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1 **Estimation of the elastic modulus of child cortical bone specimens**  
2 **via microindentation**

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31 **ABSTRACT**

32 *Purpose:* Non-pathological child cortical bone (NPCCB) studies can provide clinicians with vital  
33 information and insights. However, assessing the anisotropic elastic properties of NPCCB remains a  
34 challenge for the biomechanical engineering community. For the first time, this paper provides elastic  
35 moduli values for NPCCB specimens in two perpendicular directions (**longitudinal** and **transverse**) and  
36 for two different structural components of bone tissue (osteons and interstitial lamellae).

37 *Materials and Methods:* Microindentation is one of the reference methods used to measure bone  
38 stiffness. Here, 8 adult femurs (mean age  $82\pm 8.9$  years), 3 child femurs (mean age  $13.3\pm 2.1$  years) and 16  
39 child fibulae (**mean age  $10.2\pm 3.9$  years**) were used to assess the elastic moduli of adult and children  
40 bones by microindentation.

41 *Results:* For adult specimens, the mean moduli measured in this study are 18.1 (2.6) GPa for osteons,  
42 21.3 (2.3) GPa for interstitial lamellae and 13.8 (1.7) GPa in the **transverse** direction.  
43 For child femur specimens, the mean modulus is 14.1 (0.8) GPa for osteons, lower than that for interstitial  
44 lamellae: 15.5 (1.5) GPa. The mean modulus is 11.8 (0.7) GPa in the **transverse** direction. Child fibula  
45 specimens show a higher elastic modulus for interstitial lamellae 15.8 (1.5) than for osteons 13.5 (1.6),  
46 with 10.2 (1) GPa in the **transverse** direction.

47 *Conclusion:*

48 For the first time, NPCCB elastic modulus values are provided in **longitudinal** and **transverse** directions  
49 at the microscale level.

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51 **KEYWORDS:** Pediatrics; Cortical bone; Elastic modulus; Microindentation

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## 62 **Introduction**

63  
64 Bone is a multi-scale living tissue engaged in a continuous remodeling process of successive resorption  
65 and formation phases. This enables it to respond to mechanical stimuli, to fix the microdamaged matrix  
66 and to adjust the homeostasis of calcium (1).

67 At microscale level, this remodeling forms osteons or Haversian systems, the main structural units in  
68 cortical bone. An osteon, a cylinder roughly 200 or 250  $\mu\text{m}$  in diameter, is formed of mineralized  
69 collagen fibrils: hydroxyapatite crystals and collagen molecules (2). The crystalline apatite mineral  
70 component plays a role in bone strength (3), and the collagen component in plastic deformation (4). The  
71 **non-osteonal bone is interstitial bone** from previous generation of osteons, subperiosteal and  
72 subendosteal circumferential lamellae. **With age, the portion of osteonal bone increases with respect**  
73 **to the proportion of interstitial bone, as more and more osteons become remodeled** (5). Sobol et al.  
74 (2015) state that the proportion of osteons with a large diameter of Haversian canals increases with age,  
75 due to the activity of osteoclasts and the resorption of the osteon's lamellae during the resorption phase  
76 (6). **Furthermore, the proportion of osteonal bone to interstitial bone also changes with anatomic**  
77 **site (7)**. Different osteon characteristics play a role in the microscale elastic properties of bone: collagen  
78 fibril orientations (8, 9), levels of mineralization (10, 11) and variations in collagen crosslinking (12).  
79 Furthermore, the elasticity of cortical bone is known to be anisotropic at tissue scale, also called  
80 mesoscale (13). To gain insight into how microscale elastic properties contribute to mesoscale anisotropy,  
81 one of the reference techniques used is microindentation. This allows the elastic modulus to be  
82 determined in several directions at microscale level (14, 15, 16, 17). Microindentation involves applying a  
83 force on a polished specimen surface by a Berkovich diamond-tip indenter, enabling indentation modulus  
84 assessment. The literature reports wide use of microindentation on adult bone (9, 18, 19, 20, 21, 22) to  
85 investigate microscale anisotropy by assessing the elastic moduli in different directions.  
86 However, the few existing studies on child bone limited their explorations to the **longitudinal** direction  
87 (23, 24, 25, 26). The reasons for this limitation are the lack of bone pieces available and their irregular

