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Microstructure and nanomechanical properties in osteons relate to tissue and animal age

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Abstract

Material property changes in bone tissue with ageing are a crucial missing component in our ability to understand and predict age-related fracture. Cortical bone osteons contain a natural gradient in tissue age, providing an ideal location to examine these effects. This study utilized osteons from baboons aged 0 to 32 years (n=12 females), representing the baboon lifespan, to examine effects of tissue and animal age on mechanical properties and composition of the material. Tissue mechanical properties (indentation modulus and hardness), composition (mineral-to-matrix ratio, carbonate substitution, and crystallinity), and aligned collagen content (aligned collagen peak height ratio) were sampled along three radial lines in three osteons per sample by nanoindentation, Raman spectroscopy, and second harmonic generation microscopy, respectively. Indentation modulus, hardness, mineral-to-matrix ratio, carbonate substitution, and aligned collagen peak height ratio followed biphasic relationships with animal age, increasing sharply during rapid growth before leveling off at sexual maturity. Mineral-to-matrix ratio and carbonate substitution increased 12% and 6.7%, respectively, per year across young animals during growth, corresponding with a nearly 7% increase in stiffness and hardness. Carbonate substitution and aligned collagen peak height ratio both increased with tissue age, increasing 6 to 12% across the osteon radii. Indentation modulus most strongly correlated with mineral-to-matrix ratio, which explained 78% of the variation in indentation modulus. Overall, the measured compositional and mechanical parameters were the lowest in tissue of the youngest animals. These results demonstrate that composition and mechanical function are closely related and influenced by tissue and animal age.

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Keywords

ageing; primate; osteon; nanoindentation; tissue properties

1. Introduction

The incidence of osteoporotic fracture increases dramatically with ageing (Burge et al., 2007; Schneider, 2008). Measures of bone mass and architecture alone are inadequate for predicting age-related fracture risk (Cummings et al., 2002; Delmas and Seeman, 2004; Garnero, 2008). Tissue-level material properties are a missing component in our knowledge of ageing and bone fragility, but are clearly critical to understanding skeletal mechanical integrity. Nano-scale variability of the elastic modulus in healthy bone hinders crack propagation and acts as a toughening mechanism (Fratzl and Gupta, 2007; Tai et al., 2007). In addition, osteoporosis alters the nano-scale heterogeneity of the material properties and composition of bone tissue (Boskey and Mendelsohn, 2005; Gadeleta et al., 2000; Roschger et al., 2008).

To understand the skeletal changes leading to osteoporosis and age-related fracture, one must first understand the natural variations that occur in healthy bone tissue with ageing (Lin and Lane, 2004).¹¹ Due to the remodeling process, bones from a single animal contain tissue of varying ages; thus, tissue age within a given animal is not the same as the animal's age. In primates, the Haversian remodeling process creates a natural gradient in tissue age across the osteon (Gourion-Arsiquaud et al., 2009). In this process, osteoclasts travel along the Haversian canal and resorb a cylindrical volume of bone surrounding the blood vessel. Osteoblasts follow the osteoclasts and form layers of bone to refill the resorbed cylinder, starting at the periphery and finishing at the center near the blood vessel 2 to 4 months after the process was initiated (Parfitt, 1994). Hence, in a section transverse to the Haversian canal, osteons appear as concentric ring-like structures, with the newest tissue located at the center nearest to the blood vessel, and the oldest tissue located at the periphery of the osteon.

Tissue-level variations in composition and mechanical properties with either tissue or animal age have been studied individually, producing mixed results (Akkus et al., 2003; Gupta et al., 2006; Rho et al., 1999; Rho et al., 2002; Paschalis et al., 1996; Paschalis et al., 1997). As a consequence, relationships between these properties and ageing in humans are still unclear. Even fewer studies have related tissue composition to mechanical function (Busa et al., 2005; Donnelly et al., 2010; Miller et al., 2007). Furthermore, material variations have only been examined over limited age ranges (Akkus et al., 2003; Carden and Morris, 2000; Paschalis et al., 1996; Paschalis et al., 1997). As tissue composition plays a crucial role in determining mechanical function, understanding these relationships will improve our understanding of normal and pathological bone function and may enable us to improve upon current therapies for skeletal diseases such as osteoporosis. In rodent models of ageing, tissue composition and mechanical properties varied systematically with tissue age in newly formed tissue (Donnelly et al., 2010; Miller et al., 2007); however, cortical microstructure and skeletal ageing processes of rodents differ markedly from those of humans.

