INTERSPECIMEN STUDY OF BONE TO RELATE MACROMECHANICAL, 
NANOMECHANICAL AND COMPOSITIONAL CHANGES
ACROSS THE FEMORAL CORTEX OF BONE

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Mechanics of bone is widely studied and researched, mainly for the study of fracture. This has been done mostly on a macro scale. In this work hierarchical nature of bone has been explored to investigate bone mechanics in more detail. Flexural test were done to classify the bones according to their strength and deflection. Raman spectroscopy analysis was done to map the mineralization, collagen crosslinking changes across the thickness of the bone. Nanoindentation was done to map indentation hardness and indentation modulus across femoral cortex of the bone. The results indicate that the composition of the bone changes across the thickness of the femoral cortex. The hypothesis is confirmed as increase in mineralization, carbonate to phosphate ratio and collagen crosslinking shows the effect as increased indentation hardness and modulus and decreased deflection.
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CHAPTER 1

SCOPE OF THESIS

In past much research have already been conducted on mice bones using techniques such as three point bending, Raman spectroscopy and nanoindentation. But no work has been done to relate them on macro, micro and nano scale, all the three techniques together give a better picture of mineralization and collagen crosslinking affecting the nano as well as macro mechanical properties.

In this thesis following hypothesis has been confirmed:
Hypothesis 1: The increase in indentation hardness and indentation modulus across the thickness of the bone is co-related and paralleled to the increase in mineral to matrix ratio, carbonate to phosphate ratio and collagen crosslinking.
Hypothesis 2: Stiffness increases due to increase in mineralization and increase in collagen crosslinks, thereby decreasing the deflection.

The objective of this thesis is to relate two groups of mice bones deflection with hardness, mineralization and collagen crosslinking. These mechanical properties are related to chemical compositions like mineralization and collagen crosslinking with nano-mechanical property like indentation hardness and elastic modulus. The proposed hypothesis is that as the bone ages (the tissue gets aged from endosteal to periosteal, the bones that are studied in this work are mature mice model they all are of same age.), the tissue mineralizes and therefore the hardness increases, crosslinking of collagen takes place and the stiffness of bone is increased. This can be seen on nano level, the bone grows inside out and therefore the tissues which are outside are young and have lower mineralization and therefore lower hardness, collagen
crosslinking is lower and therefore the stiffness is lower. Methods used for this are three point bending, microCT, nanoindentation and Raman spectroscopy. microCT is used to calculate the diameter and CSMI values to be input into the calculation of three point bend test.

- Three point bend test gives strength and deflection which is macro mechanical property of the bone.
- Raman spectroscopy gives chemical composition changes and influence on the micro level.
- Nanoindentation is used to measure indentation hardness and elastic moduli on the nano level. The measurements thus obtained are correlated and compared for four quadrants viz; anterior, lateral, medial and posterior.

Donnelly and colleagues characterized the nanomechanical properties and composition of regions of differing tissue age in the femoral cortices of growing rats [1]. Their results show a very sharp and early increase in tissue modulus, hardness, and mineral:matrix ratio with increasing distance for the first 35 microns from the periosteum (youngest tissue). Kavukcuoglu et al. studied the effect of bone aging and deficiency of osteopontin on the nanomechanical properties of femur of young, mature and old mice. Many previous studies shows change in toughness and modulus with change in age [2, 3, 4, 5]. This work is a unique attempt to co-relate mechanical properties on nano, micro and macro scale to give a broader idea about their influence.
CHAPTER 2
INTRODUCTION AND BACKGROUND

2.1 Introduction

Bone is a hierarchical composite composed of approximately 65% mineral and 35% organic matrix and serves as the primary structural element providing a framework for skeletal motion. It differs from other tissues due to its hardness and rigidity. These characteristics in bone are mainly due to inorganic salts that are impregnated in the organic matrix, noncollagenous proteins and mineral. Moreover, bone is a living composite that optimizes its structure to adapt to fluctuations in its mechanical environment.

2.2 Morphology: Macroscopic and Microscopic Structures

The femur which is one of the long bones other than humerus and tibia, serves as ideal model for the study of macroscopic structure of bone. A long bone consists of cylindrical shaft which forms the majority of the bone and is located in the center this is also called as diaphysis, and the ends are rounded which are termed as epiphysis. The ends are wider and rounded because these are articulated and larger areas are required to carry loads. The diaphysis is mainly composed of cortical bone. This is solid mass highly dense with microscopic channels. Almost 80% of the bone is made of cortical bone and is responsible for support and protect skeletal system. Rest 20% is cancellous bone.
2.2.1 Bone Nomenclature

The bone has three major parts viz diaphysis, metaphysis and epiphysis (Figure 2.1). Diaphysis is mainly made up of cortical bone. Metaphysis and epiphysis on the other hand is made up of cancellous bone. Periosteum is the outer surface of the bone and is made up of fibrous connective tissues. Periosteum develops as into bone during the growth of the bone and also provides healing of fractures. Endosteum is the inner surface of the bone and is lined on the marrow cavity of the diaphysis.

Figure 2.1. Schematic showing the location of femur, the nomenclature for the femur parts and the cross section of femur showing the four quadrants.
Figure 2.2. Schematic of bone cross section showing four quadrants, direction of bone growth and change in hardness, stiffness, modulus across the thickness of bone with change in mineralization and collagen crosslinks.

The above Figure 2.2 shows schematic of cross section of the right femur of mature mice model with four quadrants marked viz anterior, lateral, posterior and medial. The blue arrow shows the direction of bone growth, which is from inside to the out of the bone. Hence the tissues at the inner side of the bone are matured and old, whereas tissues which are at the outer surface of the bone are young. At the inner surface the tissues the mineral size grows as a part of mineral absorption process on the hydroxy
apatite crystals. At the young tissue sites the nucleation takes place to form small crystals of minerals. The hardness therefore is lower at the outer surface of the bone as compared to the inner surface. There is more number of collagen crosslinks at the inner surface of the bone than the outer surface. Moreover the reducible collagen crosslinks reduces as the bone tissue matures thereby making the bone stiffer and less flexible and hence lower deflection. At the outer surface the collagen crosslinks are reducible and are labile, this crosslinks can be broken and again joined to remake the collagen bonds. Hence the younger cells have lower collagen crosslinks and therefore lower stiffness and higher deflection.

2.2.2 Composition of Bone

Hydroxyapatite, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, is the primary mineral of bone with elements including carbonate, citrate, magnesium, fluoride, and strontium also found on the crystal lattice, but to a much lesser extent. Bone mineral varies with age, in content, composition and crystal size. Bone may be hypomineralized when there is rapid growth and hypermineralized during senescent periods [6,7,8]. As the bone ages there is an increase in crystal size [9,10], mineralization, and strength, but also a reduction in its toughness [11] and ultimate strain which may lead to microcrack development [12]. Developed microcracks, which do not heal in a timely manner, may accumulate and progress leading to increased fracture risk [13,14]. In contrast, when mineralization levels are low, stiffness and strength are reduced [15,16,17,18]. The organic matrix, composed of approximately 90% collagen and 10% of other noncollagenous proteins, also plays a role in the mechanical behavior of bone [19, 20, 21]. Aging has been shown
to change the quality of the collagen network leading to reductions in bone toughness [22, 23, 24, 25, 26].

