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HETEROGENEITY OF TIME-DEPENDENT MECHANICAL PROPERTIES OF HUMAN CORTICAL BONE AT THE MICRO SCALE

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ABSTRACT

Background: Remodeling process affects the mineral content of osteons and imparts heterogeneity through secondary mineralization; the aim of the present study is to assess the elastic and plastic time-dependent mechanical properties of osteons reflecting different mineral content as well as interstitial tissue of human femoral cortical bone by nanoindentation. **Methods:** Four trapezoiform blocks approximately 3 mm thick were cut from the distal end of different human femoral diaphysis. Osteons with different apparent mineral degrees were classified by means of gray levels imaging using Environmental Scanning Electron Microscopy (ESEM). Nanoindentation tests were performed in the longitudinal direction of the bone axis using a four-stage protocol (load-hold-unload-hold) and the experimental curves were fitted by a mechanical model allowing the determination of the time-dependent mechanical properties. **Results:** Apparent low mineral content impact negatively the mechanical response of bone material at the micro-scale. Mechanical response varies among osteons

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exhibiting different mineral degrees. The values of the apparent elastic modulus double when the strain rate is analyzed at the extreme values ($\dot{\varepsilon}$ = zero and infinity) whatever the bone component. **Conclusions:** These results evidence the mechanical heterogeneity of bone microstructure due to remodeling process. The quantification of the time-dependent mechanical properties could be useful to improve numerical models of bone behavior and provide new insights to build up original biomimetic materials.

Keywords: Human; Cortical-bone; Osteons; Time-dependent; Nanoindentation.

INTRODUCTION

Human cortical bone is a hierarchical and multiphase biological material that has been widely studied at the mechanical, structural and physiological level.[3–5,11,12,15,30,31]

The structure of human cortical bone is composed by interstitial tissue and concentric lamellar systems call osteons (also known as Haversian system).[31] Those osteons are the basic structural elements of the secondary haversian bone.[29] The spatial organization of the osteons, orientation of the fiber bundles and mineral content were studied by many authors[1,2,7,23] with different, often contradictory conclusions.

Bensamoun *et al.*[6] characterized the structural and mechanical properties of osteons reflecting different apparent mineral content. In that work, Environmental Scanning Electron Microscopy (ESEM) was used to perform images of bone microstructure with the aim to classify osteons by their decreasing mineralization degree. Using these images osteons were categorized in White osteons for a high mineral content, Gray osteons for an intermediate mineral level and Dark osteons for the lowest mineral content. Concerning the mechanical properties, the result reported showed that high values of the elastic and plastic mechanical properties are positively correlated to high mineral content.

In recent times, nanoindentation technique has been widely used to assess the local elastic–plastic mechanical properties of bone at the micro scale.[14,16,17,32–35,39,40] Nevertheless, few of these works have ever considered the influence of the mineral content among the Haversian systems during their mechanical assessments.

Using nanoindentation technique, the studies of Fan and Rho[14] and Vanleene *et al.*[37] had reported the variation of the mechanical properties as a function of the strain rate. Their results have demonstrated the viscoelastic characteristics of bone material. Nevertheless, the classical Oliver and Pharr mechanical model used to compute the elastic–plastic properties from nanoindentation data are not perfectly adjusted to characterize the time-dependent mechanical properties of viscous materials. This fact provides an opportunity for new models to compute these properties.

The study reported by Oyen and Cook[27] proposed one of the first elastic–plastic–viscoplastic mechanical models used to quantify the time-dependent mechanical properties from nanoindentation data. However, the quantification of the time-dependent mechanical properties in human cortical bone has been rarely reported in the literature.[38] The studies of Oyen *et al.*[26,28,36] quantified the viscous, elastic and plastic indentation responses on different types of bones. Later, Isaksson *et al.*[18,19] proposed a different mechanical model adding a Burger component to the classical elastic–plastic model in order to assess the viscoelasticity of rabbit cortical bone. However, these mechanical models allow assessing only the viscoelastic or the viscoplastic mechanical properties but not both simultaneously.

Recently, Mazeran *et al.*[24] proposed and new four stages protocol (load–hold–unload–hold) combined with a time-dependent mechanical model allowing the calculation of the elastic, viscoelastic, plastic and viscoplastic properties from a single nanoindentation test. This method has been successfully tested on polymers[24] and bones.[20,21] In addition, quantified elastic and viscoelastic properties allow the calculation of an apparent elastic modulus as a function of the strain rate. This assessment allows one to compute and to predict the variation of the elastic moduli while an increasing strain rate from zero to infinity.