The present study utilized the natural gradient in relative tissue age across osteons in female baboons to study tissue age and animal age-related variations in tissue composition and mechanical properties over the entire lifespan of the animal. Baboon bone is an excellent model for human bone because baboons have a similar Haversian microstructure resulting from secondary remodeling and also experience a similar ageing process, including increased skeletal fragility (Havill et al., 2003; 2008). The maximum lifespan of a baboon is approximately 1/3 that of the human, with sexual maturity occurring around five years of

age (Bronikowski et al., 2002; Martin et al., 2002). This lifespan is sufficiently long to elicit reproductive senescence and menopause, accompanied by changes in hormone levels that are important in bone metabolism and loss (Bellino and Wise, 2003; Havill et al., 2003; Martin et al., 2003).

The goals of this study were to characterize changes in nanomechanical properties and tissue composition with both tissue age (relative) and animal age over the entire lifespan of the baboon and to examine relationships between tissue composition and mechanical properties. In a previous study of the baboon samples utilized here, preliminary analyses with Raman spectroscopy and more extensive analyses with Fourier transform infrared spectroscopic imaging (FTIRI) showed variations in compositional parameters with both tissue and animal age (Gourion-Arsiquaud et al., 2009). However, variations in mechanical parameters were examined only with tissue age in one age group (13 years old). We hypothesized that tissue mechanical properties would vary with changes in mineral and aligned collagen content as a function of both tissue and animal age in baboon cortical bone, an established non-human primate model for human cortical bone (Bellino and Wise, 2003; Havill et al., 2003; 2008).

2. Materials and Methods

Samples and specimen preparation

Femoral bone samples were obtained from twelve female baboons examined previously (Gourion-Arsiquaud et al., 2009), aged 0.28 to 32.45 years, representing the majority of the baboon lifespan (Bronikowski et al., 2002). All animals were housed outdoors in group housing at Southwest National Primate Research Center/Southwest Foundation for Biomedical Research (SNPRC/SFBR, San Antonio, TX) and had no evidence of metabolic bone disease. The Institutional Animal Care and Use Committee approved all procedures at SNPRC/SFBR, and no animals were euthanized specifically for this study.

After necropsy, the femurs were wrapped in saline soaked gauze and stored at -20° C. The femurs were fixed, dehydrated, and embedded in polymethylmethacrylate (Erben, 1997). Transverse sections from the midshaft were polished anhydrously to achieve a root mean square surface roughness less than 15 nm on a 5 μm $\times$ 5 μm AFM scan (Dimension 3100, Veeco Metrology Group) (Donnelly et al., 2006a).

Three osteons per sample were selected for characterization by nanoindentation, Raman spectroscopy and SHG, and three radial lines were characterized within each osteon (Figure 1). To sample a consistent anatomical area between samples, osteons were selected from the postero-lateral quadrant of the femurs, within 2 mm of the endosteal surface (Figure 1). The osteons did not have widened Haversian canals and lamella near the canals were well-formed and not eroded, suggesting that these osteons were not remodeling at the time of animal death.

Nanoindentation

Nanoindentation was used to probe the tissue nanomechanical properties. As nanoindentation tests volumes of material at a length scale less than that of individual microstructural features in bone, this technique avoids confounding factors such as microstructure and porosity that affect tissue properties at larger length scales (Burstein et al., 1976; McCalden et al., 1993). A 20 μm $\times$ 20 μm surface topography scan was made with the scanning nanoindenter (TriboIndenter, Hysitron, Inc.) prior to each indentation to place them at the center of lamellae, ensuring that comparable material was sampled between measurements (Figure 1) (Donnelly et al., 2006a). Each osteon contained between 5 and 15 lamellae that were characterized along 3 radial lines, resulting in 15 to 45 indentations per osteon ($\sim$ 45 to 135 per animal). The tip was loaded into the sample at a rate of 50 $\mu\text{N/s}$,

held at a maximum load of 700 μN for 10 s, and then unloaded at a rate of 50 $\mu\text{N/s}$ (Donnelly et al., 2006a,b), resulting in ~ 150 nm deep indentations. The indentation modulus and hardness were calculated from the unloading portion of the load-displacement curve (Oliver and Pharr, 1992).