2.3 Ageing of Bone

With age the structure of bone deteriorates and therefore the mechanical properties changes. These changes happen for the overall complete bone as well as on the nano, micro scale as well. Since bone has a hierarchical structure, changes in chemical composition like carbonate, collagen etc exhibits a change in overall quality of the bone. For example the quality of collagen deteriorates with age and that affects the toughness of bone by decreasing to 35%, elastic modulus was decreased by 30%. The decrease in toughness also affected the bones ability to dissipate energy and eventually fracture by 50% [27]. The old tissues in bone have higher collagen crosslinks as compared to young tissues, this affect the bones response to applied load, higher crosslinks gives low deflection due to higher stiffness and vice versa [28]. Bone mineral varies in content, composition and crystal size. With age mineral content varies, bone tends to hypomineralized (less mineralization) when there is rapid growth which is oseen in young tissues while hypermineralized in old tissues [29, 30, 31].

Hypermineralization in older bones have a tendency for developing macro and dangerous crack [32]. As the bone ages there is increase in mineralization which reduces toughness [33]. The aged bones develop microcracks which do not heal, this has a potential to crack when loaded [34,35]. The mineral content increased with age and the crystal size also showed increase in young human iliac crest was shown by Handshin and Stein [36, 37]. The ash weight and mineral-to-matrix ratio is linearly related [38]. Reduction in stiffness and strength have been attributed to low
mineralization levels, while lower one’s ultimate strain which increases the fragility is attributed to high levels of mineralizations [39, 40, 41, 42, 43, 44, 45]. The effect of increase age can be seen in quality of collagen network that has been shown to decrease toughness in bone [46, 47, 48, 49, 50]. The organic matrix is composed of 90% collagen and 10% other noncollagenous proteins [51, 52, 53]. Although the roles of noncollagenous proteins are unclear they can be used to clinically assess bone turnover [54,55,56].

2.4 Bone Hardness and Bone Mineral Crystal Size

The increase in mineral size increases the hardness of the bone. The content of carbonate in hydroxy apatite increases and phosphate decreases. This is seen from carbonate to phosphate ratio calculation from raman spectra. The process of crystal formation is a dynamic physiochemical process. As the osteoclasts resorb the crystals, they get deposited on the osteoid. The smaller crystals are easy to resorb, so they gets dissolved first. The larger crystals remain. The ions which are liberated due to the resorption of the smaller crystals gets deposited on the existing larger crystals to make them even larger in size. This process helps in ordered stucture formation.
CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Bone

The mature male C57BL/6 mice were allowed to acclimate for two-weeks, and then sacrificed. These are commonly used inbred mouse strain and has been shown to be a valid model for studying age-related bone loss [57, 58, 59]. A 12:12 hour light-dark cycle and NIH-31 diet was maintained during the two-week acclimation period. Immediately after the mice was euthanized, the right femurs were dissected free, cleaned of soft tissue, and wrapped in phosphate-buffered saline (PBS) soaked gauze to maintain moisture.

3.1.2 Phosphate Buffered Solution (PBS)

The phosphate buffer solution used was water based salt solution which has a controlled mixture of various salts like ACS reagent grade dibasic sodium phosphate, and monobasic potassium phosphate. The phosphate buffer solution used had a concentration of 0.1M and pH of 7.4±0.01 at 25C. The solution was bought from Ricca Chemical Company (Arlington, TX). This solution helps to simulate the pH of the human body and also the osmolarity and ion concentrations of the human body.
3.2 Sample Preparation

3.2.1 X-ray Micro Tomography

After dissection, the right femoral bone of the mouse is wrapped in gauze soaked with phosphate buffered saline (PBS), placed in a labeled vial, and kept in a freezer at -23 °C. Once removed from the freezer, the bone sample is kept at room temperature for 30 min to allow it to partially defrost. The gauze is then separated carefully from the bone and the bone is ready for microCT imaging.

3.2.2 Three Point Bend Test

The femurs were preserved in phosphate buffer solution wrapped in gauze and frozen after dissection. Prior to three point testing these frozen samples were removed and allowed to thawed for 30 min. The gauze was removed carefully to make sure that the bone is not damaged. This femur is then ready for testing. After the testing the bone breaks into two pieces one is termed as proximal end and the other is termed as distal end. The proximal end is wrapped in PBS soaked gauze and again frozen. The distal end is dried at room temperature for 24 hrs and then used for preparing sample for nanoindentation and Raman spectroscopy. This procedure was repeated for all the other twenty-four bones.

3.2.3 Nanoindentation and Raman

These dried distal femur ends were taken and embedded in a 25 mm height x 28 mm diameter polypropylene mold cup supplied by Electron Microscopy Sciences. In a separate container, EpoFix epoxy is mixed with the EpoFix hardener supplied by Electron Microscopy Sciences in 15:2 parts by volume, mixed thoroughly, and kept
aside to remove bubbles. The epoxy is then poured in the mold and allowed to cure for 24 hrs. Once the epoxy is cured, the plastic mold is cut open to get the mounted specimen. The sample is then polished using a series of 400, 600, 800, 1200 silicon carbide paper and later with 0.05 micron alumina suspension at 60 rpm. The polished sample is then sonicated in deionized water for 15 minutes to remove all the debris collected during the polishing process. Figure 3.1 shows the embedded distal end in epoxy and polished sample ready to test for nanoindentation and Raman.

![Image](image_url)

*Figure 3.1. Distal end embedded in epoxy and polished.*

3.3 **Methods**

3.3.1 **X-ray Micro Tomography**

The machine used to microCT is the Skyscan 1172. The partially defrosted sample is wrapped in the parafilm and placed inside the hollow polystyrene foam. The polystyrene foam is also wrapped with parafilm to ensure minimum loss of moisture.
Care is taken to align the bone vertically so that there is minimal misalignment in axis while the sample rotates during imaging. With the help of clay the polystyrene foam is fixed to the sample holder which is then screwed onto the stage. Preliminary trials were done to set the following imaging parameters: voltage was adjusted to 48 kV and current of 204 µA. A 0.5 mm Al filter was employed to give better contrast between the bone and background. Before the actual scan is performed the flat field correction is done for dark and bright field. The scan is then performed, with medium resolution settings of 5 microns, with 180° for the region of interest.

To selecting the regions of interest, a microCT phantom image projection of the whole specimen was first acquired. This image is used to (1) accurately measure the bone length (confirmed using a micrometer); (2) accurately measure the mid-diaphyseal bone diameter; and (3) to select a the approximate regions of interest, slightly larger than needed so that all the images from the complete region of interest are available for selective post-processing. The first region of interest was the distal femoral end which was chosen such that the 1800 microns (for a 17 mm total femoral length) was adjusted according to the individual length of each bone sample upwards from the growth plate. The second region of interest was at the mid-diaphysis of the femur using a 1 mm thick centered volume. These regions were reconstructed and analyzed. The raw images were corrected for ring artifacts and beam hardening.