88 shape and small size, an additional challenge in terms of preparing experimentally usable samples.  
89 Moreover, all the studies on child bone using microindentation were conducted on pathological specimens  
90 embedded in epoxy resin, to facilitate the achievement of a flat and smooth surface (23, 25). However,  
91 resin has been demonstrated to affect microindentation measurements by increasing the indentation  
92 modulus (27).

93 This article is the first to provide elastic moduli values for NPCCB specimens in two perpendicular  
94 directions (**longitudinal** and **transverse**) and for two different structural components of bone tissue  
95 (osteon and interstitial lamellae).

96

## 97 **Materials and methods**

98

### 99 *Specimen preparation*

100 8 adult femurs (mean age  $82\pm 8.9$  years) were collected from corpses at the Timone Hospital (Marseille,  
101 France). **19** child bone pieces from **16 fibulae (mean age  $10.2\pm 3.9$  years)** and 3 femurs (mean age  
102  $13.3\pm 2.1$  years) were recovered during bone-lengthening surgery in the Timone hospital. Both adult and  
103 child specimens were collected from the distal 1/3 of the femur, and child specimens from the distal 1/3  
104 of the fibula. The children were free of pathologies affecting bone quality and were not bedridden. The  
105 National Commission for Data Protection and Liberties (CNIL-France) accepted the experimental  
106 protocol and ethical approval was granted by the anatomy laboratory. Informed consent was obtained  
107 from the children's legal representatives.

108 The specimens of child bone, **the longest being under 2 cm**, were cut into rectangular parallelepipeds  
109 by a water-cooled low-speed diamond saw (Buehler Isomet 4000, Buehler, Lake Bluff, IL, USA).  
110 Throughout cutting, the bone was hydrated with buffered saline (28). The mean dimensions of these  
111 specimens along the radial, circumferential and **longitudinal** directions, respectively, are the following:  
112 adult femurs:  $2.2\pm 0.5 \times 5.8\pm 0.6 \times 10.7\pm 1.9$  mm<sup>3</sup>, child fibulae:  **$1.4\pm 0.6 \times 2.9\pm 0.8 \times 4.5\pm 1.5$  mm<sup>3</sup>** and child  
113 femurs:  $0.9\pm 0.4 \times 3\pm 0.3 \times 6.4\pm 3$  mm<sup>3</sup>. To ensure the parallelism of bone specimens, a specimen holder

114 was especially developed for the cutting phase and adapted to the diamond saw. The direction of the  
115 medullary canal, assumed to be parallel to the Haversian canals in long bones, was considered as  
116 **longitudinal** and the **perpendicular direction to this canal as the transverse direction**. The osteons  
117 and interstitial lamellae were only distinguishable from each other in planes perpendicular to the  
118 **longitudinal** direction (Figure 1). Consequently, the elastic modulus of the osteon and interstitial lamellae  
119 were measured separately only in the **longitudinal** direction (Tables 1 and 2).

120 Specimens were stored at -20°C (29) after cutting. The specimens' surfaces were smoothed using silicon  
121 carbide abrasive paper (grit # 1200) under deionized water and then polished using a 1µm diamond  
122 suspension. After each side (**longitudinal** and **transverse**) was polished, the specimen was cleaned with  
123 water in an ultrasonic cleaner system (Elma/DE-78224 Singen, Germany) to remove the polishing dust.  
124 During microindentation measurement, they were fully hydrated and maintained at room temperature  
125 (~23°C).

## 126 ***Microindentation***

127 The microindentation equipment used (Tester *NHT*<sup>2</sup>, Anton Paar, Switzerland and Austria) provides an  
128 elastic indentation modulus at microscale level with a Berkovich diamond tip displaced in the vertical  
129 direction.