Raman Spectroscopy

Raman spectroscopy was used to characterize the tissue composition. Spectra were collected using a near-infrared, 785 nm laser focused through a 50 $\times$, 0.75 numerical aperture air objective (InVia microRaman, Renishaw), producing 2 μm diameter beam. Peak heights were identified after subtracting background fluorescence using WiRETM V2.0 software (Renishaw). Spectra were taken on 5 lamellae spaced across the radii of the osteons, resulting in 15 measurements per osteon (45 per animal). Spectra were acquired along the same radial lines characterized with nanoindentation and centered on lamellae when visible to match the nanoindentation locations as closely as possible.

Three bone parameters were calculated: mineral-to-matrix ratio, carbonate-to-phosphate ratio, and crystallinity (Akkus et al., 2003; Carden and Morris, 2000; Freeman et al., 2001; Gourion-Arsiquaud et al., 2009; Penel et al., 1998; Tarnowski et al., 2004). Mineral-to-matrix ratio, a measure of the degree of mineralization of the tissue, was calculated as the ratio of the phosphate ν_1 (~ 965 cm^{-1}) to the CH_2 wag (~ 1450 cm^{-1}) peaks (Carden and Morris, 2000). The CH_2 wag peak was used due to noise around the Amide I peak. Carbonate-to-phosphate ratio, a measure of the level of carbonate substitution into the hydroxyapatite crystal lattice, was calculated as the ratio of the carbonate ν_1 (~ 1070 cm^{-1}) to the phosphate ν_1 peak (Penel et al., 1998). Crystallinity, a measure of crystal size and perfection, was estimated as the reciprocal of the full width at half maximum of the phosphate ν_1 peak (FWHM) (Freeman et al., 2001). For this measure, a sharper phosphate ν_1 peak (smaller FWHM) indicates greater mineral crystal size and perfection.

Second harmonic generation microscopy (SHG)

SHG microscopy utilizes nonlinear scattering of photons from aligned collagen fibers within bone to create a map of aligned collagen content. These measurements distinguish whether the collagen contained in the plane of measurement, in this case the transverse plane, is aligned within that plane. The aligned collagen within the plane is proportional to the square root of SHG intensity (Boyd, 2003; Campagnola et al., 2003; Moreaux et al., 2000; Williams et al., 2005; Zipfel et al., 2003). A 2.5 W Ti:Sapphire (Mai Tai Deep See, Spectra Physics), with a pulse rate of 80 MHz at 780 nm was focused onto the samples with a 25 $\times$, 1.05 numerical aperture water objective (Olympus). SHG photons were collected in epi mode and optically filtered to remove backscattered incident light. In-plane and out-of-plane focal volume dimensions (FWHM) were approximately 497 nm and 1500 nm, respectively. SHG images of the three osteons analyzed with Raman spectroscopy and nanoindentation were taken as the Kalman average of 3 scans.

The three radial lines per osteon characterized with nanoindentation and Raman spectroscopy were located on the SHG images. For each pixel along the radial lines, the square root of the SHG intensity was averaged for a window 1 pixel wide and 4 pixels high (497 nm $\times$ 1988 nm, Image J, National Institutes of Health). For each lamella, maxima corresponding to lamellar aligned collagen and minima corresponding to interlamellar aligned collagen were recorded. Each osteon contained between 5 and 15 lamella, hence 15 to 45 maxima and minima were recorded per osteon (~ 45 to 135 per animal). The relative degree of aligned collagen for each lamellae was calculated as the peak height ratio of lamellar to interlamellar aligned collagen (Donnelly et al., 2006b; Martin et al., 2002). To isolate the contributors to changes in peak height ratio, lamellar and interlamellar values

were determined individually and normalized by the mean aligned collagen content of the osteon to correct for intensity variations.

Data Analysis

All statistical testing for variations with animal and tissue age were performed on the raw, unaveraged data using linear models (JMP 7.0, SAS Institute, Inc.), with animal age (0.28-32.45 years, n=12 animals), osteon (1–3, nested variable), radial line (1–3, nested variable), and distance from the center of the osteon (0-80 μm) as factors. First, multi-factor ANOVAs tested whether relationships between parameters and animal age or tissue age depended on maturity (young, 0–5 years old, or mature, >5 years old) (Bronikowski et al., 2002). If maturity was not significant, a multi-factor ANOVA was performed on the combined data. If maturity was significant, separate multifactor ANOVAs were run for young and mature data. P-values less than 0.05 were considered significant.

To correlate nanomechanical properties with compositional measures, the mean values of the outcome measures were calculated for each baboon. Single-factor ANOVAs tested for the individual effect of each compositional measure on indentation modulus and hardness. Then, multi-factor ANOVAs determined the combination of compositional parameters with greatest predictive power for indentation modulus and hardness. A change of 0.05 in the coefficient of variation (R^2) was considered an improved model fit.

