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THE EFFECT OF VARIOUS PATHOLOGIES ON BONE QUALITY

Daniel S. Porter
University of Kentucky, danielshawporter@yahoo.com

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Daniel S. Porter, Student

Dr. David Pienkowski, Major Professor

Dr. Abhijit R. Patwardhan, Director of Graduate Studies

THE EFFECT OF VARIOUS PATHOLOGIES ON BONE QUALITY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Engineering
at the University of Kentucky

By
Daniel Shaw Porter
Lexington, KY
Director: Dr. David Pienkowski, Associate Professor of Biomedical Engineering
Lexington, KY
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ABSTRACT OF DISSERTATION

THE EFFECT OF VARIOUS PATHOLOGIES ON BONE QUALITY

Bone's ability to resist fracture is often ignored until a low-energy fracture occurs. Patients with Chronic Kidney Disease (CKD) or osteoporosis are at an increased risk of low-energy fracture. Generally, fracture risk is evaluated by using a bone mineral density (BMD) test. BMD values; however, do not fully predict bone's ability to resist fracture. This suggests that other parameters may be involved. Bone quality is the term used to describe these parameters, which are categorized into three groups: structural, material, and microdamage. The aim of this dissertation research was to examine whether bone quality was altered in patients who: 1) had abnormal bone turnover (high or low) due to CKD, 2) suffered a low-energy fracture despite normal BMD, or 3) had osteoporosis and were treated with bisphosphonates. These studies used iliac crest bone specimens from Caucasian females aged 21 to 87 years. Bone's material parameters were measured by Fourier transform infrared spectroscopy. The key finding from the turnover study was that high and low turnover was associated with altered bone quality. Specifically, bone with high turnover had a lower mineral-to-matrix ratio compared to normal and low turnover ($p<0.05$), while low turnover had a lower cancellous bone volume and trabecular thickness compared to normal or high turnover ($p<0.05$). The key finding from the fracture study was that patients with normal BMD and low-energy fractures had altered bone quality (greater collagen crosslinking ratio) compared to patients who had low- BMD with low-energy fractures and healthy subjects (controls) ($p<0.05$). Lastly, the key findings from the bisphosphonate studies were that osteoporosis patients treated with these drugs had altered bone quality (specifically, greater ($p<0.05$) mineral-to-matrix ratio) compared to untreated turnover-matched osteoporotic patients, and that were several positive linear correlations with the nanoindentation derived Young's modulus and hardness of cortical and trabecular bone and the duration of bisphosphonate treatment ($p<0.05$). The findings presented provide further evidence that bone quantity is not the sole factor in determining bone's ability to resist fractures and that bone quality is an essential factor.

Keyword: Bone Quality, Bone Quantity, Chronic Kidney Disease, Osteoporosis, Bisphosphonate

Daniel S. Porter
Student's Signature
4-24-14
Date

THE EFFECT OF VARIOUS PATHOLOGIES ON BONE QUALITY

BY

DANIEL SHAW PORTER

Dr. David Pienkowski
Director of Dissertation

Dr. Abhijit R. Patwardhan
Director of Graduate Studies

4-24-14

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 GLOBAL INTRODUCTION

Bone's ability to resist fracture is often ignored until a low-energy fracture has occurred. These fractures are classified as fractures occurring from a fall at standing height or less.[1] Patients with osteoporosis or chronic kidney disease (CKD) are at an increased risk of suffering a low-energy fracture compared to the general population.[2, 3] Osteoporosis and CKD are associated with aging, and as more Americans live over the age of 65, the number of patients with osteoporosis or CKD is expected to rise along with the number of fractures and cost associated with these fractures.[4, 5]

1.1 Osteoporosis and Chronic Kidney Disease

"The Silent Disease" is an epithet for osteoporosis as it is typically symptomless until a low-energy fracture occurs.[6] The National Institute of Health defines osteoporosis as a skeletal disorder that is characterized by compromised bone strength which leads to an increased risk of fracture,[7] while the World Health Organization defines osteoporosis as a bone mineral density (BMD) t-score less than -2.5 standard deviations below the mean value in young adults.[8, 9] Osteoporosis currently affects an estimated 10 million Americans while another 34 million have low bone mass also known as osteopenia.[10] Approximately 2 million fractures per year are due to osteoporosis.[5] The costs associated with osteoporosis and osteoporotic-related fractures in 2005 were approximately $17 billion.[5]

CKD affects 26 million Americans.[11] A study by Coresh *et al.* found that 75% of patients older than the age of 75 had decreased kidney function.[11] Patients with CKD have an increased fracture risk compared to the general population[2, 12, 13] and often have similar incidence rates of fracture as non-CKD individuals who are 10 to 20 years older.[14, 15]