3.3.2 Three Point Bend Test

The instrument used is RSA3 from TA Instruments. For 3-pt bend test sample mid-diaphyseal diameter and total length is measured from the phantom image of the femur bone which was taken before region of interest. The span length of the lower
portion of the 3-pt bend fixture is 10 mm. The sample is removed from the PBS and immediately setup for mechanical testing. The sample is placed in such a way that the posterior side is in compression as shown in Figure 3.2. The test is done in displacement control at room temperature, and the force – displacement and stress – strain curve are plotted. The rate of displacement is set to 0.01 mm/sec (0.6 mm/min) to ensure a high data acquisition rate and maximum deflection is set to 5 mm. Before the test, extreme care is taken to align the bone samples properly and consistently (confirming the posterior-anterior and proximal-distal placement) (see Figure 3.3) and ensuring that while lowering the upper fixture the sample is just barely touched (for correctly estimating the deformation and strain). After the sample breaks, the proximal end is re-wrapped in PBS soaked gauze and kept in the freezer at -23 °C. The distal end is prepared for nanoindentation and Raman spectroscopy as discussed in section 3.2.3.

Stress, strain, Young’s modulus and modulus of toughness can be calculated from the force-displacement data recorded during the 3-pt bending tests and microCT imaging results as follows:

Stress: \( \sigma = F \left( \frac{Lc}{4I} \right) \)

Strain: \( \varepsilon = d \left( \frac{12c}{L^2} \right) \)

Young’s modulus: \( E = S \left( \frac{L^3}{48I} \right) \)
Modulus of toughness: \[ u = U \left( \frac{3c^2}{IL} \right) \]

Where:

- \( F \): applied force,
- \( L \): span,
- \( c \): distance from center of mass of the cross section,
- \( I \): cross sectional moment of inertia,
- \( d \): displacement,
- \( S \): stiffness,
- \( U \): work to failure.

*Figure 3.2. Placing of femur on three point bend fixture.*
3.3.3 Nanoindentation

The MTS nanoindenter XP was used following the Constant Stiffness Method Standard Hardness/Modulus with a Berkovich tip. In order to provide environmental isolation a combination of a minus k vibration isolation table and a thermal sound
vibration isolation cabinet were utilized. Prior to testing, the indenter system was calibrated on a sample of fused silica.

The mounted specimen was fixed to the sample holder stage and with the help of a microscope the anterior, posterior, medial and lateral quadrants were identified. The indentations were done from the outer periosteum edge towards the inner endosteum surface. A total of six indents were made in a straight line across the thickness of the cortical bone, illustrated with a yellow line in Figure 3.4. Each indent was spaced 10 microns apart. The percent unload in the stiffness calculation was kept to 50% and allowable drift correction was kept at 0.05 nm/s with a drift correction of 1. The depth limit was restricted to 600 nm. Unloading percent was 90 and the strain target was 0.05 1/s. Hardness and indentation modulus were calculated using the Oliver-Pharr method which assumes isotropic material behavior \[60\]. The elastic properties of the diamond indenter were: \(v_i = 0.07\) and \(E_i = 1140\) GPa and the Poisson’s ratio for bone was assumed to be 0.3.

The Oliver–Pharr method for determining the elastic modulus has been previously described (Oliver and Pharr, 1992). This method assumes isotropic material behavior. The primary variables are contact area \(A_C\), peak force \(P_{\text{max}}\), and contact stiffness \(S\) of the initial portion of the unloading curve. From these the reduced modulus \(E_r\) of the specimen– indenter combination is determined.
\[ E_r = \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A_c}} \]  

\[ E_s = \frac{1 - \nu_s^2}{\frac{1}{E_s} - \frac{1 - \nu_i^2}{E_i}} \]  

\[ H = \frac{P_{\text{max}}}{A_c} \]  

Where, \( E_s \) is modulus of bone and \( H \) is hardness, \( \nu \) is Poisson’s ratio and the subscripts \( s \) and \( i \) refer to the bone specimen and the indenter, respectively. The elastic properties of the diamond indenter are: \( \nu_i=0.07 \) and \( E_i=1140\text{GPa} \). The Poisson’s ratio of bone is assumed to be 0.3. The indenter system was calibrated on a sample of fused silica.
Figure 3.4. Optical cross sectional bone view of the: (A) anterior quadrant, (B) lateral quadrant, (C) posterior quadrant, and (D) medial quadrant.

3.3.4 Raman Spectroscopic Characterization for Compositional Changes

The specimen, as used for nanoindentation, was kept on the microscope stage and the anterior portion of the femoral bone cortex was setup for analysis. Raman spectroscopy was done between the nanoindentation lines along the cross section of the bone cortex with point mapping. The thickness of the cortical cortex was divided into six equidistant points and Raman spectra were collected, as illustrated with a red line in Figure 3.5. A 780 nm intensity laser was used at 1 % power. This was used because it
results in very little florescence as compared to the 532 nm intensity setting. Aperture was set to 100 µm slit with a spot size of 1.6 µm and a resolution of 25 to 33.8 1/cm. The scan was done from 300 to 2000 1/cm. The exposure time was 18 sec and background and sample exposure was performed 3 times. Background was collected before every sample. This background was subtracted from the Raman spectroscopy results and a baseline correction was performed. Twenty-four spectral lines were collected from the sample. Six scans were done on each quadrant (anterior, lateral, posterior and medial) directed from the endosteal to periosteal surface. Peak and corrected area analyses were then performed.

The characteristics band areas were determined from the Raman spectra for the calculations of mineral:matrix ratio, carbonate:phosphate ratio, collagen cross-linking ratio. Mineral:matrix ratio was calculated from band area ratio of 958 to 1660. Carbonate:phosphate ratio was calculated from band area ratio of 1070 to 958. Collagen cross-linking was calculated from band area ratio of 1660 to 1690.

Raman spectroscopy is used to characterize the compositional changes on the micron level as it gives a good comparison with the nanoindentation results as nanoindentation is done on nanometer scale. Moreover Raman spectroscopy is a non-destructive method for characterization.
Figure 3.5. Optical cross sectional bone view of the: (A) anterior quadrant, (B) lateral quadrant, (C) posterior quadrant, and (D) medial quadrant. Nanoindentation locations are shown as yellow lines and Raman spectroscopy locations as a red line.
CHAPTER 4

RESULTS

4.1 Three Point Bend Test

A total of twenty-four right femurs were tested for elastic modulus and ultimate stress refer Figure 4.1. From three point bend test two clusters have been identified. Group A (blue) is high elastic modulus and low ultimate stress. Group B (green) is low elastic modulus and high ultimate stress. The samples from group A are termed as A1, A2 and A3 where 1, 2 and 3 denote the specimen number under the group A. Similarly B1, B2 and B3 are specimens 1, 2 and 3 from group B. The average value for group A is $0.28 \pm 0.028$ Gpa and $38.01 \pm 4.149$ Mpa for elastic modulus and ultimate strength respectively. The average value for group B is $0.44 \pm 0.027$ Gpa and $26.39 \pm 6.847$ Mpa for elastic modulus and ultimate strength respectively.
Figure 4.1. Elastic modulus and ultimate stress plot showing the range of twenty-four right femurs three point bend test to form two clusters.

