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**ASSESSMENT OF MECHANICAL PROPERTIES OF HUMAN OSTEON
LAMELLAE EXHIBITING VARIOUS DEGREES OF MINERALIZATION
BY NANOINDENTATION**

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Running title: mechanical assessment of osteons

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ABSTRACT

Background: Cortical bone analysis was investigated at the macroscopic level with mechanical tests and imaging techniques while few studies were done at the microscopic level (osteons). The purpose of this study is to measure the elastic modulus of thick lamellae of osteons exhibiting different degrees of mineralization. This study might provide for the clinician a better understanding of bone remodeling and help assessing the different stages of bone healing.

Methods: Six femoral human samples (5x4x4mm) were cut transversally along the length of a Human femur. Scanning electron micrographs were produced in order to have a mapping of back scattered electron reflecting the composition of the microstructure. Three types of osteons were selected: white (mineralized osteon), grey (intermediate mineralization) and dark (low mineralization) osteons. Nanoindentation tests were performed on three locations of thick lamellae located in the middle of each osteon. The mechanical test induces 3 holdings and unloadings with a constant holding during 10 seconds. The maximal force was 2500 μ N, which induced a maximal depth of about 400nm.

Results: Elastic modulus (E) and hardness (H) for the white (N=61), grey (N=17) and dark osteons (N=39) were $E_{\text{white}} = 21.30 \pm 3.00\text{GPa}$ ($H_{\text{white}} = 0.55 \pm 0.15$), $E_{\text{grey}} = 19.27 \pm 1.78\text{GPa}$ ($H_{\text{grey}} = 0.41 \pm 0.09$) and $E_{\text{dark}} = 12.95 \pm 2.66\text{GPa}$ ($H_{\text{dark}} = 0.30 \pm 0.10$), respectively. The variation of elastic properties within a lamella was approximately 2.4 GPa, depending on the level of mineralization.

Conclusions: These results demonstrate the inhomogeneity of the lamella suggesting that both orientation of collagen fibers and degree of mineralization may vary within the lamella. Our study shows a large range of elastic properties and hardness reflecting different degrees of osteons mineralization.

Keywords

Nanoindentation – cortical Human bone – bone remodeling – osteons lamellae – Elastic modulus.

1. INTRODUCTION

The mechanical properties of Human cortical bone were investigated at the tissue (haversian system) and microstructural (osteon, osteon lamellae) levels.

At the microstructural level, three types of osteons were identified based on the collagen fibers orientation and classified as transversal (type I), alternated (type II) and longitudinal (type III)¹⁻⁴. The mechanical properties of isolated osteons were determined using different mechanical tests such as compressive, torsion and bending tests.

Compressive tests were performed on osteons from two human femoral bones (30-80 years old) with two different degree of calcification. The authors found that the values of the elastic properties for the osteons increased from type III to I for calcified and initial stage of calcification. Furthermore, the range of values of initial stage of calcification was found to be lower than the fully calcified ones². Tensile tests were performed on dry and wet osteons (of various types) of a human femur (30 years old) with two stages of calcification. Dry specimen exhibited higher mechanical properties and the degree of calcification increased the elastic properties of osteons, which were more significant for the wet specimen¹. Torsional tests were also applied on fully calcified femoral osteons and it was showed that the shear elastic modulus for the osteons type III was higher than the osteons type II⁴. The bending properties of the single osteon (type II, III) were investigated and the elastic properties of the osteon type II were found to be higher than the third type³. Thus the alternate structure seems to the authors to be more adapted to the bending stresses. To summarise, according to the author each osteon type exhibited specific mechanical properties related to the collagen fibers orientation to support different types of stresses.

First investigations on the mechanical properties of the osteon lamellae (wide between 3 to 7 μ m) were performed using nanoindentation technique by Rho et al. (1997)⁵. Young's

modulus of osteon and interstitial lamellae were measured on different human bones (femur and tibia) and different categories of bone (cortical and spongy)⁵⁻¹¹. Values of the interstitial lamellae were found to be significantly higher (range about 26GPa) than those of the secondary osteon lamellae (19-22.5GPa). The range of hardness found for the secondary osteon in the literature varied between 0.42 and 0.65GPa⁹⁻¹⁰. The elastic properties of the osteon lamellae varied also from the center of the osteon to the outer showing higher value in the center (about 2GPa). This result was not found to be consistent for all the osteons¹². In addition to the osteon area, the elastic properties of osteon lamellae were dependant on the anatomical location. For instance, values which were measured from the diaphysis to the femoral neck varied about 16%¹³. According to the author, the elastic moduli found for the femoral neck were lower than those of the mid diaphysis. This last observation could explain the fragility of the femoral neck at the macroscopic scale.