Therefore, using the mechanical model proposed by Mazeran *et al.*,[24] the aims of this work are (i) to quantify the time-dependent mechanical properties of different microstructural components of human bone cortical bone: Haversian systems exhibiting different mineral degrees as well as interstitial lamellae and (ii) to demonstrate the mechanical heterogeneity of bone microstructure due to the differences in the apparent mineral content.

MATERIALS AND METHODS

Sample Preparation

Four small trapezoiform blocks approximately 3 mm thick were cut form the distal end of different human femoral diaphysis with a diamond saw (Microcut, BROT Technologies) under deionized water irrigation. For each sample, one of the longitudinal surface was fixed to a steal disk with cyanoacrylate glue, while the other face was ground with a semiautomatic polishing machine (PRESI-Mecatech 234) with successive grades of abrasive papers (P800, P1200, P2400, P4000) under abundant irrigation of deionized water. The exposed surface was then polished with successive grades of alumina suspension with particle size of 1, 0.3 and 0.04 μm. Finally, the samples were ultrasonically cleaned in ultrapure water (Ultrasonic cleaner Bransonic®200, Branson Ultrasonics Corp;). Three cycles of 5 min each were performed changing the water at each cycle in order to remove all alumina particles and other wear debris.

Environmental Scanning Electron Microscopy Images

ESEM (Philips XL30 ESEM-FEG) was used to identify the mineral degrees of osteons by means of gray level imaging.[6] This technique allows avoiding the desiccation and coating with gold–palladium and thus their original characteristics may be preserved for the nanoindentation testing. The images were obtained from the perpendicular face to the transversal direction of the dry sample under lower-pressure gaseous environment (1.4 Torr) using an acceleration of 20 kV and a magnification of 600×. Osteons exhibiting different gray levels could be distinguished from the ESEM images (Fig. 1). In this study, osteons were classified in White Osteons for a light gray and Black Osteons for a dark gray. These differences in the gray levels are commonly associated to variations in the material composition of the

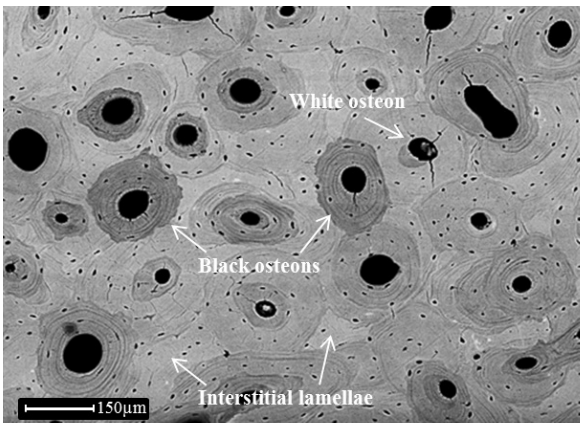


Fig. 1 ESEM image of the longitudinal axis reflecting different structural composition of the Haversian systems as well as interstitial tissue. In this image, using the gray distribution it is possible to recognize and to differentiate the Black Osteons, White Osteons and Interstitial Lamella.

osteons and they could be extrapolated to a difference in the mineral content.[6]

Nanoindentation Protocol

Nanoindentation tests were conducted in air under ambient conditions with a Nano Indenter G200 (Agilent Technologies) using a Berkovich tip (Micro Star Technologies) and the measurement of the contact stiffness via the Continuous Stiffness Measurement (CSM) method. The nanoindenter tip was calibrated using a fused silica sample. From ESEM images, 20 White Osteons, 20 Black Osteons and 20 zones of Interstitial Lamellae were selected. For this study, which comprises 600 indents including 10 indentations tests per typology performed in the longitudinal direction of the femoral section. Specifically, indentation tests are based on four stages (loading, hold load plateau, unloading and hold load plateau) protocol in order to differentiate the elastic, viscoelastic, plastic and viscoplastic behaviors. Indeed, the loading and unloading stages exhibit the viscoelastic and viscoplastic behaviors in two different conditions. The hold load plateaus allow exhibiting the reversible and irreversible viscous behaviors and thus viscoelasticity and viscoplasticity. In detail, the four stages are (1) a loading stage at constant load rate/load ratio until an indentation depth of 3000 nm; (2) a hold time of 300 s; (3) an unloading stage at constant unload rate/load ratio until 50% of the maximal load value and (4) a second hold time of 300 s. This protocol has been used in previous studies to determine the time-dependent mechanical properties of polymers[24] and bones tissues.[20,21]

Calculation of the Mechanical Properties

The mechanical model used to describe the material is based on a combination of one elastic modulus (E_{elast}), two-viscoelastic moduli (E_{ve} and η_{ve}), a hardness (H) and one viscoplasticity η_{vp} in series.