Figure 4.2. Comparison of elastic modulus, ultimate stress and displacement for randomized bone samples.
Table 4.1

Comparison of elastic modulus and ultimate stress from microCT and micrometer

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Elastic Modulus (Gpa)</th>
<th>Ultimate stress (MPa)</th>
<th>Elastic modulus (Gpa)</th>
<th>Ultimate stress (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From microCT</td>
<td></td>
<td></td>
<td>From micrometer</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.27</td>
<td>42.73</td>
<td>1.16</td>
<td>44.22</td>
</tr>
<tr>
<td>2</td>
<td>0.31</td>
<td>34.92</td>
<td>1.05</td>
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</tr>
<tr>
<td>3</td>
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<td>42.57</td>
</tr>
<tr>
<td>GROUP B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From microCT</td>
<td></td>
<td></td>
<td>From micrometer</td>
<td></td>
</tr>
<tr>
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<td>0.51</td>
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<tr>
<td>2</td>
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<td>2.25</td>
<td>23.06</td>
</tr>
<tr>
<td>3</td>
<td>0.42</td>
<td>25.46</td>
<td>5.74</td>
<td>38.82</td>
</tr>
</tbody>
</table>

The above Table 4.1 shows comparison between calculated elastic modulus and ultimate stress. While calculating the elastic modulus and ultimate stress from microCT, the values of diameter and CSMI are directly taken from the skyscan 1172; whereas from micrometer the diameter of bone is measured using micrometer and used for calculation for CSMI values. The difference is values from microCT and micrometer is observed. This is because the microCT calculates the diameter and CSMI values from the crosssection image obtained from the scan and is therefore highly accurate as compared to the values obtained from the micrometer. Henceforth the values from microCT will be taken into consideration for the analysis of the data.
Figure 4.2 and 4.3 shows comparison between elastic modulus, ultimate stress and displacement for twenty four bone samples and for group of three samples for the two clusters identified from Figure 4.1. Group A shows lower elastic modulus than group B, and higher displacement as compared to group B, and higher ultimate stress than Group B. This also can be seen from the stress – strain curve.

Figure 4.4 shows modulus versus displacement plot for the average values for both the groups. Group A shows higher displacement and lower elastic modulus whereas Group B shows lower displacement and higher elastic modulus. Figure 4.5 shows stress – strain curve for the two groups of bone. It can be seen that the Group A shows higher strain than group B with lower stress value than group A.

Figure 4.3. Comparison of group A (A1, A2 and A3)and group B (B1, B2 and B3) samples for elastic modulus, ultimate stress and displacement.
Figure 4.4. Groups A and B comparison on basis of modulus and displacement.

Figure 4.5. Stress – strain curve for group A and B.
4.2 Hypothesis 1

The increase in indentation hardness and indentation modulus across the thickness of the bone is co-related and paralleled to the increase in mineral to matrix ratio, carbonate to phosphate ratio and collagen crosslinking.

4.3 Nanoindentation

An upward trend was observed for the modulus (Figure 4.3). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values were 16.149, 19.99, 31.956, and 25.412 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 45% in the anterior, 51% in the posterior, 53% in the lateral, and 43% in the medial. The posterior quadrant had the highest average modulus across the cortex (31.956 GPa) while the anterior quadrant had the lowest (16.149 GPa).

Moreover, the average increase in hardness with advancing position from the periosteal to the endosteal surface for the four quadrants were observed (Figure 4.4). The average for both the medial and lateral quadrants was 0.786 GPa and 0.754 GPa respectively. The posterior quadrant had the highest average (1.361 GPa) while the anterior had the lowest (0.665 GPa). A maximum hardness of 1.361 GPa was measured at the location nearest to the endosteum on the posterior quadrant. The other three quadrants were not far behind however, with maximums values of 0.786, 0.754, and 0.665 GPa for the medial, lateral, and anterior quadrants, respectively, at the same location closest to the endosteal surface. A 156.7% increase in cortical bone hardness was found across the cortex of the anterior quadrant, from the maximum at the
endosteum to the minimum at the periosteum. This was the largest for any of the quadrants. The lateral increased by 45.2%, and the medial and posterior increased by 40.1% and 123.1%, respectively.

Figure 4.6. Indentation modulus values versus the distance from perioseal to endosteal surface of A1 for four quadrants investigated.

Figure 4.7. Hardness values versus the distance from perioseal to endosteal surface of A1 for four quadrants investigated.
A similar upward trend was observed for the modulus (Figure 4.5). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values were 13.5, 3.07, 35.9 and 36.4 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 101% in the anterior, 140% in the posterior, 629% in the lateral, and 214% in the medial. The medial quadrant had the highest average modulus across the cortex (36.4 GPa) while the lateral quadrant had the lowest (3.07 GPa).

The average for both the medial and posterior quadrants was 1.192 GPa and 2.022 GPa respectively. The lateral quadrant had the lowest average (0.118 GPa) while the anterior had 0.162 GPa. A maximum hardness of 2.022 GPa was measured at the location nearest to the endosteum on the posterior quadrant. The other three quadrants with maximum values of 1.192, 0.118 and 0.162 GPa for the medial, lateral, and anterior quadrants, respectively, at the same location closest to the endosteal surface. This was the largest for any of the quadrants. The anterior increased by 116%, and the lateral increased by 306% from the maximum at the endosteum to the minimum at the periosteum.
Figure 4.8. Indentation modulus values versus the distance from perioseal to endosteal surface of A2 for four quadrants investigated.

Figure 4.9. Hardness values versus the distance from perioseal to endosteal surface of A2 for four quadrants investigated.

An upward trend was observed for the modulus (Figure 4.5). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values
were 8.651, 32.195, 7.064, and 36.485 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 168.41% in the anterior, 202.84% in the posterior, 328.12% in the lateral, and 142% in the medial. The medial quadrant had the highest average modulus across the cortex (19.065 GPa) while the posterior quadrant had the lowest (3.532 GPa).

Moreover, the average increases in hardness with advancing position from the periosteal to the endosteal surface for the four quadrants were observed (Figure 4.6). The average for both the posterior and lateral quadrants was 0.16 GPa and 0.37 GPa respectively. The medial quadrant had the highest average (0.55 GPa) while the posterior had the lowest (0.16 GPa). A maximum hardness of 0.84 GPa was measured at the location nearest to the endosteum on the medial quadrant. A 290% increase in cortical bone hardness was found across the cortex of the medial quadrant, from the maximum at the endosteum to the minimum at the periosteum. This was the largest for any of the quadrants. The lateral increased by 170%, and the anterior and posterior increased by 201% and 68%, respectively.
Figure 4.10. Indentation modulus values versus the distance from perioseal to endosteal surface of A3 for four quadrants investigated.

Figure 4.11. Hardness values versus the distance from perioseal to endosteal surface of A3 for four quadrants investigated.
An upward trend was observed for the modulus (Figure 4.9). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values were 29.274, 21.605, 22.9, and 28.189 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 107.32% in the anterior, 32.31% in the posterior, 100.14% in the lateral, and 78.51% in the medial. The anterior quadrant had the highest average modulus across the cortex (21.5944 GPa) while the medial quadrant had the lowest (17.7059 GPa).