130 A fused silica reference sample was used to calibrate the tip contact surface. Each indentation was  
131 performed with a maximum load of 40 mN and at identical loaded and unloaded rates of 80 mN/min,  
132 separated by a 10 s pause. The hold period of 10 s was intended to stabilize any creep in the specimens  
133 and to avoid the formation of the common 'nose'-shaped load displacement curve responsible for  
134 inaccurate calculations of the unloading curve section (30). The distance between two indentations was 20  
135 µm, with a maximum (contact) depth of 2 µm.

## 136 ***Theory***

137 The Oliver-Pharr method is a well-documented way of determining the elastic modulus from the  
138 unloading curve, using the assumptions for a linear elastic isotropic material (31). Stiffness (*S*) reflects

139 the measurement of bone resistance to an elastic deformation (16). It characterizes the force-displacement  
140 relation:

$$141 \quad S = \frac{dF}{dh} = \frac{2}{\sqrt{\pi}} \beta E_r \sqrt{Ac}, \quad (1)$$

142 where F is the force applied, h is the total displacement at any time during loading,  $\beta$  is a correction factor  
143 for the unaxisymmetric indenter and is equal to 1.034,  $E_r$  is the reduced modulus,  $Ac$  is the projected area  
144 of contact ( $Ac=24,5 hc^2$  for Berkovich indenter,  $h$  is the contact depth of the indenter with the sample at  
145  $F_{max}$ ). The measurement of  $E_r$  from Equation (1) leads to the elastic modulus of the bone,  $E^*$ :

$$146 \quad E^* = \frac{1-\nu_s^2}{\frac{1}{E_r} - \frac{1-\nu_i^2}{E_i}}, \quad (2)$$

147 where  $\nu_s$  is the Poisson ratio of the bone ( $\nu_s=0.3$ ),  $E_i$  is the elastic modulus of the indenter tip ( $E_i = 1141$   
148 GPa) and  $\nu_i$  is the Poisson ratio of the indenter ( $\nu_i= 0.07$ ).

### 149 *Maps of indentations*

150 In this study, 80 indentations per specimen were distributed in the **longitudinal** direction as follows: 20 in  
151 an osteon, 40 in two different interstitial lamellae and 20 in a neighboring osteon. Thus, 50 indentations  
152 per specimen were performed in the **transverse** direction without distinguishing between osteon and  
153 interstitial lamellae, which cannot be differentiated from each other in the **transverse** direction (32).

154 Typical indentations in the **longitudinal** direction and in the **transverse** direction for a 15-year-old child  
155 are shown in Figure 1. In summary, 130 indentations were performed on each of the **27** bone specimens.

156 Of these **3510 indentations**, 20% were excluded from the analysis, mainly because they could not be  
157 clearly identified as belonging to either the osteon or the interstitial regions in the **longitudinal** direction.

158 Some were excluded because their load-depth curve showed irregularities (9).

### 159 *Statistical analysis*

160 Data are reported as mean and standard deviations unless otherwise stated. The analyses were based on  
161 three different categories: adult (femur), child (femur) and child (fibula). Statistical analyses were  
162 performed using Xlstat (v 2014.1.08, Addinsoft. 2016. XLSTAT, 2016: Data Analysis and Statistical

163 Solution for Microsoft Excel, Paris, France (2016)). Normality was tested via the Shapiro-Wilk procedure  
164 and equality of variances via the Fisher test. Differences between adult (femur) and child (femur) groups  
165 were tested using the Mann-Whitney test. **Bivariate correlations between the elastic moduli of child**  
166 **fibulae and age were tested by the Spearman's rank correlation test (r).**  
167 Differences between locations and directions (**longitudinal** osteon, **longitudinal** interstitial lamellae and  
168 **transverse**) were tested using the Wilcoxon test. A two-tailed significance level of 0.05 was used.