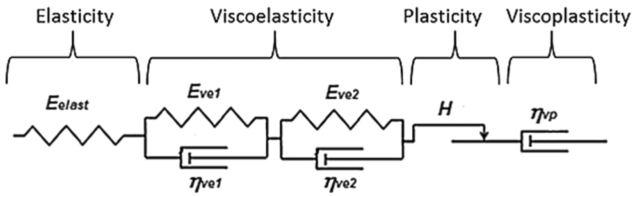


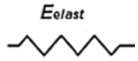
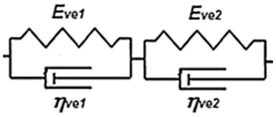
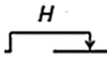
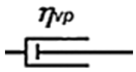
Fig. 2 Material Mechanical model is used to describe the mechanical behavior of the human femoral cortical bone. This model is composed of a pure elastic element, two viscoelasticities with different time-constants, plastic and viscoplastic components.

Two viscoelasticities are enough to describe the viscoelastic behavior of the sample. To compute these mechanical properties, a nanoindentation mechanical model (Fig. 2) composed of different mechanical elements (spring, Kelvin–Voigt elements, slider and dashpot) is used to fit the experimental indentation depth versus time curves. These elements have a quadratic response (square root of the load proportional to displacement and/or displacement velocity). After the experimental curves have been correctly fitted to the nanoindentation mechanical model, the mechanical properties can be computed from the equations summarized in Table 1 using the stiffness (k), the displacements of the different elements (d and $\dot{d}$), the parameter connecting the load and displacement (p) and the contact area (A). More details about this mechanical model are provided in the work of Mazeran *et al.*[24] Using the purely elastic (E_{elast}) and the viscoelastic properties (E_{ve} and η_{ve}), the apparent elastic modulus for different strain rates $\dot{\varepsilon}$ is computed using the following equations:

First, Eq. (1) is used to compute a reduced apparent elastic modulus E^*_{app}, i.e. the apparent value of the elastic modulus when the sample is strain at a given strain rate $\dot{\varepsilon}$.

$$\frac{1}{E^*_{\mathrm{app}}} = \frac{1}{E_{\mathrm{elast}}} + \frac{1}{E_{ve1} + \dot{\varepsilon} \cdot \eta_{ve1}} + \frac{1}{E_{ve2} + \dot{\varepsilon} \cdot \eta_{ve2}}. \tag{1}$$

Table 1 Equations used to Assess the Mechanical Properties of the Nanoindentation Model. The Values of the Displacement of the Different Elements are Taken at the Beginning of the Unloading Curve, Point of the Curve from Which the Contact Area is Computed.

Mechanical Behavior	Rheological Element	Mechanical Parameters of Each Element	Mechanical Properties
Elasticity		$\sqrt{F} = k_e \cdot d_e$	$E_{\mathrm{elast}} = k_e^2 \cdot d_e \cdot \sqrt{\frac{\pi}{A}}$
Viscoelasticity		$\sqrt{F} = k_{ve1} \cdot d_{ve1} + \eta_{ve1} \cdot \dot{d}_{ve1}$ $\sqrt{F} = k_{ve2} \cdot d_{ve2} + \eta_{ve2} \cdot \dot{d}_{ve2}$	$E_{ve1} = k_{ve1}^2 \cdot d_{ve1} \cdot \sqrt{\frac{\pi}{A}}$ $\eta_{ve1} = 2 \cdot k_{ve1} \cdot \eta_{ve1} \cdot d_{ve1} \cdot \sqrt{\frac{\pi}{A}}$ $E_{ve2} = k_{ve2}^2 \cdot d_{ve2} \cdot \sqrt{\frac{\pi}{A}}$ $\eta_{ve2} = 2 \cdot k_{ve2} \cdot \eta_{ve2} \cdot d_{ve2} \cdot \sqrt{\frac{\pi}{A}}$
Hardness		$\sqrt{F} = p \cdot d_P$ $d_P(t + \Delta t) \geq d_P(t)$	$H = p^2 \cdot d_p^2 / A$
Viscoplasticity		$\sqrt{F} = \eta_{vp} \cdot \dot{d}_{vp}$ $d_{vp}(t + \Delta t) \geq d_{vp}(t)$	$\eta_{vp} = \eta_{vp}^2 \cdot d_{vp} \cdot \dot{d}_{vp} / A$