Thick and thin osteons lamellae underwent nanoindentation tests with different experimental configurations (different indentation depth) and different physiological conditions (dry and wet)⁷. The thick lamellae showed higher mechanical properties (indentation modulus and hardness) for low depth indentation while the mechanical properties decreased for higher depth (more significantly than for the thick lamellae). The results are consistent whatever physiological conditions are. According to the author, it supports that the thick and thin lamellae had different composition and ultrastructure properties.

The question of the influence of age on the elastic properties of lamellae was analysed by Rho et al. (2002)¹¹. They concluded no significant difference of elastic properties was observed between the young and old people due probably to the low number of samples¹¹.

The nanoindentation measurements were able to show variations of elastic properties of osteon lamellae related to the anatomical location, experimental conditions and anisotropy.

Moreover, different types of lamellae (thick, thin, interstitial) showed specific elastic properties related to different chemical compositions. At the osteon level, influence of decalcification was demonstrated whatever osteon type is but no investigation was performed at the osteon lamellae.

The purpose of this study is to identify the degree of mineralization of the osteons in order to measure the mechanical properties of the osteons lamellae in process of mineralization by nanoindentation technique.

2. MATERIALS AND METHODS

2.1 Specimen preparation

A cadaver femur (natural death, male 70 years of age) was obtained from the anatomical laboratory of Hospital University of Amiens. Six samples (5*4*5mm) were cut parallel to the axis of the femur with a low-speed diamond saw (MICROCUT 2). These samples were extracted in the lateral and medial sides of the femur between 40% and 70% of the total length. Each sample were ground with different sizes of sandpaper (#600, #800 and #1200) in order to obtain a smooth surface and then polished with 0.5 μ m aluminum powder. The control of the surface was performed through an optical microscope in reflection (NIKON 198477 Japan) with a magnitude of $\times 10$ at the Integrated Microscopy Center at the University of Memphis.

2.2 Calibration

Fused silica which is an elastic isotropic material was used in order to calibrate the tip shape function of the indenter. This material was currently used for this kind of technique calibration because the ratio between the modulus and the hardness was very low.

2.3 Scanning electronic microscope

A back scattered environmental scanning electron micrographs (XL 30 ESEM Philips) was produced under high vacuum at 20KV with a magnification of 45. All the surface of the samples was scanned with an electron beam in order to have a mapping of back scattered electron reflecting the mineralization constituting the surface. This study was performed in the Integrated Microscopy Center at the University of Memphis. The advantage of this technique was that none of the specimen surface was treated. Then, three different types of osteons was

identified based on an image preprocessing. An histogram on the grey level (0-255) of the native images allowed to identify three ranges of grey level (89-107, 110-122, 120-127) corresponding to the white, grey and dark osteons, respectively (fig. 1).

Thus the white osteons appeared with a lighter grey level (representing a high degree of mineralization) with an unclear outer border compared to the grey osteons represented with a darker grey level (representing an intermediate degree of mineralization) and a well defined external border. The dark osteons (representing a low degree of mineralization) reflected a very dark grey level compared to the two other ones.

2.4 Nanoindentation technique

The measurements of the Young's modulus were determined through a mechanical technique which applied compressive tests at the microstructural level. The mechanical equipment was composed of a Berkovich diamond indenter which has a pyramidal shape and an optical microscope (fig. 2). All the indented regions were previously selected and memorized through the optical microscope, then the indenter was optically driven on the target which needed to be indented. The load – time cycle was constituted of seven segments (fig. 3): firstly two loadings and unloadings were applied in order to decrease the plasticity of the material and to reduce the viscoelasticity which was revealed through the hysteresis and indicated the viscoelasticity of the material. Furthermore the viscoelasticity was also present during the creep displacements at the peak load, thus a third loading followed by a constant holding during a time of ten seconds was performed to decrease these viscoelasticities. Lastly the tip was withdrawn and the elasticity of the material was measured on the last unloading. Thus, the load – displacement (fig. 4) was performed with a maximum peak load at 2500 μ N which induced impressions with a depth of about 400nm. On the last unloading based on the load-deformation curve, the stiffness was measured experimentally between 50% and 95% of the

peak load. The elastic moduli of the sample (E_s) was related to the stiffness (S) and the contact area (A) by the method of Oliver and Pharr (1992):

$$\frac{\sqrt{\pi}}{2} \times \frac{S}{\sqrt{A}} = \left[\frac{1-\nu_i^2}{E_i} + \frac{1-\nu_s^2}{E_s} \right]^{-1} \quad (1)$$

ν is the poisson's ratio and the subscript "s" corresponds to the sample and the subscript "i" is related to the indenter. The indenter is characterized by $E_i = 1140\text{GPa}$ and by $\nu_i = 0.07$.