169

## 170 **Results**

171

172 **For child femur specimens, the mean modulus is 14.1 (0.8) GPa for osteons, which is lower than**  
173 **interstitial lamellae, 15.5 (1.5) GPa. The mean modulus is 11.8 (0.7) GPa in the transverse direction**  
174 **(Table 1). The fibula also has a lower modulus in osteonal bone 13.5 (1.6) GPa compared to**  
175 **interstitial 15.8 (1.5) GPa. And the fibula modulus is 10.2 (1) GPa in the transverse direction (Table**  
176 **1). The osteons seemed to be stiffer in the femur than in the fibula, possibly because the fibula is not**  
177 **a load-bearing bone.**

178 **For adult specimens, the mean moduli are: 18.1 (2.6) GPa for osteons, 21.3 (2.3) GPa for**  
179 **interstitial lamellae and 13.8 (1.7) GPa in the transverse direction (Table 1).**

180 **The elastic moduli for the adults' femurs and for both child bone types (femurs and fibulae) are**  
181 **classified in each of the following directions: longitudinal (osteon and interstitial lamella) and**  
182 **transverse (Table 1). A significant difference between the elastic moduli of adult and child femur**  
183 **specimens in different directions is shown in Table 1.**

184 **For both adults and children, interstitial bone has a significantly higher modulus than osteonal**  
185 **bone, and the longitudinal modulus of both osteonal and interstitial bone is higher than the**  
186 **transverse modulus (Table 2).**

187

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189

## 190 **Discussion**

191 **We have assessed the tissue level mechanical properties of non-pathologic cortical bone in children.**  
192 **Bone is known to be a multiscale tissue, with each scale playing a role in bone quality and bone**  
193 **disorders (34). The Haversian structure of long human bones contributes to the anisotropy of**  
194 **mesoscale elastic properties of the cortical part of the bone. However, this elastic anisotropy is also**  
195 **a microscale characteristic of bone tissues. This paper focuses on the microscale level by measuring**  
196 **the mechanical properties of NPCCB specimens in two perpendicular directions.**

197 **In the literature, indentation protocols vary and entail different parameter values. In most**  
198 **studies, the bone specimens are embedded in epoxy resin, nevertheless it has been proven that the**  
199 **resin, constricts the movement of the collagen fibrils and increases connectivity within the bone**  
200 **material (27). Therefore it changes the bone mechanical properties providing greater bone elastic**  
201 **moduli values. Bone specimens are best prepared without using chemicals for cleaning and without**  
202 **dehydration, thereby preserving the elastic properties of the microstructure features (9). In the**  
203 **present study, the specimens were not embedded in resin. For adult bone, the elastic moduli for**  
204 **osteons 18.1 (2.6) GPa and for interstitial lamellae 21.3 (2.3) GPa in longitudinal direction were**  
205 **lower than those obtained by Rho et al. (2002) (17), 21.8 (2.1) GPa for osteon and 23.8 (1.8) GPa for**  
206 **interstitial lamellae. This difference could be due to the fact that the bone specimens in Rho's study**  
207 **were embedded in epoxy resin (15), whereas the bone specimens in the present work were not**  
208 **embedded and were kept hydrated.**

209 **In addition to that, the elastic modulus,  $E^*$ , depends also on the maximum load, the**  
210 **loading/unloading rate and the contact depth,  $h$ . For example, Rho et al. (1997) (16) used a load of**  
211 **13.5 mN with a contact depth of 1000 nm, whereas Bala et al. (2011) (22) chose a maximum load of**  
212 **500 mN with a contact depth of 5000 nm; both were addressing embedded bone specimens. In this**  
213 **study, the maximum load of 40 mN induced contact depths of roughly 2000 nm, within the range of**

214 these literature values. The load chosen in the present work was higher than many other loads in  
215 different protocols in the literature (20, 22, 23), since we were seeking to measure a mean value for  
216 the elastic moduli in the same way as Bala et al. (2011) (22), avoiding lamellar heterogeneity.