1.2 Determining Fracture risk in Patients with Osteoporosis or CKD

Patients with osteoporosis or CKD typically undergo a BMD (bone quantity) scan to determine their risk for suffering a fracture. BMD is the amount of mineral per cross section area of bone and is measured by using dual-energy x-ray absorptiometry (DXA). A BMD t-score below -2.5 is associated with an increased risk for suffering a low-energy fracture.[3, 16, 17] There is growing evidence, however, that a BMD t-score below -2.5 is not the sole factor for suffering a low-energy fracture as these fractures have also been reported in patients with a BMD t-score above -2.5.[17, 18] Additionally, a few studies have shown that bone's ability to resist fracture is incompletely predicted by BMD.[19, 20] These findings suggest that other parameters besides bone quantity may affect bone's ability to resist fracture.

1.3 Bone Quality

Bone quality is a term coined in the early nineties to describe parameters other than bone quantity that influence bone's ability to resist fracture (Figure 1.1).[21-27] These parameters have been categorized into three groups: structural, material, and microdamage.

1.3.1 Bone's Structural Parameters

Bone on the micro- and macro-levels consists of cortical and cancellous bone (Figure 1.2 A-E). Cortical is the denser of the two types and occurs on the perimeter. Cancellous bone, also known as trabecular bone, exists in the center of bone. Bone structural parameters on the macro- and micro-levels come from the distribution and arrangement of cortical and cancellous bone.

The macro-level parameters are the shape and size of the bone. The micro-level parameters are trabecular thickness, trabecular separation, cortical thickness, and cortical porosity. Changes in the macrostructural and microstructural parameters have been linked to changes in bone's mechanical properties or greater fracture risk.[19, 28-45]

On the macro-level, the outer and inner diameters of bone are important as the bending stress of bone increases by the diameter of the bone raised to the fourth power (equations 1 and 2).[44]

Two-dimensional and three-dimensional modeling of trabecular bone found that loss of trabecular bone (increased trabecular separation) and reduced trabecular thickness resulted in lower bone strength.[40, 41] These studies found that trabecular separation had a more profound effect on bone strength than thinner trabeculae. Another study found that Young's modulus, yield stress, and ultimate stress of bone were associated with bone volume and trabecular separation, but trabecular thickness was not associated with bone's mechanical properties.[42]

1.3.2 Bone's Material Parameters

Bone on the nano-level is a composite material containing matrix (primarily consisting of type 1 collagen) and mineral (hydroxyapatite) (Figure 1.2 E).[47] The collagen provides bone with flexibility and the ability to absorb energy.[48] Collagen consists of three polypeptide chains that form a single triple helix structure. Collagen crosslinks are formed between these structures to provide stability. There are two types of crosslinks: enzymatic and non-enzymatic. The enzymatic crosslinks are formed due to the actions of lysyl oxidase (LOX).[49] The process of collagen crosslinking is initiated by the conversion of telopeptidyl lysine and hydroxylysine residue to aldehyde.[48] LOX is an extracellular copper enzyme that needs pyridoxal phosphate (vitamin B6) and lysine tyrosyl-lysine quinone as co-factors.[48] The enzymatic crosslinks are either mature (non-reducible trivalent crosslinks) or immature (reducible divalent crosslinks). The non-enzymatic collagen (advanced glycation end-products, AGE) crosslinks are due to attraction of glucose to collagen. The crosslinks are shown in Figure 1.4.[50] Additionally, the collagen and collagen crosslinks provide the scaffold for the deposit of the mineral (hydroxyapatite).

The mineral component provides bone its ability to withstand compressive loading. The hydroxyapatite crystals [$Ca_{10}(PO^4)_6(OH)_2$] in the bone are smaller and not as crystalline as compared to naturally occurring hydroxyapatite. The crystals found in bone often contain numerous impurities such as carbonate (CO_3^{2-}) substituted for PO_4^- or OH^- ions. These impurities can have a positive effect on bone by making bone more soluble and allowing it to act as a reservoir for mineral homeostasis. Not all impurities, however, are good for bone as fluoride was found to reduce bone strength.[51, 52]

The material parameters are the amount of mineral with respect to matrix (mineral-to-matrix ratio), mineral composition, mineral size, collagen crosslinking, and collagen quality. The amount of mineral with respect to matrix affects bone mechanical properties.[28, 53-60] Hyper-mineralized bone has a greater stiffness and force at failure, but lower ultimate displacement. Alternatively, hypo-mineralized bone has a lower stiffness and force at failure but a greater ultimate displacement (Figure 1.5).[60] In either case, however, there is a reduction in the work to failure of bone.[60] The amount of mineral with respect to collagen is analogous to the Goldilocks effect. This effect is when something must fall within a certain range, as opposed to reaching extremes.