The hardness (H) was measured with the ratio between the maximum load performed and the contact area (A).

Usually the equation (1) is applied at a macroscopic level for homogeneous and isotropic material but it is well known that the bone material is anisotropic, therefore the effective modulus is an average of the anisotropic elastic constant.

Then, nanoindentation (Hysitron Inc., Minneapolis, MN, USA) tests were performed on the three types of osteons. Each indent was performed three times on a thick lamellae (defined by a lighter grey level compared to the darker thin lamellae) located in the center of osteon considered as the most representative area of the entire osteon⁷ (fig 5). A total of 351 indentations from dry samples were performed on 61 white, 17 grey and 39 dark osteons. The reproducibility was obtained by performing three repeated tests executed three different times on the same osteon .

2.5 Statistical analysis

Anova test was performed with the software Statgraphics 5.0 (Sigma Plus, Maryland, USA) in order to study firstly the variation of elastic properties (E) and hardness (H) within a same thick lamellae, and the difference of elastic moduli and hardness between different types of osteons.

3. RESULTS

3.1 Calibration of the nanoindentation technique

The elastic moduli of the fused silica measured with the nanoindentation technique is about 72.1GPa \pm 0.5GPa. This experimental value was close to the theoretical technique which was 72GPa. This properly calibration enables to confirm the future results found by this technical experimentation.

3.2 Reproducibility of the nanoindentation technique

The reproducibility of elastic modulus and hardness obtained with three repeated tests performed on the same thick lamellae of one osteon is about $E = 1.33\text{GPa}$ and $H = 0,03\text{GPa}$.

3.3 Variation of Elastic properties and hardness within a thick lamellae

The intra lamellae variation of elastic moduli and hardness were summarized in Table 1. The approximately variation of elastic properties and hardness within a lamella were 2.6GPa (with a range of variation from 0.2 to 8 GPa) and 0.11GPa (with a range of variation from 0.01 to 0.43GPa), respectively. These values were dependant on the types of osteons.

3.4 Comparison of Elastic properties and hardness between the different types of osteon lamellae

The inter lamellae elastic moduli and hardness were summarized in Table 2. The elastic properties and hardness for the white and grey osteons were within the same range, but significantly (fig. 6 - 7) ($P < 0.001$) higher than the dark osteons. The white osteons had a higher moduli and hardness compared to the grey ones ($\Delta E = 2\text{GPa}$ and $\Delta H = 0.13$, $P < 0.05$) and the dark ones ($\Delta E = 8\text{GPa}$ and $\Delta H = 0.27$, $P < 0.001$). Furthermore, the elastic moduli

and hardness of the grey osteons were significantly higher ($\Delta E = 6\text{GPa}$ and $H = 0.14\text{GPa}$, $P < 0.001$) than the dark osteons.

4. DISCUSSION

In the present study, the variation of mechanical properties within a lamella was approximately $E = 2.6\text{GPa}$ (with a range of variation from 0.2 to 8 GPa) and $H = 0.11\text{GPa}$ (with a range of variation from 0.01 to 0.43GPa), for the different types of osteons. This result demonstrates the inhomogeneity of the lamella suggesting that both orientation of collagen fibers and degree of mineralization may vary within the lamella. This last result was not in agreement with the study of Ascenzi et al. (1968) who considered the lamellae as homogeneous whatever type it is (I, II, III)².

Three different categories of osteons were characterized by their grey level reflecting different degree of mineralization. The quantitative measurements showed an increased of the elastic moduli and hardness with the grey level of the osteon confirming the presence of different degree of mineralization. The increases of the mechanical properties are in agreement with the data found at the osteon level by Ascenzi et al. (1968) on any osteon type². Our ratio between elasticity of dark and white or grey osteon (factor 2) is close to that found by Ascenzi et al. (1968) for the alternate osteon (type II)². The ratio for the other types of osteons (I, III) would be around 1.3. Our data are statistically significant and provide a quantitative range of elasticity which could be used as reference values for fully and less mineralized osteon lamellae.

In the present study, the range of the elastic moduli and hardness for the white and grey osteons are within the same range than those published in the literature^{5,9-11,13}.