Moreover, the average increase in hardness with advancing position from the periosteal to the endosteal surface for the four quadrants were observed (Figure 4.10). The average for both the medial and lateral quadrants was 0.41933 GPa and 0.506 GPa respectively. The anterior quadrant had the highest average (0.6526 GPa) while the posterior had (0.5206 GPa). A maximum hardness of 1.089 GPa was measured at the location nearest to the endosteum on the anterior quadrant. The other three quadrants were not far behind however, with maximums values of 0.854, 0.609, and 0.717 GPa for the medial, lateral, and posterior quadrants, respectively, at the same location closest to the endosteal surface. A 234% increase in cortical bone hardness was found across the cortex of the anterior quadrant, from the maximum at the endosteum to the minimum at the periosteum. This was the largest for any of the quadrants. The lateral increased by 77%, and the medial and posterior increased by 132% and 198%, respectively.
Figure 4.12. Indentation modulus values versus the distance from perioseal to endosteal surface of B1 for four quadrants investigated.

Figure 4.13. Hardness values versus the distance from perioseal to endosteal surface of B1 for four quadrants investigated.
An upward trend was observed for the modulus (Figure 4.11). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values were 28.502, 29.874, 53.16, and 36.58 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 98.75% in the anterior, 59.79% in the posterior, 135% in the lateral, and 466% in the medial. The posterior quadrant had the highest average modulus across the cortex (26.6752 GPa) while the anterior quadrant had the lowest (17.8786 GPa).

Moreover, the average increase in hardness with advancing position from the periosteal to the endosteal surface for the four quadrants were observed (Figure 4.12). The average for both the anterior and lateral quadrants was 0.6898 GPa and 0.6924 GPa respectively. The posterior quadrant had the highest average (0.7058 GPa) while the medial had the lowest (0.5005 GPa). A maximum hardness of 1.19 GPa was measured at the location nearest to the endosteum on the posterior quadrant. The other three quadrants were not far behind however, with maximums values of 0.9175, 0.976, and 1.035 GPa for the medial, lateral, and anterior quadrants, respectively, at the same location closest to the endosteal surface. A 318% increase in cortical bone hardness was found across the cortex of the medial quadrant, from the maximum at the endosteum to the minimum at the periosteum. This was the largest for any of the quadrants. The lateral increased by 97%, and the anterior and posterior increased by 170% and 102%, respectively.
Figure 4.14. Indentation modulus values versus the distance from perioseal to endosteal surface of B2 for four quadrants investigated.

Figure 4.15. Hardness values versus the distance from perioseal to endosteal surface of B2 for four quadrants investigated.
An upward trend was observed for the modulus (Figure 4.13). The indentation modulus peaked near the endosteal surface for all four quadrants. The maximum values were 28.7415, 26.0878, 24.6524, and 12.7167 GPa for the anterior, lateral, posterior and medial quadrants, respectively. The modulus decreased by 97.17% in the anterior, 37.86% in the posterior, 73.47% in the lateral, and 15.0077% in the medial. The anterior quadrant had the highest average modulus across the cortex (28.7415 GPa) while the medial quadrant had the lowest (12.71 GPa).

Moreover, the average increase in hardness with advancing position from the periosteal to the endosteal surface for the four quadrants were observed (Figure 4.14). The average for both the anterior and lateral quadrants was 0.797 GPa and 0.8294 GPa respectively. The posterior quadrant had the highest average (0.902 GPa) while the medial had the lowest (0.3076 GPa). A maximum hardness of 1.767 GPa was measured at the location nearest to the endosteum on the posterior quadrant. The other three quadrants were not far behind however, with maximum values of 0.497, 1.073, and 1.128 GPa for the medial, lateral, and anterior quadrants, respectively, at the same location closest to the endosteal surface. A 473% increase in cortical bone hardness was found across the cortex of the posterior quadrant, from the maximum at the endosteum to the minimum at the periosteum. This was the largest for any of the quadrants. The lateral increased by 35%, and the medial and anterior increased by 25% and 141%, respectively.
Figure 4.16. Indentation modulus values versus the distance from perioseal to endosteal surface of B3 for four quadrants investigated.

Figure 4.17. Hardness values versus the distance from perioseal to endosteal surface of B3 for four quadrants investigated.
4.4 Raman Spectroscopy

As seen with the nanomechanical properties, the mineral to matrix ratio and carbonate to phosphate ratio as well as collagen crosslinking increases from the periosteal surface to the endosteal surface of the cortical cortex. Hence, the maximum values for each quadrant were measured on the inner side of the bone cortex and the minimum values were near the outer edge. The plots for mineral to matrix ratios, carbonate to phosphate ratio and collagen crosslinking plotted for anterior, posterior, lateral and medial quadrants for all the groups. The measured values are summarized in Table 4.2 for percentage increase for all groups against all four quadrants. As can be seen from Table 4.2, the percentage shows increase in the values of all the variables suggesting the increase in mineral content, carbonate content and collagen crosslinking as compared to matrix, phosphates and reducible crosslinks respectively.

![Mineral:Matrix A1](image)

**Figure 4.18.** Mineral:Matrix ratio values versus the distance from periosteal to endosteal surface of A1 for four quadrants investigated.
Figure 4.19. Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of A1 for four quadrants investigated.

Figure 4.20. Collagen cross-linking values versus the distance from perioseal to endosteal surface of A1 for four quadrants investigated.
**Figure 4.21.** Mineral:Matrix ratio values versus the distance from perioseal to endosteal surface of A2 for four quadrants investigated.

**Figure 4.22.** Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of A2 for four quadrants investigated.
Figure 4.23. Collagen cross-linking values versus the distance from perioseal to endosteal surface of A2 for four quadrants investigated.

Figure 4.24. Mineral:Matrix ratio values versus the distance from perioseal to endosteal surface of A3 for four quadrants investigated.
Figure 4.25. Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of A3 for four quadrants investigated.

Figure 4.26. Collagen cross-linking values versus the distance from perioseal to endosteal surface of A3 for four quadrants investigated.
Figure 4.27. Mineral:Matrix ratio values versus the distance from perioseal to endosteal surface of B1 for four quadrants investigated.

Figure 4.28. Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of B1 for four quadrants investigated.
Figure 4.29. Collagen cross-linking values versus the distance from perioseal to endosteal surface of B1 for four quadrants investigated.

Figure 4.30. Mineral:Matrix ratio values versus the distance from perioseal to endosteal surface of B2 for four quadrants investigated.
Figure 4.31. Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of B2 for four quadrants investigated.

Figure 4.32. Collagen cross-linking values versus the distance from perioseal to endosteal surface of B2 for four quadrants investigated.
Figure 4.33. Mineral:Matrix ratio values versus the distance from perioseal to endosteal surface of B3 for four quadrants investigated.

Figure 4.34. Carbonate:Phosphate ratio values versus the distance from perioseal to endosteal surface of B3 for four quadrants investigated.
Figure 4.35. Collagen cross-linking values versus the distance from perioseal to endosteal surface of B3 for four quadrants investigated.