The values of the reduced apparent elastic modulus have been calculated for three values of strain rate (1) $\dot{\varepsilon} = 0$ in order to assess the value of the apparent elastic modulus in quasi-static conditions, (2) $\dot{\varepsilon} = 0.05\,\mathrm{s}^{-1}$ corresponding to the typical strain rate used in the nanoindentation tests and (3) $\dot{\varepsilon} =$ infinity. It should be noted that an infinity strain rate cannot be reached experimentally. This value is used to highlight the maximum value reached by the apparent elastic modulus in high strain rate condition.

Second, in order to assess the apparent elastic modulus E_{app} of the sample Eq. (2) is used:

$$E_{\mathrm{app}} = E^*_{\mathrm{app}} \times (1 - v_s^2), \tag{2}$$

where E_{app} is the apparent elastic modulus of the sample calculated considering a Poisson's ratio for cortical bone $v_s = 0.3$.[34]

Statistical Analysis

Statistical tests were performed using statistical analysis and graphics software SYSTAT version 2012 (SYSTAT Software Inc., Chicago, USA). Nonparametric Kruskall and Wallis test and Dwass–Steel–Chritchlow–Fligner Test (DSCF) with a significance p-value ($p < 0.05$) were carried out to stablish the possible significant statistical differences between the different typologies and their mechanical properties.

RESULTS

The time-dependent mechanical properties have been computed for White Osteons, Black Osteons and the Interstitial Lamellae. The values obtained are summarized in Table 2. The results have been expressed as the mean ± standard deviation (SD). These values are illustrated graphically in box plot graphs in order to represent the variation of the mechanical properties (Figs. 3–6).

The elastic modulus E_{elast} is represented in Fig. 3. The values obtained are different for all the microstructural components with statistically significant differences ($p < 0.05$).

Table 2 Values of the Mechanical Properties of the Human Femoral Cortical Bone Computed for the Different Components of the Cross Sectional Area.

Mechanical Property	Interstitial Lamellae	White Osteons	Black Osteons
E_{elast} (GPa)	35.2 ± 5.1	32.2 ± 5.4	26.8 ± 5.1
E_{ve1} (GPa)	76.3 ± 18.1	79.7 ± 24.0	52.0 ± 16.1
$\eta_{ve1} \times 10^2$ (GPa · s)	31.6 ± 13.8	35.9 ± 16.8	23.2 ± 10.9
E_{ve2} (GPa)	137.3 ± 24.0	130.4 ± 32.3	84.7 ± 17.5
$\eta_{ve2} \times 10^3$ (GPa · s)	68.1 ± 22.0	70.1 ± 22.4	48.5 ± 11.4
H (GPa)	0.84 ± 0.07	0.78 ± 0.08	0.62 ± 0.06
η_{vp} (GPa · s)	308.8 ± 43.0	288.0 ± 36.5	230.4 ± 25.3

Note: Mean ± standard deviation (SD).

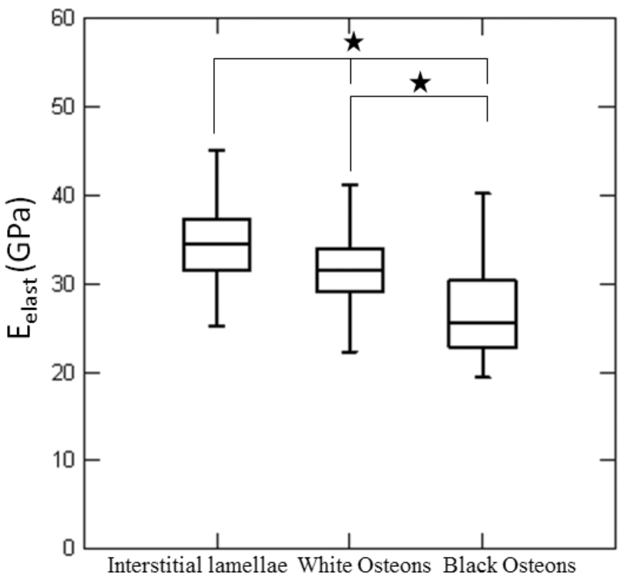


Fig. 3 Variation of the purely elastic modulus E_{elast} of the human femoral cortical bone as a function of the indentation zone: Interstitial Lamellae; White Osteons and Black Osteons. Statistically significant differences were found between all the microstructural typologies. (★ means statistically significant differences from the DSCF test $p < 0.05$).