The elastic properties measured in the present study exhibit a variation about 40% (from 13.33GPa to 21.59GPa) between the different types of osteons. It is interesting to note that

this variation of modulus at the microscopic scale is more important than that of the variation found at the macroscopic level i.e. at the tissue level (11-20%)¹⁴. A previous work performed on macro and micro measurements using ultrasound and nanoindentation technique showed that elastic modulus measured at the macroscopic scale represents the homogenized elastic properties measured at the microstructural level¹⁵.

To summarize, the mechanical properties of thick lamellae of different types of osteons in process of mineralization were characterized. Our data provide a range of values of elastic properties of bone at a microstructural level, reflecting the remodelling process. The bone quality assessment using nanoindentation provided helpful information about the level of mineralization and maturation of osteons. The cortical bone analysis described in this study might give to the clinician a better knowledge of bone remodelling and help assessing the different stages of bone healing. Further studies are encouraged using nanoindentation technique in order to improve the pathogenesis understanding of bone development disease.

5. ACKNOWLEDGEMENTS

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Figure 1 : Back scattered image of the surface of the cortical sample. The three different types of osteons are represented with 1: white osteon, 2: grey osteon and 3: dark osteon

Figure 2 : Representation of the coupling between the optical microscope and the indenter.

Figure 3. The load cycle applied to the indenter.

Figure 4. Representation of the typical curve representing the load – displacement performed.

Figure 5. Localizations of the indentations

Figure 6. Range of values of elastic modulus for different types of osteons.

(*** : $P < 0.001$ and ** : $P < 0.05$).

Figure 7. Range of values of hardness for different types of osteons

(*** : $P < 0.001$ and ** : $P < 0.05$).

Table 1. Variability of elastic modulus and hardness (minimum, maximum, median and mean with standard deviation) measured within a thick lamellae from white, grey and dark osteons

Table 2. Comparison of elastic modulus and hardness (minimum, maximum, median and mean with standard deviation) measured within a thick lamellae from white, grey and dark osteons

