Compositional Changes in the Microstructure of Cortical Bone with Increasing Tissue Age
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INTRODUCTION
Age-related decreases in toughness have been observed in cortical bone [1-3]. However, the specimens studied have consisted of a mixture of osteonal and interstitial tissues and therefore represent bulk tissue properties. Compression tests of micro-cores prepared from interstitial tissues and secondary osteons have demonstrated increased toughness in the latter tissue with no age or gender effect noted [4]. This suggests there are changes in the mineral and/or collagen phases of secondary osteons as they age to eventually become interstitial tissues. Though it is not possible to determine their exact ages, secondary osteons, old osteons, and interstitial tissues represent three distinct stages in a chronological transition from newly remodeled to old tissue. Fourier transform infrared spectroscopy (FTIR) is a technique well suited for determining the compositional properties of the mineral and collagen phases at the micro-structural level [5]. The present study utilizes FTIR to investigate changes in the mineral and collagen phases of cortical bone as it transitions from secondary osteons to interstitial tissue.

MATERIALS AND METHODS
Cadaveric femurs from twelve male donors were divided into two age groups (n = 6 per group): Middle-Aged (49 to 55 y.o.) and Old (67 to 87 y.o.). One cross-section (~2 mm thick) was cut from the subtrochanteric region of the femoral diaphysis of each donor. A randomly oriented strip (~5 mm wide) was then cut from the medial quadrant. The specimens were then dehydrated through a series of increasing concentrations of EtOH, cleared with xylene, and finally infiltrated and embedded in polymethylmethacrylate (PMMA). For FTIR analysis, 2 µm thick sections were cut from the embedded undecalcified bone blocks with a Leica microtome (SM 2500; Leica). The sections were then transferred onto BaF2 windows (SpectraTek, Hopewell Junction, NY, USA).

Utilizing a light microscope (Meiji, Japan), three distinct tissue types from each section were classified as representing chronological stages of bone remodeling. Secondary osteons (SO) were classified as Haversian systems exhibiting concentric lamellae encompassed by an intact cement line. Old osteons (OO) were classified as lamellar Haversian systems with a cement line interrupted by the presence of a secondary osteon(s) or interstitial tissue. Interstitial tissues (INT) were classified as regions between osteons which were the product of old remodeling events, i.e. exhibited lamellar osteonal remnants. For each donor, three samples of each of the three tissue types were selected for FTIR analysis.

Spectra were acquired with a Spectrum Spotlight 300 Imaging System (Perkin Elmer Instruments, Shelton, CT, USA), consisting of a step-scanning FT-IR spectrometer with an MCT (mercury-cadmium, telluride) focal plane array detector placed at the image focal plane of an IR microscope. Single spectra and images were collected in the transmission mode at a spectral resolution of 4 cm⁻¹ in the frequency region between 2000 and 800 cm⁻¹ with an IR detector pixel size of 6.25×6.25 μm.

FTIR parameters were calculated using ISYS software. Mineral to matrix ratio (M/M) was calculated by integrating the phosphate area peaks between 916 to 1180 cm⁻¹ and the amide I mode from 1592 to 1712 cm⁻¹. Carbonate-to-phosphate ratio (C/P) was calculated through the integrated area of the v2 carbonate peak (852-890 cm⁻¹) and that of the phosphate. Crystallinity was calculated as the ratio of relative peak height subbands at 1030 and 1020 cm⁻¹ within the phosphate contour. The ratio of mature to immature collagen cross-links (XLR) was determined by the intensity ratio of amide I subbands at 1660 and 1690 cm⁻¹. Data for all parameters are presented as the mean of results from 3 samples of each tissue site from each donor within the same age group.

RESULTS
ANOVA (Table 1) revealed age to have a significant effect on the collagen crosslink ratio. Tissue site had a significant effect on the mineral to matrix ratio. Post-hoc analysis (Table 2) revealed the Old group exhibited a higher collagen crosslink ratio than the Middle-aged group. Interstitial tissues exhibited higher mineral content compared to both secondary and old osteonal tissues.

<table>
<thead>
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<th>Factor</th>
<th>M/M</th>
<th>C/P</th>
<th>XLR</th>
<th>Crystallinity</th>
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<td>Age</td>
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<td>NS</td>
<td>0.0440</td>
<td>0.0992</td>
</tr>
<tr>
<td>Site</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
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(Age*Site NS NS 0.0440 0.0992
(Significance p < 0.05

Table 2: Post-hoc Analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>M/M</th>
<th>C/P</th>
<th>XLR</th>
<th>Crystallinity</th>
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<tbody>
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<td>NS</td>
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<tr>
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<td>0.0685*</td>
<td>0.0879+</td>
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</table>

(Significance p < 0.05; +INT > OO; *INT > SO; -OO > SO)

DISCUSSION
FTIR analysis revealed interstitial tissue exhibited a higher quantity of mineral compared to either osteonal group, while no differences were noted between the osteonal groups. This is indicative of continued mineralization as bone transitions from osteonal to interstitial tissue and is consistent with the observation that secondary mineralization may continue for years after primary mineralization [6]. Additionally, as increases in ash content have been related to decreases in cortical bone toughness [7], the present findings are also consistent with microcompression tests which demonstrated interstitial tissue absorbs less energy to failure compared to osteonal tissue [4].

The results also indicated older tissue to exhibit a higher collagen crosslink ratio compared to middle-aged tissue. Age-related increases in the collagen crosslink ratio have been observed in homogenized bone collagen, though the trend is most pronounced up to 25 years of age [8]. A trend was observed for increases in the collagen crosslink ratio as tissue aged from old osteons to interstitial tissue. Collagen crosslink maturation has been related to bone age in the newly formed cortical bone of baboons [9]. Increases in the collagen crosslink ratio have also been related to increased fracture risk in human cortical bone [10], consistent with the decreased toughness of interstitial tissue observed in micro-compression tests [4]. A trend toward age-related increases in mineral crystallinity was also observed in this study. Considered with the age-related increases in the collagen crosslink ratio, this is indicative of the need for collagen stabilization through crosslink maturation in order for mineral formation and growth to proceed.

ACKNOWLEDGEMENTS
This work was supported by NIH grants AG022044 and AR046121. We thank Mr. Siyuan Ding for preparation of the cross-sections and Ms. Patricia Morales for sectioning of the cross-sections.

REFERENCES:

Poster No. 2208 • ORS 2011 Annual Meeting