The viscoelastic response of bone was assessed by two Kelvin–Voigt components, which are composed of two elastic components (E_{ve1} and E_{ve2}) and two viscous components (η_{ve1} and η_{ve2}). The viscoelastic response is represented in Figs. 4(a)–4(d). It should be noted that similar viscoelastic response was found among the interstitial tissue and the White Osteons. Contrary, a clear variation of the values including statistically significant differences ($p < 0.05$) is noticed between osteons.

Hardness (H) and Viscoplasticity (η_{vp}) values are represented in Figs. 5(a) and 5(b), respectively. The results show that the diminution in the apparent mineral content leads to a decrease in the values of hardness and viscoplasticity. Values are different among osteons as well as interstitial tissue. Statistical tests show significant differences between all components ($p < 0.05$).

From the elastic and viscoelastic properties E_{elast}, E_{ve} and η_{ve}, the apparent elastic modulus as a function of the strain rate could be computed using Eqs. (1) and (2). Strain rates has been chosen to be 0, 0.05 s^{-1} and infinity. The apparent modulus for a strain rate equal to zero represents the quasi-static response, 0.05 s^{-1} represents a typical value of the strain rate used in nanoindentation experiments, and infinity represents a strain rate in case of chock. The apparent elastic modulus E_{app} for each zone and for a given strain rate is represented in Fig. 6 and values were summarized in Table 3. One should note that values increase close to a factor of two when the strain rate increases from zero to infinity. In all the cases, there are statically significant differences ($p < 0.05$) between White and Black Osteons.

DISCUSSION

In this study, the quantification of the time-dependent mechanical properties of microstructural