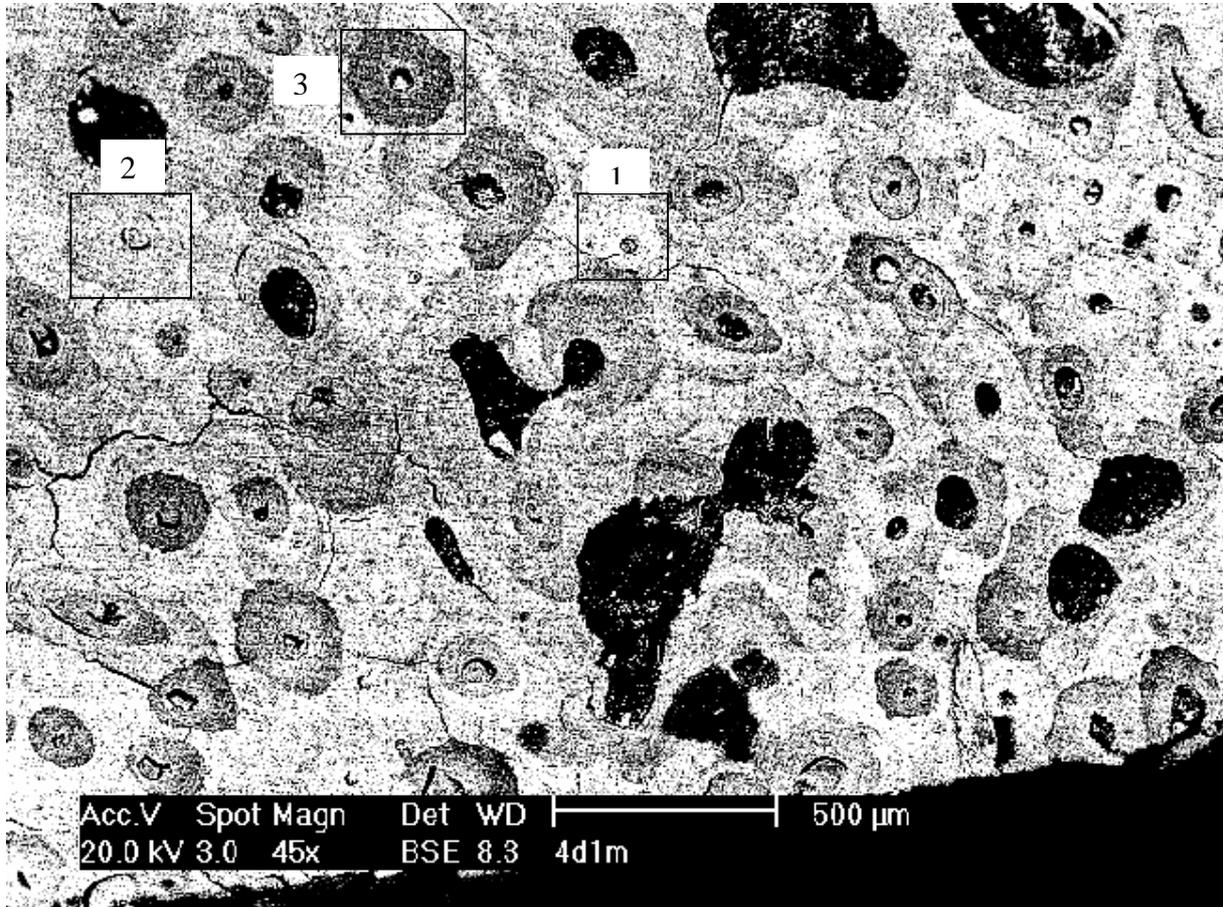


Figure. 1 : Back scattered image of the surface of the cortical sample. The three different types of osteons are represented with 1: white