To visualize the tissue age data, results for a single animal were averaged by binning in 6 μm increments across the 3 radii of the 3 sampled osteons. Specifically, the mean distance from the center of the osteon and mean parameter values were calculated for each 6 μm bin of the indentation modulus and Raman data.

3. Results

Sexual maturity (age ≤ 5 years) significantly influenced indentation modulus but tissue age did not. Indentation modulus increased steeply with animal age across young, sexually immature animals and increased gradually across sexually mature animals (Figure 2). The magnitude of the increases were 1.85 GPa, or about 6.6%, per year across young animals ($p < 0.0001$, ages 0.28 to 2.81 years, n=6), and only 0.08 GPa, or 0.2% per year, across sexually mature animals ($p = 0.0058$, ages 6.21 to 32.45 years, n=6) (Table I). Indentation modulus did not vary with tissue age across the osteon (Figure 2).

Hardness was significantly influenced by both sexual maturity and tissue age (Figure 2). Hardness increased steeply across young animals but showed no correlation with animal age in sexually mature animals (Figure 2). In young animals, hardness increased 0.08 GPa, or about 6.8%, per year ($p < 0.0001$, Table I). Hardness did not vary with tissue age across the osteon radii in young animals, but decreased with tissue age in mature animals, decreasing by 0.3% per micron for a total decrease of 9-18% across the osteon radii ($p = 0.0008$).

Mineral-to-matrix ratio was affected by animal age but not tissue age. Mineral-to-matrix ratio increased by 0.88, or about 12%, per year across young animals ($p < 0.0001$), but was independent of age in mature animals (Table I, Figure 3). Mineral-to-matrix ratio was not correlated with tissue age across the osteon radii.

Carbonate-to-phosphate ratio was affected by both animal age and tissue age. Carbonate-to-phosphate ratio increased by 0.01, or 6.7%, per year across young animals ($p < 0.0001$) and was independent of animal age after sexual maturity (Table I, Figure 3). Carbonate-to-phosphate ratio increased with tissue age across the osteon radii in both young and mature animals, increasing by 0.2% per micron ($p < 0.0001$, Figure 4).

Crystallinity varied with both animal age and tissue age. Crystallinity increased by 0.08% per year with animal age regardless of maturity ($p < 0.0001$, Table I, Figure 3), while the increase in crystallinity with tissue age was greater in young than in mature animals. In young animals, crystallinity increased by 0.1% per micron or 3-6% total across the osteon radii ($p < 0.0001$). In mature animals, the magnitude of the increase with tissue age was less than half that which occurred in young animals, 0.04% per micron or 1.2-2.4% total across the osteon radii ($p < 0.0001$, Table I, Figure 4).

Aligned collagen content varied with tissue and animal age (Figure 5). The peak height ratio of lamellar to interlamellar aligned collagen increased by 4.6% per year with animal age in young animals, and by 0.45% per year in mature animals ($p < 0.0001$) (Table I). This increase with animal age was due to both increased lamellar aligned collagen (+2.7% per year in young animals, and +0.22% per year in mature animals) and decreased interlamellar aligned collagen with animal age (-1.5% per year in young animals, -0.20% per year in mature animals). With tissue age, collagen peak height ratio increased by 0.19% per micron, for a total increase of 6 to 12% across the osteon radii, regardless of animal age ($p < 0.0001$). This increase was primarily due to increased lamellar aligned collagen (+0.30% per micron in all animals).

When correlations between nanomechanical properties and composition were examined, indentation modulus and hardness were most influenced by mineral-to-matrix ratio (Figure 6). Mineral-to-matrix ratio alone explained 78% of the variation in indentation modulus ($p < 0.0001$) and 70% of the variation in hardness ($p < 0.0004$). The only other significant relationship was with lamellar aligned collagen, which explained 30% of the variation in indentation modulus ($p = 0.0386$), but did not significantly predict the variation in hardness ($p = 0.0939$). Combining lamellar aligned collagen with mineral-to-matrix ratio did not improve the predictive power for indentation modulus.