Table 4.2

Comparison of nano-mechanical properties of group A and B for endosteal and periosteal region

<table>
<thead>
<tr>
<th>Groups</th>
<th>Quadrants</th>
<th>Nano-mechanical property</th>
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<td></td>
<td>Elast modulus (GPa)</td>
<td>Hardness (GPa)</td>
<td>Elast modulus (GPa)</td>
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<tr>
<td>A1</td>
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<td></td>
<td>Mineral to matrix ratio</td>
<td>Carbonate to phosphate ratio</td>
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Table 4.3
Comparison of micro-chemical properties of group A and B for endosteal and periosteal region
Table 4.2 and 4.3 we can see that the indentation hardness as well as modulus has increased from periosteal to endosteal region. This is observed in all the quadrants for both the groups. The chemical composition changes too from endosteal to periosteal region. We can see that the mineral to matrix ratio has increased. This is because the mineralization increases as the tissue ages. This happens because of the deposition of carbonate and hence can be seen that the carbonate to phosphate ratio increases too. Collagen crosslinking is increased indicating the increase in stiffness of the bone, thus attributing to the increase in elastic modulus.

4.5 Hypothesis 2

Stiffness increases due to increase in mineralization and increase in collagen crosslinks thereby decreasing the deflection.

From Figures 4.33, 4.34, 4.35 and 4.36 it can be seen that for the anterior and lateral quadrant the indentation modulus and hardness values for group B show steady increase in the values from periosteal to endosteal surface. This group has lower deflection as compared to group A. The anterior quadrant is under compression during the three point bend test and higher indentation hardness and modulus contribute to
resistance to deflection. Group A shows lower elastic modulus as well as strength than group B on macro level, refer to Figures 4.37, 4.38, 4.39 and 4.40.

**Figure 4.36.** Comparison of indentation modulus for anterior group versus the distance from perioseal to endosteal surface.

**Figure 4.37.** Comparison of hardness for anterior group versus the distance from perioseal to endosteal surface.
Figure 4.38. Comparison of indentation modulus for lateral group versus the distance from perioseal to endosteal surface.

Figure 4.39. Comparison of hardness for lateral group versus the distance from perioseal to endosteal surface.
Figure 4.40. Comparison of indentation modulus for medial group versus the distance from perioseal to endosteal surface.

Figure 4.41. Comparison of hardness for medial group versus the distance from perioseal to endosteal surface.
Figure 4.42. Comparison of indentation modulus for posterior group versus the distance from perioseal to endosteal surface.

Figure 4.43. Comparison of hardness for posterior group versus the distance from perioseal to endosteal surface.

When mineral to matrix ratio, carbonate to phosphate ratio and collagen crosslinking from raman spectroscopy is compared on basis of quadrants for all the
groups along the distance from periosteal to endosteal we can see that there is a steady increase in the ratios and collagen crosslinking for all the groups for anterior quadrant. Refer Figures 4.41 to 4.52, we can conclude that the chemical composition shows higher values for group B in comparison with group A. The mineral to matrix ratio increase shows that the mineral content increases towards endosteal surface with decrease in matrix content giving increase in hardness value. Similar increase in property is observed for carbonate to phosphate ratio as well as collagen crosslinking.

This confirms the hypothesis that the increase in mineralization and collagen crosslinking in group B has led to stiffer bones as compared to group A. This has led to lower deflection of bone.

As the bone tissue ages from endosteal to periosteal it can be seen that the elasticity of the tissues decreases as the collagen crosslinks giving rigid bonds which are difficult to flex, thereby increasing the macro as well as nano-mechanical properties.
Figure 4.44. Comparison of mineral to matrix ratio for anterior group versus the distance from perioseal to endosteal surface.

Figure 4.45. Comparison of carbonate to phosphate ratio for anterior group versus the distance from perioseal to endosteal surface.
Figure 4.46. Comparison of collagen crosslinking for anterior group versus the distance from perioseal to endosteal surface.

Figure 4.47. Comparison of mineral to matrix ratio for lateral group versus the distance from perioseal to endosteal surface.
Figure 4.48. Comparison of carbonate to phosphate ratio for lateral group versus the distance from perioseal to endosteal surface.

Figure 4.49. Comparison of collagen crosslinking for lateral group versus the distance from perioseal to endosteal surface.
Figure 4.50. Comparison of mineral to matrix ratio for medial group versus the distance from periosseal to endosteal surface.

Figure 4.51. Comparison of carbonate to phosphate ratio for medial group versus the distance from periosseal to endosteal surface.
Figure 4.52. Comparison of collagen crosslinking for medial group versus the distance from perioseal to endosteal surface.

Figure 4.53. Comparison of mineral to matrix ratio for posterior group versus the distance from perioseal to endosteal surface.
Figure 4.54. Comparison of carbonate to phosphate ratio for posterior group versus the distance from perioseal to endosteal surface.

Figure 4.55. Comparison of collagen crosslinking for posterior group versus the distance from perioseal to endosteal surface.
### Table 4.4

**Comparison of macro and nano-mechanical properties**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Macro-mechanical property</th>
<th>Nano-mechanical property</th>
<th>Quadrants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elastic Modulus (Gpa)</td>
<td>Ultimate Strength (Mpa)</td>
<td>Displacement (mm)</td>
</tr>
<tr>
<td>A1</td>
<td>0.27</td>
<td>42.73</td>
<td>1.543</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>0.31</td>
<td>34.92</td>
<td>0.969</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>0.26</td>
<td>36.38</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>0.28±0.026</td>
<td>38.01±4.15</td>
<td>1.37±0.35</td>
</tr>
<tr>
<td>B1</td>
<td>0.47</td>
<td>33.66</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>0.44</td>
<td>20.06</td>
<td>0.475</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>0.42</td>
<td>25.46</td>
<td>1.245</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>0.44±0.025</td>
<td>26.39±6.84</td>
<td>0.75±0.43</td>
</tr>
</tbody>
</table>
Table 4.5