osteon, 2: grey osteon and 3: dark osteon

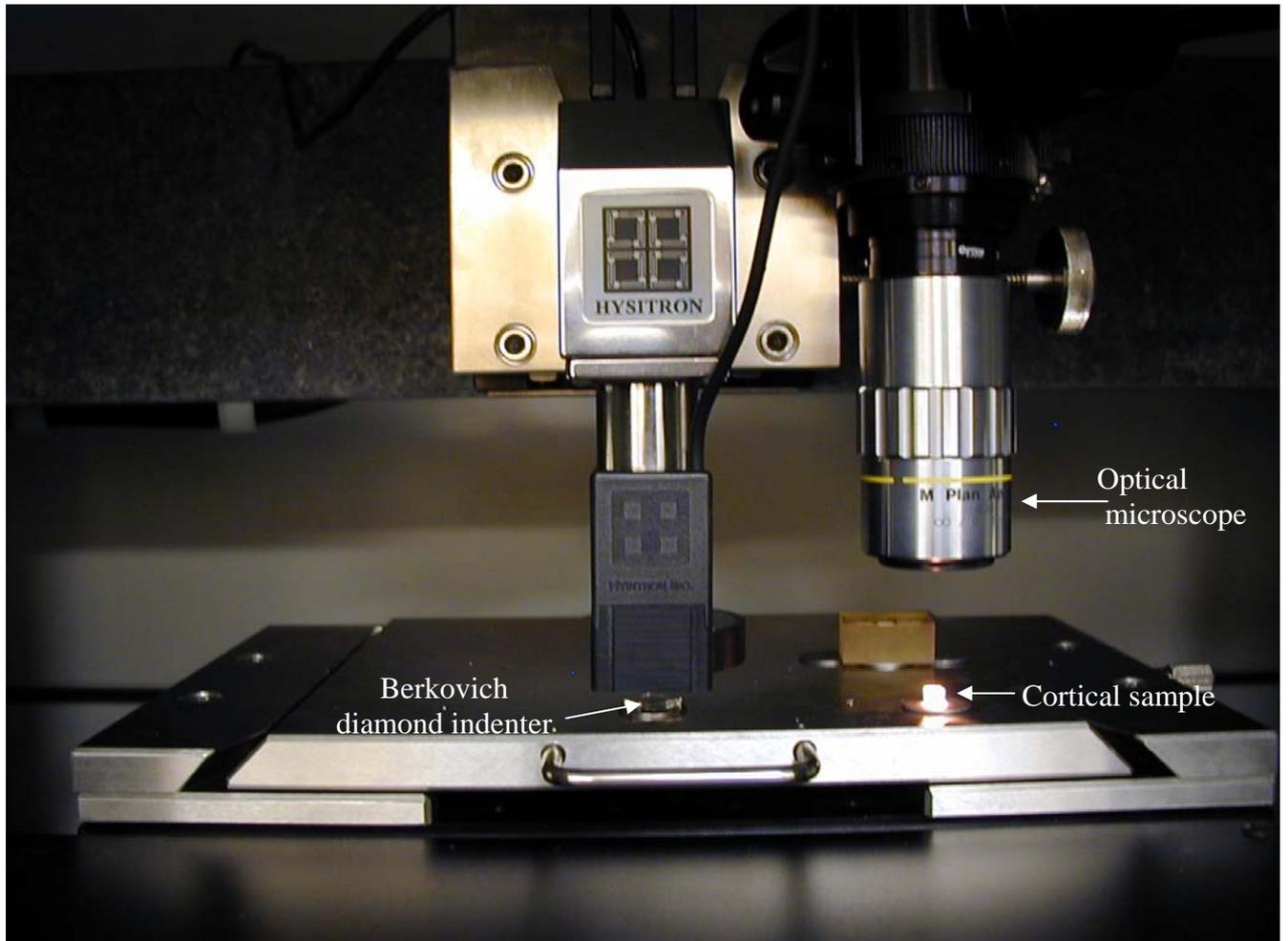


Figure 2. Representation of the coupling between the optical microscope and the indenter.

