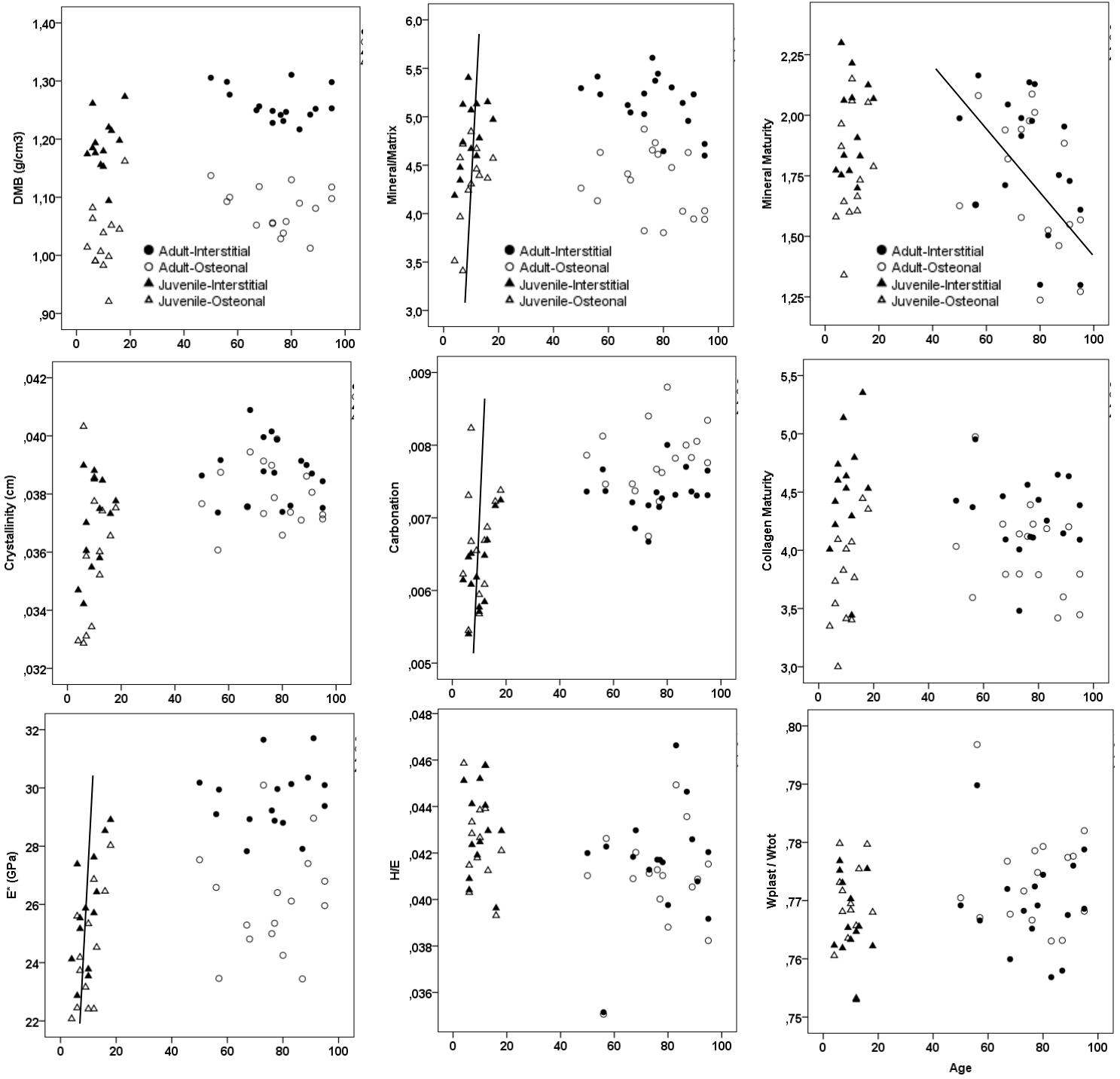


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940 **Figure 5.**

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