4. Discussion

We hypothesized that tissue mechanical properties would vary with changes in mineral and aligned collagen content as a function of both tissue and animal age. Indeed, variations in the tissue mechanical properties correlated with mineral-to-matrix ratio and aligned collagen content. Variations in mineral-to-matrix ratio were the most important predictor of variations in indentation modulus and hardness. The addition of other parameters to mineral-to-matrix ratio did not add significant predictive value to the ANOVA models. Mineral-to-matrix ratio effectively captured the variations explained by carbonate substitution and crystallinity (measures of the mineral), and lamellar aligned collagen (measure of the matrix).

When the behavior of individual mechanical and compositional parameters was examined, indentation modulus, hardness, mineral-to-matrix ratio, carbonate substitution, and aligned collagen peak height ratio, followed biphasic relationships with animal age, increasing sharply in the first years of life, and then remaining constant with age after sexual maturity. Based on the linear fits, mineral-to-matrix ratio increased nearly twice as fast as indentation modulus and hardness in young, growing animals. Of course, other microstructural factors such as mineral crystal size and orientation, collagen alignment, and noncollagenous matrix proteins may also contribute to the tissue mechanical properties (Busa et al., 2005; Donnelly et al., 2010). Indeed, similar to stiffness, peak height ratio, a measure of aligned collagen content, followed a bi-phasic relationship with animal age. Furthermore, after sexual maturity, stiffness increased at a gradual rate almost identical to that of the lamellar aligned collagen. Although crystallinity showed a significant increase with animal age in mature

animals, the percent changes were extremely small and may not be physiologically significant.

Carbonate substitution, crystallinity, and aligned collagen peak height ratio increased with tissue age, whereas mineral-to-matrix ratio and the mechanical parameters did not. Carbonate substitution increased at a gradual, constant rate throughout the lifespan of the baboon, suggesting that carbonate ions are substituted into the crystal lattice at a constant rate. Crystallization increased nearly an order of magnitude more rapidly in young than in mature animals, although again the percent changes were small.

In rodent models of ageing, increased mineralization, carbonate substitution, and crystallinity corresponded with increased stiffness and hardness, and similar to the current study, stiffness and hardness increased more gradually than did tissue mineralization (Donnelly et al., 2010; Miller et al., 2007; Tarnowski et al., 2002). Based on our data, these relationships hold true in a non-human primate model that more closely parallels human skeletal ageing than other animal models. Furthermore, variations in composition and nanomechanical properties were functions of tissue and animal age, a novel finding because prior studies have focused only on mature animals and showed variable relationships with ageing (Akkus et al., 2003; Gupta et al., 2006; Rho et al., 1999; Rho et al., 2002). The mechanical properties also related to collagen content and organization, as previously reported in a small sample of human vertebrae (Donnelly et al., 2006b). This relationship held over the entire lifespan of the baboon, and reflected that both stiffness and aligned collagen content increased as functions of animal age. Mineral-to-matrix ratio, carbonate substitution, indentation modulus, and hardness increased rapidly in the rat cortex to reach levels of mature tissue within the first four days after formation, but thereafter increased only slightly (Busa et al., 2005; Donnelly et al., 2010; Tarnowski et al., 2002). As baboon osteons selected for this study were not visibly remodeling, the tissue material properties would have already reached levels of the mature tissue and no steep increases in tissue material properties would be expected. Carbonate substitution and crystallinity did increase with tissue age across osteon radii, in agreement with data obtained through FTIR, a complementary spectroscopic technique (Gourion-Arsiquaud et al., 2009).

As in any experiment, certain limitations may affect the interpretation of our results. One consideration is that the exact age of the osteons could not be distinguished because fluorochrome labels were not administered to the animals. However, by sampling three non-remodeling osteons within the same anatomical region of the cortex, we hoped to reduce variability due to anatomical location. The lack of steep gradients in tissue mineralization and stiffness near the Haversian canals confirmed that the selected osteons were not remodeling at the time of animal death. Furthermore, although our composition data obtained by Raman spectroscopy is consistent with key points from that obtained by FTIR, one discrepancy is evident. Namely, carbonate-to-phosphate ratio measured by FTIR decreased with animal age, whereas our Raman data showed increasing carbonate substitution during growth, followed by no change following sexual maturity (Gourion-Arsiquaud et al., 2009; Paschalis et al., 1996). This discrepancy may arise from fundamental differences between these two techniques; for example, Raman carbonate substitution is calculated from the peak height ratio rather than the peak area ratio used for FTIR. Finally, although dehydration increases tissue modulus and hardness (Bushby et al., 2004; Hengsberger et al., 2002; Rho and Pharr, 1999), all samples were treated similarly, and therefore our ability to detect variations in these properties with tissue and animal age was presumably not compromised.