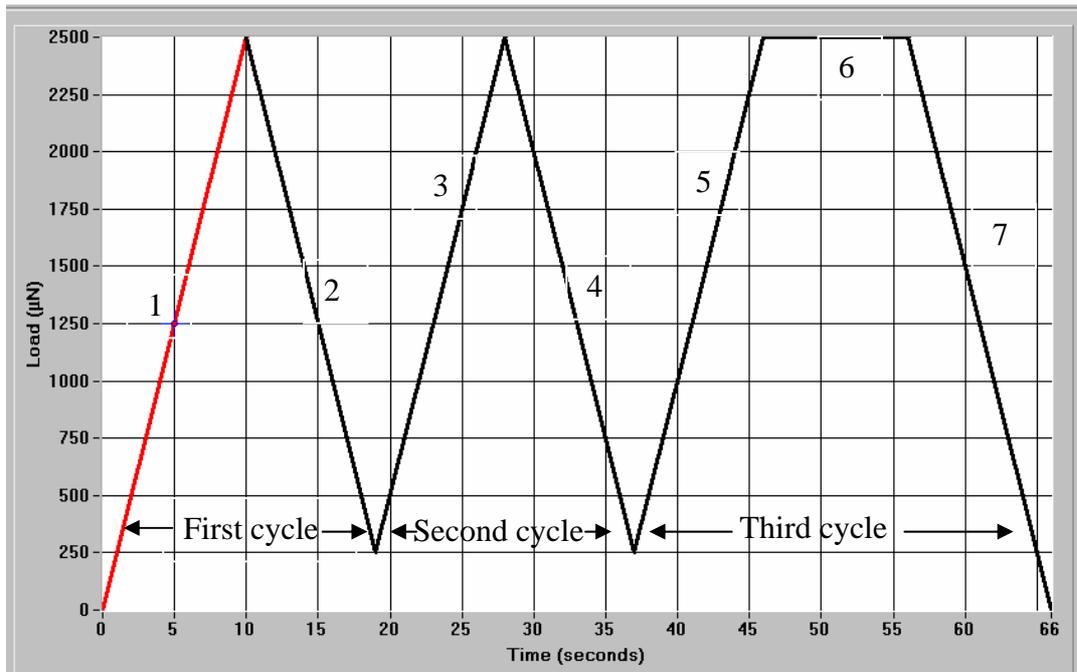


Figure 3. The load cycle applied to the indenter

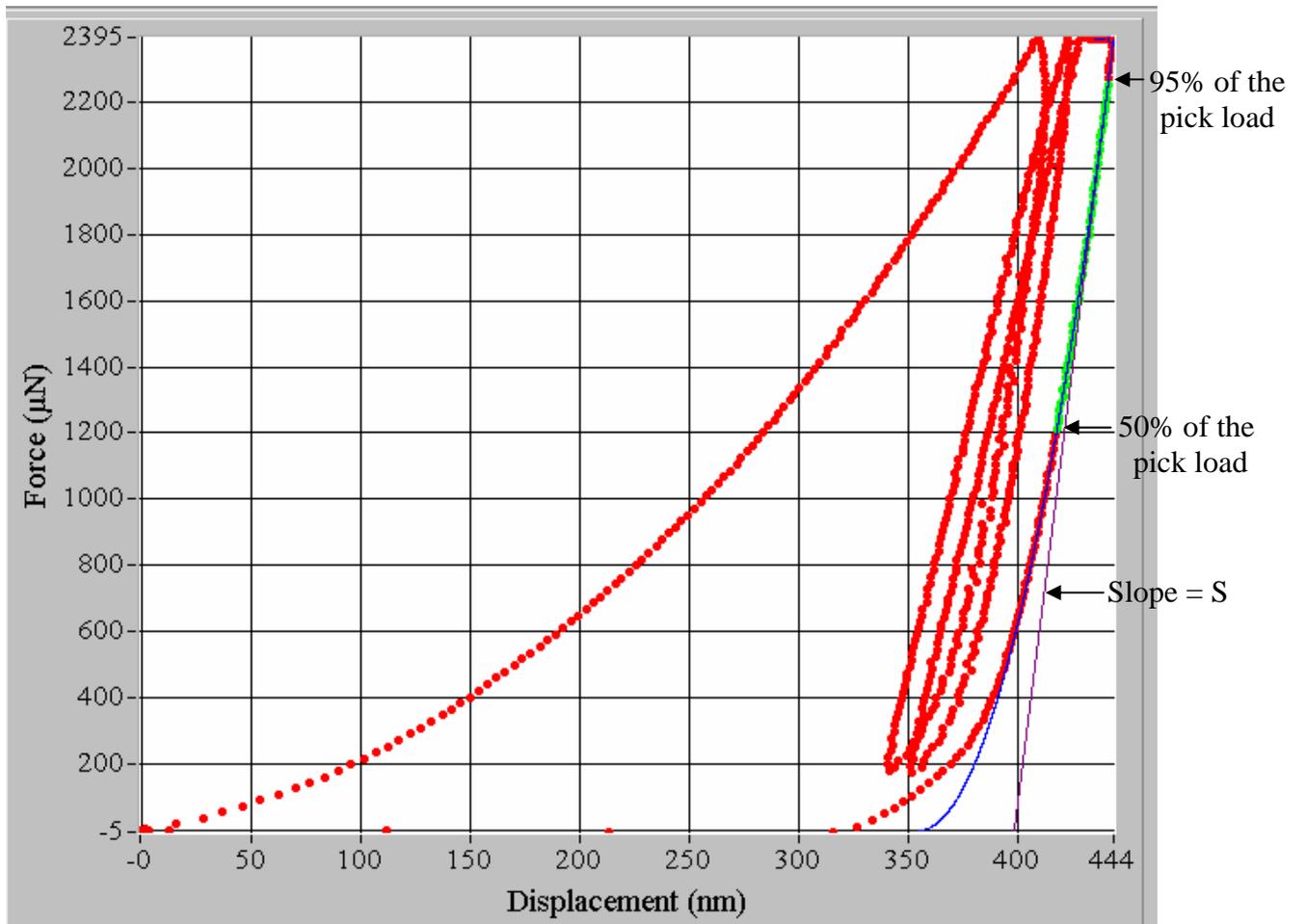


Figure 4. Representation of the typical curve representing the load – displacement performed