217 To the best of our knowledge it is the first time the elastic moduli of NPCCB are assessed by  
218 microindentation. Consequently, adults specimens were needed to ensure the reliability of the  
219 measurements. The values measured on the adult specimens were close to those reported in Zysset  
220 et al. (1999) (18): 19.1 (5.4) GPa for osteons and 21.2 (5.3) GPa for interstitial lamellae on human  
221 femur specimens which were not embedded in epoxy resin, were hydrated and have no pathological  
222 diseases. Similarly, in accordance with Reisinger et al. (2011) (9) who tested also non-resin,  
223 hydrated and non-pathological adult femur specimens, the longitudinal elastic modulus is greater  
224 than the elastic modulus measured in transverse direction.

225 Moreover, studies using microindentation as Rho et al. (1997) (16) were done on adult tibiae  
226 yielding lower moduli for osteons 22.5 (1.3) GPa than for interstitial lamellae 25.8 (0.7) GPa. These  
227 tibiae specimens were embedded in epoxy resin and cannot be directly compared to the values  
228 obtained in the present work due to the presence of resin and the different bone type. However, the  
229 ratio between the moduli of the osteonal and the interstitial lamellae of the study can be discussed  
230 as reference to the Rho's study since the resin is supposed to act in a similar way on both types of  
231 tissue. In both studies, the elastic modulus of the interstitial lamella is greater than the elastic  
232 modulus of the osteon.

233 Adult femur specimens were used in this study to compare their longitudinal and transverse  
234 elastic moduli child femur specimens. The results show a significant difference (Table 1). This  
235 different mechanical behavior could be linked to structure changes. Structural parameters varying  
236 with age include the number of osteons and osteon fragments (35), the percentage of unremodeled  
237 bone, and the number of non-Haversian canals or primary osteons (36). Non-Haversian canal  
238 osteons, primary vascular channels filled with concentric lamellae, are known to be present in child  
239 bone and almost disappear in adult bone, unlike the number of osteons, which increases with age

240 (37). All the specimens (adult and child) had a greater elastic modulus in the interstitial lamellae  
241 than in the osteon, and in the longitudinal than in the transverse direction.

242 Spearman test was used to investigate the correlation between age and elastic moduli for children  
243 fibula. Only the elastic modulus of osteon was significantly correlated to age ( $r=0.4736$  for a-  
244 risk=5%). This could be associated to the mineralization of growing bone (38). Significant  
245 correlation with age could be found for elastic moduli of interstitial lamellae and transverse but  
246 with less confidence 10% and 15% respectively. One of the reasons could be that the interstitial  
247 lamellae cannot be distinguished from woven/primary bone which is still present in growing  
248 cortical bone and could affected the elastic moduli. As the osteons are easily identified in planes  
249 perpendicular to the bone axis, the measurement of the elastic modulus is more reliable. Moreover,  
250 it is the first microindentation study on NPCCB in literature. This significant correlation is  
251 interesting and can be related it to the composition and structure of the growing bone in future  
252 works.

253 As for animal, the cortical bone becomes stiffer with age (39). The elastic modulus of rabbit  
254 cortical bone increases significantly during growth and maturation (40). For porcine femurs, the  
255 elastic modulus of osteons in young (6 months) and old (42 months) bones (17.5 GPa versus 18 GPa)  
256 is lower comparing to the interstitial bone (19 GPa versus 20 GPa). Moreover, the longitudinal  
257 elastic modulus of these porcine specimens (18.25 GPa versus 19 GPa) was always higher than in  
258 the transverse direction (9 GPa versus 14 GPa). For Casanova et al. (2017) (41), the elastic modulus  
259 for femoral mice cortical bone in the longitudinal direction is 16 GPa and is higher than that of 12.5  
260 GPa in the transverse direction. These observations agree well with the results and other studies  
261 addressing bone anisotropy using microindentation (9, 19, 42).