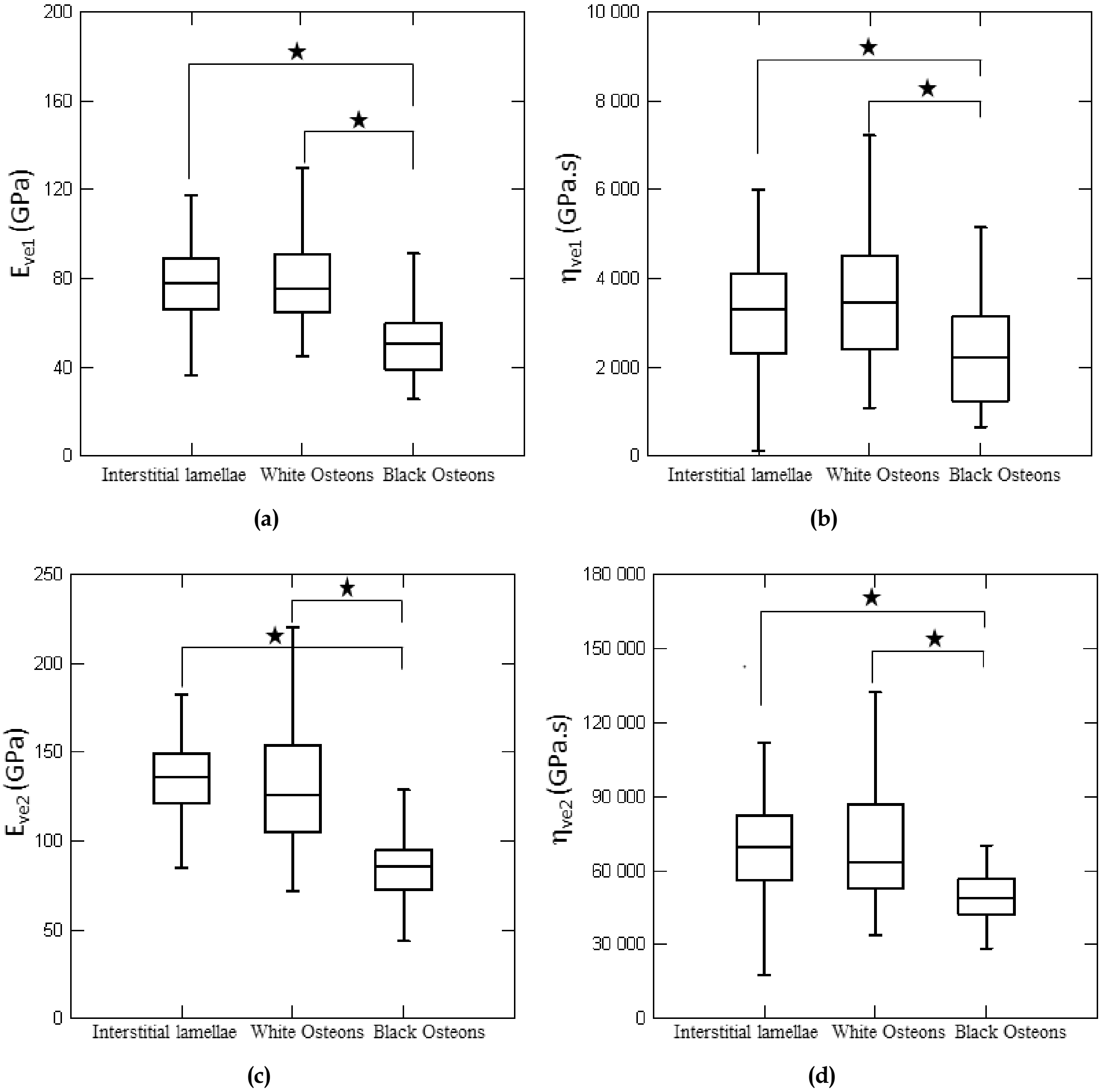
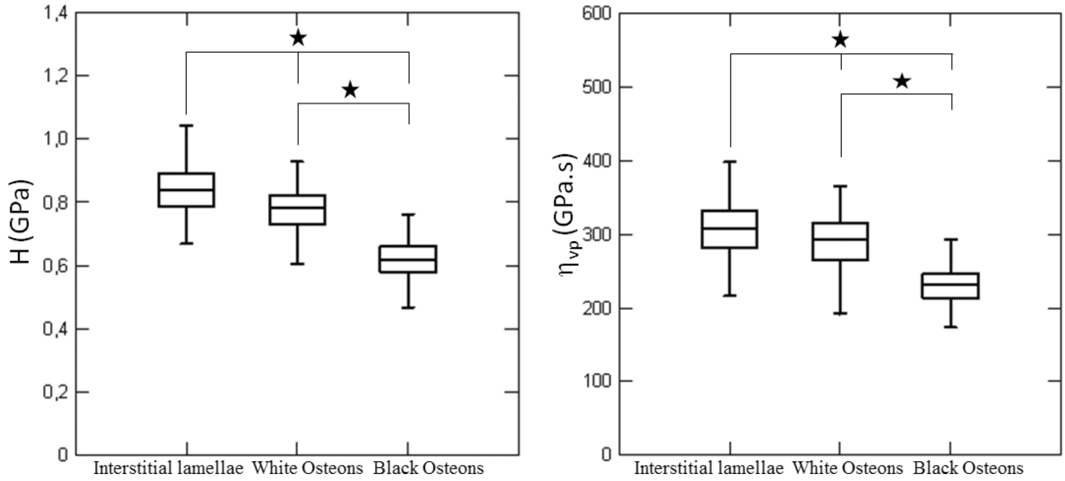


Fig. 4 Variation of the viscoelastic moduli of the human femoral cortical bone according to the indentation zone: Interstitial lamellae, White Osteons and Black Osteons. The figures **(a)** and **(b)** represent the Elastic components (E_{ve}) and the figures **(c)** and **(d)** the viscous components (η_{ve}) of the viscoelastic response. (★ means statistically significant differences from the DSCF test $p < 0.05$).

component of bone (osteons and interstitial tissue) was carried out. From the results computed, the heterogeneity of the mechanical response between osteons exhibiting different apparent mineral contents was demonstrated. There is a general trend toward increased values of the mechanical properties with increases in the apparent mineral content. Nanoindentation results show a variation in the mechanical response between White Osteons and Black Osteons of about 17% for the pure elastic modulus, close to 35% for the first and second viscoelastic modulus. Finally, the variation is around 20% for hardness and viscoplasticity.