In summary, composition and mechanical function were closely related in baboon osteonal bone and depended on tissue and animal age. When the entire lifespan of the animal was

examined, tissue from the young, growing animals had lower stiffness and hardness, associated with lower mineralization, carbonate substitution, crystallinity, and aligned collagen content than tissue from sexually mature animals. In future studies, fluorochrome labeling could help identify newly formed osteons to determine the full effect of tissue age within osteonal bone, while studies of male animals would allow identification of sex-based differences that are not age-dependent. Based on the current results, we would expect the lamellae of newly formed osteons to have tissue properties similar to young, sexually immature animals. These results provide a baseline for variations that occur in healthy bone tissue with the natural ageing process and may have clinical implications in osteoporosis, where increased bone loss is not coupled with new bone formation. However, the relationship between fracture resistance and these age-related changes in tissue composition and nanomechanical properties must be examined further.

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Figure 1.

(a) Cross section of a baboon femur embedded in polymethylmethacrylate showing the area chosen for characterization. (b) Light microscopy image of the area boxed in part (a) with specific osteons chosen for characterization circled. (c) Atomic force microscopy image of a quadrant of one of the osteons showing one radial line that was characterized with nanoindentation, Raman spectroscopy, and second harmonic generation. The residual indentations are visible in the white triangles. The insert shows a magnified view of a residual indentation.

Figure 2.

Nanoindentation results versus animal and tissue age. (a) Indentation modulus and (c) hardness increased with animal age in the young animals and were constant after sexual maturity. Each point in (a) and (c) represents the mean $\pm$ SD of all measurements for a single animal for visual presentation. The dashed lines are the ANOVA models for young and mature animals, which were performed on the raw, unaveraged data (45-135 measurements per animal). (b) Indentation modulus and (d) hardness were constant with tissue age. Increasing distance from the center of the osteon corresponds with increasing tissue age. Open symbols with dashed lines represent young animals and solid symbols with solid lines represent mature animals.

Figure 3.

Raman spectroscopy results versus animal and tissue age. (a) Mineral-to-matrix ratio and (c) carbonate substitution both increased in the young animals and were constant after sexual maturity, whereas (e) crystallinity increased with animal age independent of maturity. Each point in (a), (c), and (e) represents the mean $\pm$ SD of all measurements for a single animal for visual presentation. The dashed lines are the ANOVA models, which were performed on the raw, unaveraged data (45 measurements per animal) (b) Mineral to matrix ratio showed no change with tissue age, whereas (d) carbonate substitution increased with animal age, independent of maturity, and (f) crystallinity increased with tissue age faster in young than in mature animals. Increasing distance from the center of the osteon corresponds with increasing tissue age. Open symbols with dashed lines represent young animals and solid symbols with solid lines represent mature animals.

Figure 4.

SHG results with animal and tissue age. (a) Peak height ratio (lamellar/interlamellar aligned collagen) increased more with animal age in young than in mature animals. Each point represents the mean $\pm$ SD of all measurements for a single animal for visual presentation. The dashed lines are the ANOVA models for young and mature animals, which were performed on the raw, unaveraged data (45-135 measurements per animal) (b) Peak height ratio increased with tissue age. Increasing distance from the center of the osteon corresponds with increasing tissue age. Open symbols with dashed lines represent young animals and solid symbols with solid lines represent mature animals.

Figure 5.

Significant correlations between mean nanomechanical parameters and composition measures obtained from each animal. Open symbols represent young animals and solid symbols represent mature animals. Dashed lines and R^2 values are from the ANOVA models. (a) Indentation modulus versus mineral-to-matrix ratio. (b) Hardness versus mineral-to-matrix ratio. (c) Indentation modulus versus lamellar aligned collagen content.

Significant variations of mechanical and compositional measures with animal and tissue age. Ranges reported for percent change across the osteon reflect variable osteon size. NS = not significant, $p \geq 0.05$; young = 0 – 5 years; mature > 5 years.

Table I

	Animal Age (% Change Per Year)		Tissue Age (% Change Across Osteon)	
	Young	Mature	Young	Mature
Indentation Modulus	6.6	0.20	NS	NS
Hardness	6.8	NS	NS	-9 to -18
Mineral:Matrix	12	NS	NS	NS
Carbonate:Phosphate	6.7	NS	6 to 12	
Crystallinity	0.08		3 to 6	1.2 to 2.4
Peak Height Ratio	4.6	0.45	6 to 12	