262 Nevertheless, we were unable to further investigate the degree of anisotropy due to the low  
263 number of specimens and, above all, because osteon and interstitial lamellae cannot be  
264 distinguished in the transverse direction in the indentation protocol. Table 2 therefore compares  
265 longitudinal osteon, longitudinal interstitial lamellae and transverse, as proposed by Rho et al.

266 (1999) (32). Another limitation of this work is its use of the Oliver-Pharr method assuming that  
267 bone is locally isotropic. A useful direction for further work would be to integrate the anisotropic  
268 model (22).

269 The assessment of the elasticity modulus at the microscopic scale is part of a larger study on  
270 multimodal and multi-scale characterization of the NPCCB (33) aimed at obtaining maximum  
271 exploitable information from these valuable and rare specimens. Developing a database of multi-  
272 scale mechanical properties of children bones will allow making dedicated models to better  
273 understand certain pathological mechanisms characteristic of the growing bone (green wood  
274 fractures, osteopenia), to improve diagnostic procedures and thus adapt and develop the  
275 therapeutic choices: prostheses, orthopedic surgery, and rehabilitation.

276 An important future goal is to link these microscale parameters to the mesoscale mechanical  
277 behavior of bone, using homogenization techniques. Coupled with increasingly effective imaging  
278 techniques, this could yield relevant analytical and/or numerical models (43) for the estimation of  
279 child bone quality. In addition, both assessing the mechanical parameters and analyzing the degree  
280 of anisotropy of cortical bone will be vital to the clinical assessment of bone quality. In conclusion,  
281 the contribution of this study lies in determining the elastic moduli for NPCCB specimens at  
282 microscale level, providing the first measurements of an indentation modulus in longitudinal and  
283 transverse directions.

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#### 290 **Declaration of interest**

291 The authors report no conflicts of interest. The authors alone are responsible for the content and writing of  
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293

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**Figure 1.** Typical indentations on osteon and interstitial structures at a: **longitudinal** side for a 15-year-old child and b: **transverse** side for the same child.



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**Table 1.** Elastic moduli of all adult and child specimens in **longitudinal** and **transverse** directions. P-values for group comparison were obtained using Mann-Whitney test. Bold denotes p-values < 0.05.

	<b>Adult (femur)</b> <b>(82±8.9)</b> <i>E*</i> Mean (SD*)	<b>Child (femur)</b> <b>(13.3±2.1)</b> <i>E*</i> Mean (SD*)	<b>p-value</b> <b>Adult</b> <b>(femur)</b> <b>and</b> <b>Child (femur)</b>	<b>Child (fibula)</b> <b>(10.2±3.9)</b> <i>E*</i> Mean (SD*)
<b>Longitudinal OSTEON</b>	18.1 (2.6)	14.1 (0.8)	<b>0.024</b>	13.5 (1.6)
<b>Longitudinal INTERSTITIAL</b>	21.3 (2.3)	15.5 (1.5)	<b>0.142</b>	15.8 (1.5)
<b>Transverse</b>	13.8 (1.7)	11.8 (0.7)	<b>0.041</b>	<b>10.2 (1)</b>

465 \*=standard deviation.

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467 **Table 2.** Differences in the elastic moduli between different locations  
 468 (osteon and interstitial lamellae) and directions (**longitudinal** and **transverse**)  
 469 of adult and child (fibula) groups using Wilcoxon test.

		<b>Adult</b> <b>(femur)</b> <b>(82±8.9)</b> p-value	<b>Child</b> <b>(fibula)</b> <b>(10.2±3.9)</b> p-value
<i>E*</i> (GPa)	<b>Longitudinal OSTEON versus Longitudinal INTERSTITIAL</b>	<b>0.011</b>	<b>0.0004</b>
<i>E*</i> (GPa)	<b>Longitudinal OSTEON versus Transverse</b>	<b>0.025</b>	<b>0.0004</b>
<i>E*</i> (GPa)	<b>Longitudinal INTERSTITIAL versus Transverse</b>	<b>0.011</b>	<b>0.0004</b>

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