The values of the purely elastic (E_{elast}) mechanical response were different for all the microstructural typologies. The values reported herein show that microstructural components become



(a) (b)

Fig. 5 Variation of Hardness **(a)** and Viscoplasticity **(b)** of the human femoral cortical bone with the indentation zone: Interstitial lamellae; White Osteons and Black Osteons. (Statistically significant differences from the DSCF test ★ $p < 0.05$.)

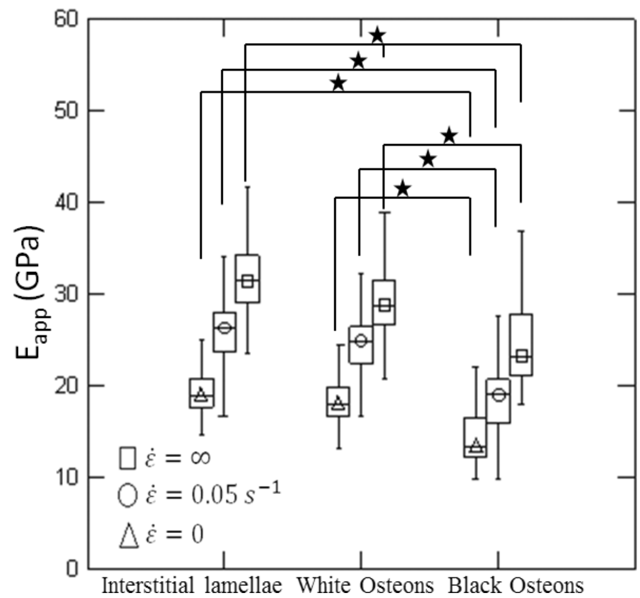


Fig. 6 Apparent elastic modulus as a function of the zone and the strain rate: from left to right the strain rate $\dot{\varepsilon} = 0$ (zero), $0.05\,\mathrm{s}^{-1}$ and ∞ (infinity). Statistically significant differences were found between White and Black Osteons whatever the value of strain rate (★ $p < 0.05$).

stiffer due to perhaps the increase of the apparent mineral content. This fact could be due to the maturation degree that leads to a probable accumulation of mineral components within the osteons' matrix. Nevertheless, the range of values computed for interstitial tissue and osteons are considerably greater than other studies. This could be linked to the special sensibility of the mechanical model used to quantify the purely elastic response.

The viscoelasticy (E_{ve} and η_{ve}) was found to be different between osteons but similar between White Osteons and interstitial tissue. This behavior suggests firstly the higher presence of mineral within the white osteons and secondly the presence of different structural arrangements within the different microstructural components that could produce a possible differentiation at the lamellar level. In an animal model, Isaksson *et al.*[18] reported that viscoelastic properties of rabbit bones are affected by the mineral, carbonate and collagen content within the bone matrix. Higher values for the elastic and viscous parameters of bone viscoelasticity were found for bones with high values of compositional parameters. However, comparisons could be handled carefully because of the structural differences between animal and human bone and because in our study neither of those structural components was measured. Nevertheless, the similarity among the mechanical models helps to understand the viscous response of bone material.

Table 3 Values of the Apparent Elastic Modulus of the Human Femoral Cortical Bone in Function of the Strain Rate and the Zones of the Cross Sectional Area.

	Apparent Elastic Modulus E_{app} (GPa)		
Strain Rate ($\dot{\varepsilon}$)	Interstitial Lamellae	White Osteons	Black Osteons
0	18.7 ± 3.9	17.7 ± 3.7	13.3 ± 3.7
$0.05\,s^{-1}$	27.6 ± 4.5	25.9 ± 4.3	20.8 ± 3.8
∞	32.0 ± 5.0	29.3 ± 5.2	24.4 ± 4.9

Note: Mean $\pm$ standard deviation (SD).

The mechanical properties of bone, specially the viscous properties could be affected by the hydrated state of the sample.[32] It has been reported that chemical bonds between collagen fibrils were suspected to be hydrogen bonds and strongly related to the water content of the bone matrix. That water content could be the layered water located between the crystal interfaces of the extrafibrillar mineral of bone.[10,13,37] The work of Black and Mattson[7] suggests that the apparent water content vary among osteons due to the mineralization process. That work predicts that water content vary from 24.81% for young osteons to 1.19% in mature osteons. This fact could help to explain the differences of the viscous response between osteons.