**Micro-chemical properties**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Quadrants</th>
<th>Mineral:matrix ratio</th>
<th>Carbonate:phosphate ratio</th>
<th>Collagen crosslinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Anterior</td>
<td>8.68</td>
<td>0.50</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>49.77</td>
<td>0.57</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>35.62</td>
<td>0.59</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>17.77</td>
<td>0.54</td>
<td>4.28</td>
</tr>
<tr>
<td></td>
<td><strong>Average</strong></td>
<td>27.96</td>
<td>0.55</td>
<td>3.12</td>
</tr>
<tr>
<td>A2</td>
<td>Anterior</td>
<td>21.14</td>
<td>0.73</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>27.96</td>
<td>0.57</td>
<td>5.10</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>41.62</td>
<td>0.78</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>14.84</td>
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<td>3.83</td>
</tr>
<tr>
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<td><strong>Average</strong></td>
<td>26.39</td>
<td>0.68</td>
<td>3.28</td>
</tr>
<tr>
<td>A3</td>
<td>Anterior</td>
<td>8.68</td>
<td>0.50</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>49.77</td>
<td>0.57</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>35.62</td>
<td>0.59</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>17.77</td>
<td>0.54</td>
<td>4.28</td>
</tr>
<tr>
<td></td>
<td><strong>Average</strong></td>
<td>27.96</td>
<td>0.55</td>
<td>3.12</td>
</tr>
<tr>
<td>Average</td>
<td><strong>Average</strong></td>
<td>27.44±0.9</td>
<td>0.59±0.07</td>
<td>3.17±0.09</td>
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<tr>
<td>B1</td>
<td>Anterior</td>
<td>46.23</td>
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<td>16.94</td>
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</tr>
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<td>Posterior</td>
<td>27.40</td>
<td>0.70</td>
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<td>28.39</td>
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<td><strong>Average</strong></td>
<td>29.74</td>
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</tr>
<tr>
<td>B2</td>
<td>Anterior</td>
<td>17.26</td>
<td>0.97</td>
<td>6.99</td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>28.62</td>
<td>0.95</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>16.88</td>
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</tr>
<tr>
<td></td>
<td>Medial</td>
<td>52.70</td>
<td>0.87</td>
<td>5.75</td>
</tr>
<tr>
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<td><strong>Average</strong></td>
<td>28.87</td>
<td>0.95</td>
<td>4.58</td>
</tr>
<tr>
<td>B3</td>
<td>Anterior</td>
<td>9.68</td>
<td>0.90</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>14.13</td>
<td>0.73</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>34.36</td>
<td>0.68</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>43.02</td>
<td>2.59</td>
<td>6.58</td>
</tr>
<tr>
<td></td>
<td><strong>Average</strong></td>
<td>25.30</td>
<td>1.22</td>
<td>3.96</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>27.97±2.35</td>
<td>0.98±0.23</td>
<td>4.06±0.47</td>
<td></td>
</tr>
</tbody>
</table>
From Table 4.4 it can be seen that group A has deflection of 1.37±0.35 which is higher than group B with deflection of 0.75±0.43. The elastic modulus of group A is 0.28±0.026 GPa and for group B it is 0.44±0.25 GPa. This indicates that the higher stiffness in bone has caused lower deflection and higher elastic modulus as can be seen from increase in collagen crosslinking in group B which is 4.06±0.47 as compared to group A which is 3.17±0.09 Table 4.5. The nano-mechanical results are parallel with the macro-mechanical results. Showing group A has lower indentation modulus than group B which are 22.23±1.14 GPa and 28.52±7.47 GPa respectively.

When values for the nanoindentation results are compared from Table 4.4 with the chemical compositional changes on micro level from Table 4.5, we can say that the elastic modulus on nanoscale can be better understood by the collagen crosslinking ratio and hence can be seen that as collagen crosslinks in group B shows higher values than group A, stiffness has affected. The bones have become more rigid and difficult to flex showing higher values in elastic modulus from the nanoindentation data. So, more collagen crosslinks in group B high stiffness and therefore high elastic modulus than group A. Carbonate to phosphate ration indicates substitution of carbonates in the hydroxyl apatite lattice and also higher mineral size due to mineralization. So, higher carbonate to phosphate ratio shows higher overall hardness from the nanoindentation. The nanoindentation as well as Raman spectroscopy probe the material properties at the nano and micro level to give accurate properties as compared to macro mechanical tests which are average of material properties.

Indentation hardness in group A is lower (0.75±0.23) than group B (1.00±0.16) can be attributed to the increase in carbonate to phosphate ratio in group B than in A.
which are 0.98±0.23 and 0.59±0.07 respectively. It is observed that the mineral to matrix ratio for both the groups are almost equal.

Table 4.6

Correlation between nanomechanical and compositional properties

<table>
<thead>
<tr>
<th></th>
<th>Elastic modulus</th>
<th></th>
<th>Indentation hardness</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Lateral</td>
<td>Medial</td>
<td>Posterior</td>
</tr>
<tr>
<td>A1 Mineral/matrix</td>
<td>0.9999</td>
<td>0.9656</td>
<td>0.8732</td>
<td>0.6242</td>
</tr>
<tr>
<td></td>
<td>Carbonate to phosphate</td>
<td>0.9666</td>
<td>0.8645</td>
<td>0.8633</td>
</tr>
<tr>
<td></td>
<td>Collagen crosslinking</td>
<td>0.8170</td>
<td>0.8281</td>
<td>0.7680</td>
</tr>
<tr>
<td>A2 Mineral/matrix</td>
<td>0.9809</td>
<td>0.7817</td>
<td>0.9706</td>
<td>0.9785</td>
</tr>
<tr>
<td></td>
<td>Carbonate to phosphate</td>
<td>0.8662</td>
<td>0.4351</td>
<td>0.9723</td>
</tr>
<tr>
<td></td>
<td>Collagen crosslinking</td>
<td>0.9504</td>
<td>0.9012</td>
<td>0.9734</td>
</tr>
<tr>
<td>A3 Mineral/matrix</td>
<td>0.7590</td>
<td>0.9046</td>
<td>0.9766</td>
<td>0.9313</td>
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<tr>
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<td>Carbonate to phosphate</td>
<td>0.8403</td>
<td>0.7584</td>
<td>0.8046</td>
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<tr>
<td></td>
<td>Collagen crosslinking</td>
<td>0.8970</td>
<td>0.8991</td>
<td>0.8905</td>
</tr>
<tr>
<td>B1 Mineral/matrix</td>
<td>0.7256</td>
<td>0.9227</td>
<td>0.7712</td>
<td>0.9153</td>
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<td></td>
<td>Carbonate to phosphate</td>
<td>0.7643</td>
<td>0.9448</td>
<td>0.8256</td>
</tr>
<tr>
<td></td>
<td>Collagen crosslinking</td>
<td>0.7952</td>
<td>0.9443</td>
<td>0.7865</td>
</tr>
<tr>
<td>B2 Mineral/matrix</td>
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<td>0.8006</td>
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<td>Carbonate to phosphate</td>
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<tr>
<td></td>
<td>Collagen crosslinking</td>
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<td>0.8963</td>
<td>0.9509</td>
</tr>
<tr>
<td>B3 Mineral/matrix</td>
<td>0.9764</td>
<td>0.8636</td>
<td>0.9550</td>
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<tr>
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<td>Collagen crosslinking</td>
<td>0.9765</td>
<td>0.7981</td>
<td>0.9936</td>
</tr>
</tbody>
</table>

For the two groups the nanomechanical properties like elastic modulus and indentation hardness are compared with the compositional variables like mineral/matrix ratio, carbonate/phosphate ratio, and collagen crosslinking. Group A shows strong
correlation with the elastic modulus for anterior, lateral and medial quadrants as
compared to posterior for indentation hardness for all the compositional variables, but
on an average the elastic modulus shows more strong correlation with the
compositional variables than the indentation hardness. For group B the elastic modulus
shows strong correlation in all four quadrants as compared to indentation hardness.
The plots for all the correlations are appended at the end of the document in appendix.
The correlation $r^2$ values for elastic modulus for group A and B are 0.8478 and 0.8611
respectively as compared to the less moderately correlated values of $r^2$ for indentation
hardness for group A and B which are 0.8306 and 0.7969 respectively.
CHAPTER 5

DISCUSSION

The hypothesis of relating nano-mechanical properties and micro-chemical properties is confirmed. The results show that as the carbonate to phosphate ratio increases the hardness increases. Noteworthy is other observation that as the collagen crosslinking increases the modulus increases. It is also seen that the young tissues which are on the periosteal shows lower nano-mechanical properties than the matured and older endosteal region.