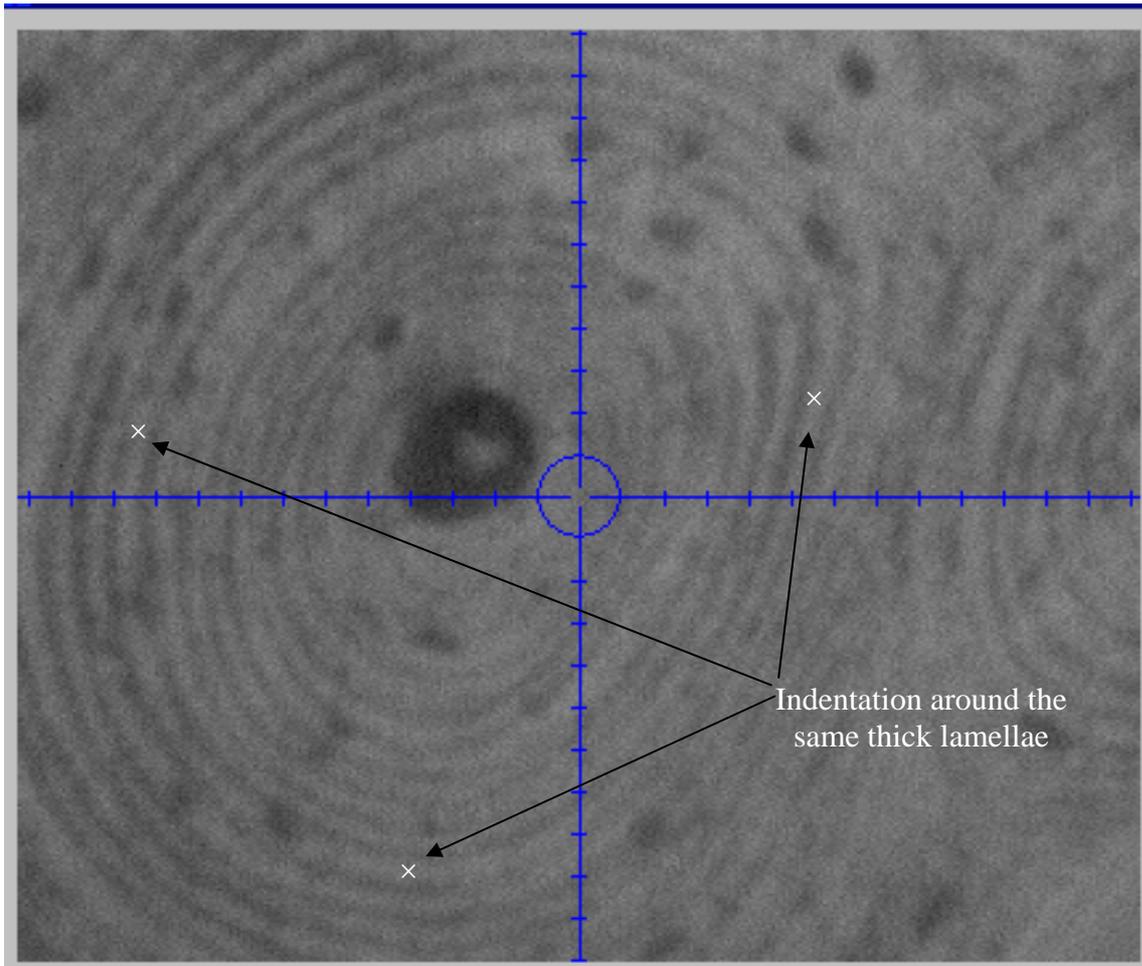


Figure 5. Localizations of the indentations

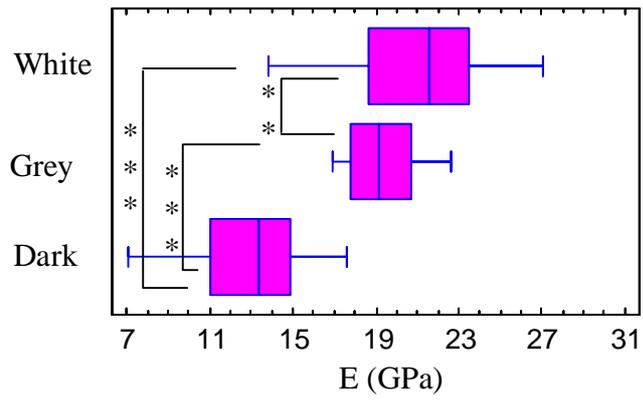


Figure 6. Range of values of elastic modulus for different types of osteons

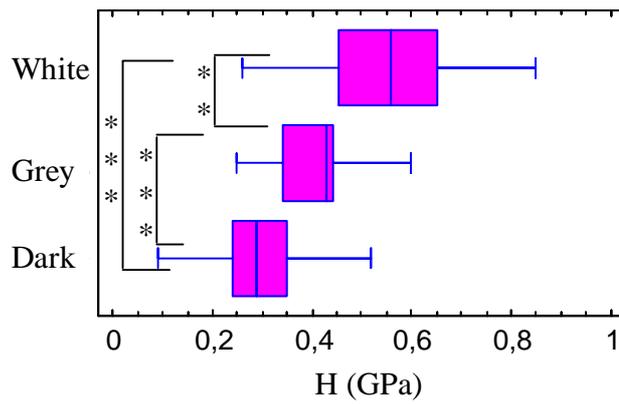


Figure 7. Range of values of Hardness for different types of osteons

White osteons (N=61)		Grey osteons (N=17)		Dark osteons (N=39)	
E (GPa)	H(GPa)	E (GPa)	H(GPa)	E (GPa)	H(GPa)
0.19 – 7.76	0.02 – 0.43	0.93 – 5.93	0.02 – 0.26	0.24 – 8.48	0.01 – 0.31
2.81	0.11	2.42	0.06	2.12	0.05
2.93 ± 1.83	0.15 ± 0.10	2.69 ± 1.57	0.10 ± 0.08	2.45 ± 1.70	0.09 ± 0.08

Table 1. Variability of elastic modulus and hardness (minimum, maximum, median and mean with standard deviation) measured within a thick lamellae from white, grey and dark osteons.

White osteons (N=61)		Grey osteons (N=17)		Dark osteons (N=39)	
E (GPa)	H(GPa)	E (GPa)	H(GPa)	E (GPa)	H(GPa)
13.87 – 27.06	0.26 – 0.85	16.88 – 22.63	0.25 – 0.6	7.04 – 17.59	0.09 – 0.52
21.59	0.56	19.15	0.43	13.33	0.29
21.30 ± 3	0.55 ± 0.15	19.27 ± 1.78	0.41 ± 0.09	12.95 ± 2.66	0.30 ± 0.10

Table 2. Comparison of elastic modulus and hardness (minimum, maximum, median and mean with standard deviation) measured within a thick lamellae from white, grey and dark osteons