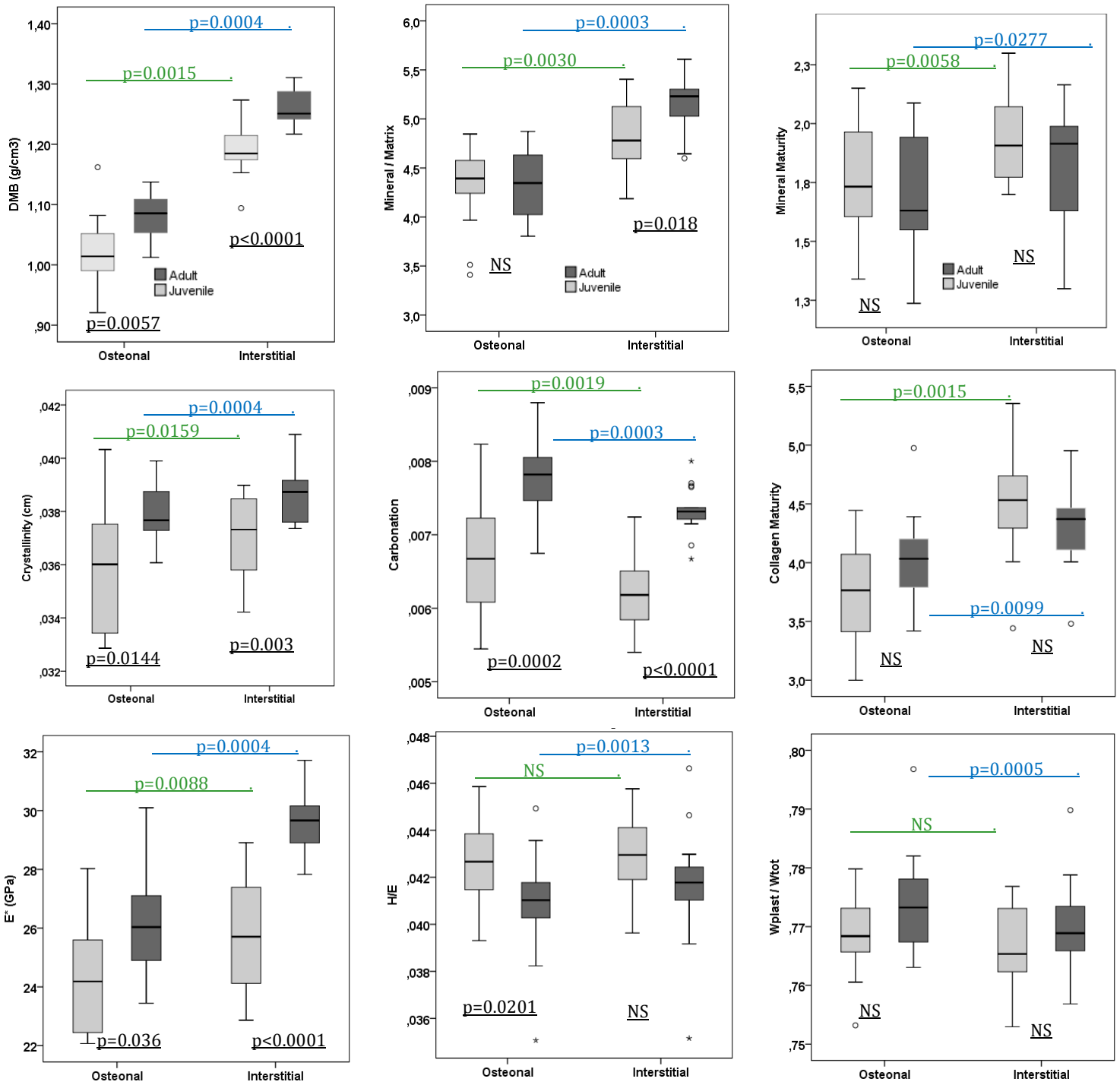
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946 **Figure 6.**
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