The results obtained for hardness (H) show different values for each microstructural element. However, the values computed for White and Black Osteons are considerably higher than those reported for osteons with similar characteristics by Bensamoun *et al.*[6] This fact could be due to a possible hardening process that could occur within the bone matrix with time. Another explanation to that difference is the indentation depth used for each study (~500 nm for Bensamoun *et al.* and ~3000 nm in this study). According to Chen *et al.*,[8] the superficial penetration of the nanoindentation tip creates lower values of hardness.

There are very few nanoindentation studies that have quantified the viscoplastic (η_{vp}) mechanical properties. This fact makes it difficult to find a reasonably clear explanation to link the biological level and the mechanical behavior that is present in all materials. Even if the mechanisms of viscoplastic deformation are still unknown, the study of Vanleene *et al.*[37] suggests that viscoplastic behavior reflects the impact of the organic component in the mechanical behavior of the bone matrix. However, the results presented in this study propose that mineral organization and their apparent quantity also affect the permanent deformation of bone microstructures.

The apparent elastic modulus (E_{app}) of cortical bone measured in this study is 27.6 ± 3.9 GPa for the interstitial lamellae, 25.9 ± 4.3 GPa for White osteons and 20.8 ± 3.8 GPa for Black osteons. These values have been compared to previous studies reported in the literature (Bensamoun *et al.*,[6] Rho *et al.*,[33,35] Zysset *et al.*[40]). The results are consistent with the upper part of these studies and most of the differences could be explained by the fact that they do not consider the influence of the strain rate when elastic modulus is computed, or they do not differentiate the mineral content of the osteons.

In addition, the apparent elastic modulus (E_{app}) as a function of the strain rate has been computed for all osteons as well as interstitial tissue. The results show that apparent elastic modulus increases by a factor close to two when the strain rate increases from zero to infinity (for interstitial tissue from 18.7 to 32.0 GPa, for White Osteons from 17.7 to 29.3 GPa and for Black Osteons from 13.3 to 24.4 GPa). This behavior is similar to that reported at the macro scale by McElhaney.[25] At the macro scale, the effects of the strain rate in the elastic response of bone material has been assessed experimentally using different methods as air-guns[25] and Hopkinson's bars.[22] The results reported show an increase of the elastic values according to the increase of the strain rate. According to the study performed by Cowin,[9] that fact could reflect bone adaptability under different stress conditions.

At the micro scale, the effects of the strain rate in the elastic response of bone have been rarely investigated. Few nanoindentation studies as those performed by Fan *et al.*,[14] Vanleene *et al.*[37] and Jaramillo-Isaza *et al.*[20,21] have reported that the elastic modulus was strain rate dependent. Those studies and the results obtained in this work highlight the importance of considering the strain rate in the assessment of the mechanical response from the nanoindentation data. Moreover, the results presented suggest the viscoelastic behavior of bone material.

Even if previous works have reported the mechanical differences between osteons and interstitial lamellae,[33,34] the results of this study suggest that at a certain point the remodeling process could create enough mineral depots that the mechanical response for certain properties will be similar between mature osteons and the interstitial tissue.

To summarize, our results demonstrate that time-dependent mechanical properties of human cortical bone could be described by an elastic–viscoelastic–plastic–viscoplastic mechanical model. The results show a variation of the time-dependent mechanical response of bone related to the mineral degree present within the different components (osteons and interstitial tissue). Values of the mechanical properties are higher for White Osteons than for Black Osteons thus mechanical properties showed to be positively correlated to the apparent mineral content. Our results suggest that the mechanical and structural differences between osteons could be a possible consequence of (i) remodeling cycles producing mineral accumulation, (ii) a possible orientation of the collagen fibers and the hydroxyapatite crystals and (iii) remaining water content. In addition, the results demonstrate that changes in the strain rate in the nanoindentation could modify the apparent elastic modulus of the bone. More precisely, it was found that the value of the apparent elastic modulus increases by a factor close to two when the strain rate rises from zero to infinity.

The data reported in this work could be helpful to improve the current understanding of bone remodeling process and the interactions between bone components at the micro and nano scales. In addition, values reported in this study could be useful to develop growth theories, to improve osseointegration models, for modeling and constitutive laws of bone behavior and remodeling process and to build up more resistant biomimetic materials.

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We represent that this submission is original work, and is not under consideration for publication with any other journal.

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