As hypothesized, deflection of bone is related with the mineralization and collagen crosslinks. The nano-mechanical properties like hardness and indentation modulus affects the deflection and elastic modulus. It is clear that as the collagen crosslink increases the deflection decreases, this implies that the bone has become stiffer with increase in crosslink. This can be explained by the measurement of collagen crosslinking which is the ratio of non-reducible to reducible crosslinks. The reducible cross links are non-stable and are divalent. As the bone grows old and matures the crosslinks transforms into non-reducible collagen by attaching one more collagen to the link of two. These links are non-reducible and as the proportion of these crosslinks increases the bone becomes stiff and thus loses its flexibility. This means that the bone deflection reduces, which can be seen from the data. The influence of increase in collagen crosslinks is evident from the elastic modulus values. As the collagen crosslink ratio increases the elastic modulus increases too. Although mineral to matrix ratio is approximately equal, the carbonate to phosphate ratio has contributed to the hardness. The mineral matures as the age of the tissue increases towards endosteal, the mineral
size increases and therefore carbonate content increases [61]. This can be seen from the carbonate to phosphate ratio in group B which is more than group A. This contributes to the increased hardness for group B than group A.

Similar to our study using a mature mice model, Donnelly and colleagues characterized the nanomechanical properties and composition of regions of differing tissue age in the femoral cortices of growing rats [62]. Their results unlike ours show a very sharp and early increase in tissue modulus, hardness, mineral:matrix ratio and carbonate to phosphate ratio with increasing distance for the first 35 microns from the periosteum (youngest tissue). However, after this region, the indentation moduli and hardness results of the older tissue plateaued and were thus not found to be significantly different. In contrast to these reported findings, our results show an increasing trend in the nanomechanical and compositional measures across the cortex. The leveling effect reported in their study may be primarily explained by the lack of intracortical remodeling in their tested rat model. Furthermore differences in size between mouse and rat femora may play a role. The larger distance from the endosteum to the periosteum for a rat femur, as compared to a mouse femur, may play a role in the overall intraspecimen tissue age. Finally, differences may simply exist because comparisons are being made at different cross-sectional locations within the femur and because a young growing rat is different that an inbred mature mouse, as in our case the comparisons are done on the same locations throughout the sample population. Moreover their correlation between nanomechanical and compositional studies show $r^2$ values of indentation modulus and hardness of 0.54 and 0.62 with mineral to matrix ratio and with carbonate to phosphate ratio as 0.36 and 0.40
respectively. Our results on the other hand show better correlation as shown in Table 4.6.
CHAPTER 6

CONCLUSION

In conclusion, the properties on all scales like macro, micro and nano have been co-related to better understand the bone properties. The three point bending results like elastic modulus and deflection were co-related to the collagen crosslinking obtained from the Raman spectra. Thus, relating macro-mechanical and micro-compositional properties. The hardness and indentation modulus were compared with the mineral to matrix and carbonate to phosphate ratio from Raman spectra co-relating nano-mechanical properties to micro-compositional properties. This thesis is an attempt to understand the hierarchical nature of bone and the influence of the properties on all the scales. The hypothesis put forth were confirmed on all the levels of hierarchy to better understand the relation between chemical changes with the nano and macro mechanical properties.
APPENDIX

SUPPLEMENTAL GRAPHS
Figure A.1. Correlation between variables and hardness for anterior quadrant for A1.

Figure A.2. Correlation between variables and elastic modulus for anterior quadrant for A1.
Figure A.3. Correlation between variables and hardness for lateral quadrant for A1.

Figure A.4. Correlation between variables and elastic modulus for lateral quadrant for A1.
Figure A.5. Correlation between variables and hardness for medial quadrant for A1.

Figure A.6. Correlation between variables and elastic modulus for medial quadrant for A1.
Figure A.7. Correlation between variables and hardness for posterior quadrant for A1.

Figure A.8. Correlation between variables and elastic modulus for posterior quadrant for A1.
Figure A.9. Correlation between variables and hardness for anterior quadrant for A2.

Figure A.10. Correlation between variables and elastic modulus for anterior quadrant for A2.
Figure A.11. Correlation between variables and hardness for lateral quadrant for A2.

Figure A.12. Correlation between variables and elastic modulus for lateral quadrant for A2.
Figure A.13. Correlation between variables and hardness for medial quadrant for A2.

Figure A.14. Correlation between variables and elastic modulus for medial quadrant for A2.
Figure A.15. Correlation between variables and hardness for posterior quadrant for A2.

Figure A.16. Correlation between variables and elastic modulus for posterior quadrant for A2.
Figure A.17. Correlation between variables and hardness for anterior quadrant for A3.

Figure A.18. Correlation between variables and elastic modulus for anterior quadrant for A3.
Figure A.19. Correlation between variables and hardness for lateral quadrant for A3.

Figure A.20. Correlation between variables and elastic modulus for lateral quadrant for A3.
Figure A.21. Correlation between variables and hardness for medial quadrant for A3.

Figure A.22. Correlation between variables and elastic modulus for medial quadrant for A3.
Figure A.23. Correlation between variables and hardness for posterior quadrant for A3.

Figure A.24. Correlation between variables and elastic modulus for posterior quadrant for A3.
Figure A.25. Correlation between variables and hardness for lateral quadrant for B1.

Figure A.26. Correlation between variables and elastic modulus for lateral quadrant for B1.
Figure A.27. Correlation between variables and hardness for posterior quadrant for B1.

Figure A.28. Correlation between variables and elastic modulus for posterior quadrant for B1.
Figure A.29. Correlation between variables and hardness for anterior quadrant for B2.

Figure A.30. Correlation between variables and elastic modulus for anterior quadrant for B2.
Figure A.31. Correlation between variables and hardness for lateral quadrant for B2.

Figure A.32. Correlation between variables and elastic modulus for lateral quadrant for B2.
Figure A.33. Correlation between variables and hardness for medial quadrant for B2.

Figure A.34. Correlation between variables and elastic modulus for medial quadrant for B2.
Figure A1.35. Correlation between variables and hardness for posterior quadrant for B2.

Figure A.36. Correlation between variables and elastic modulus for posterior quadrant for B2.
Figure A.37. Correlation between variables and hardness for anterior quadrant for B3.

Figure A.38. Correlation between variables and elastic modulus for anterior quadrant for B3.
Figure A.39. Correlation between variables and hardness for lateral quadrant for B3.

Figure A.40. Correlation between variables and elastic modulus for lateral quadrant for B3.
Figure A.41. Correlation between variables and hardness for medial quadrant for B3.

Figure A.42. Correlation between variables and elastic modulus for medial quadrant for B3.
Figure A.43. Correlation between variables and hardness for posterior quadrant for B3.

Figure A.44. Correlation between variables and elastic modulus for posterior quadrant for B3.
REFERENCES