Not only is the amount of mineral-to-matrix important, but changes in the hydroxyapatite (composition and size),[61-64] collagen crosslinking[65-73] and collagen quality (osteogenesis imperfecta)[74] have all been shown to alter bone's mechanical

properties. Increases in crystal size are associated with the decrease in deformation and increases in the brittleness of bone.[63, 64] Collagen crosslinks are thought to play a predominant role in bone's tensile strength and post-yield mechanical properties.[67] A study by Burstein *et al.* found that the removal of collagen did not affect the elastic pre-yield properties of bone but did reduce post-yield deformation.[75] A reduction in the total amount of enzymatic collagen crosslinks and an increase in the total amount of non-enzymatic crosslinks were found in female patients with hip fractures compared to gender- and age-matched controls.[70] A study by Paschalis *et al.* found that the greater ratio of mature (pyridinium) to immature (reducible collagen) crosslinks was associated with a 14% decrease in lumbar bone stiffness.[65] Greater amounts of non-enzymatic crosslinks are associated with decreases in the post-yield strain and strain energy,[71] while another study using cadaver bone found that non-enzymatic crosslinks increased bone strength by 7% but resulted in a 48% decrease in bone toughness.[72] In a canine animal model, bisphosphonate treatment was associated with increased non-enzymatic crosslinks and a reduction in energy absorption of cortical bone.[73]

1.3.3 Microdamage

Bone is constantly undergoing repetitive loading. This loading results in microcrack formation (microdamage). The occurrence of the microdamage and its repairs is a normal process. If the rate of bone turnover is lower than the rate of microdamage formation an accumulation of microdamage will occur. Microdamage accumulation is associated with changes in bone's toughness[76-79] and may explain the greater risk of atypical fractures seen in patients taking bisphosphonates.

1.4 Dissertation Outline

There is increasing interest in what happens to not only bone quantity but also the quality of the bone. The potential alterations in various parameters of bone quality may provide more information about bone's ability to resist fracture beyond that of bone quantity as measured by DXA. This is important as most treatment plans for maintaining bone's ability to resist fracture are focused on restoring or maintaining bone quantity, while the quality of the bone is often not considered; therefore, a better appreciation of bone quality may help in providing an improved treatment plan that not only looks at quantity but also the quality of the bone. The goal of this dissertation was to gain new information regarding how bone quality varied in human bone from patients with: a) kidney disease (Chapter 2), b) low energy fractures (Chapter 3), and c) osteoporosis treated with bisphosphonates (Chapters 4 and 5).

1.4.1 Renal Osteodystrophy and Bone Turnover (Chapter 2)

Bone is constantly undergoing bone remodeling to repair microdamage, to adapt to changes in mechanical loading seen on the bone, or to meet changes to mineral serum levels in an attempt to maintain mineral homeostasis. Bone remodeling is the process in which osteoclasts remove bone (resorption) and osteoblasts deposit new bone (formation). The rate at which bone remodeling occurs is known as bone turnover.

Renal osteodystrophy is the term used to describe the bone histological abnormalities that accompany CKD. Approximately 85% of patients with CKD stage-5 dialysis have abnormal bone turnover that is either higher (secondary hyperparathyroidism) or lower (adynamic bone disease) than normal turnover.[80] It is believed that bone quality may be influenced by the rate of bone turnover,[23] but the exact role bone turnover has on bone quality is unclear. Thus, the goal of this study was to gain a better understanding of how abnormal bone turnover (high and low) alters bone quality in bone specimens from patients with CKD compared to bone from patients with normal turnover and normal kidney function.

1.4.2 Fracture Despite Normal Bone Mineral Density (Chapter 3)

It is easy to understand the occurrence of low-energy fractures in patients with osteoporotic t-scores, but it remains unclear why low-energy fractures occur in premenopausal women with nonosteoporotic t-scores.[9, 81, 82] Studies have shown that BMD does not fully predict bone's ability to resist fractures,[19, 20] suggesting that bone quality may also influence bone's ability to resist fractures. Thus, the goal of this study was to determine if there were any changes in the quality of bone from patients who suffer low-energy fractures despite a non-osteoporotic BMD t-score compared to bone from patients with low BMD t-scores and low-energy fractures.

1.4.3 Bisphosphonates and Bone Quality (Chapter 4)

In 2008, an estimated 4 million women in the United States were taking bisphosphonates for treatment of osteoporosis.[83] Prolonged treatment with bisphosphonates has been associated with atypical femoral fractures.[84-89] Although atypical femoral fractures are currently a rare phenomenon, the number of reported cases of these fractures may increase in the future as more people are treated with bisphosphonates for a longer period. It has been documented that bisphosphonates suppress bone turnover,[90-92] and this suppression of bone turnover is believed to be responsible for the atypical femoral fractures. Thus, the goal of the this study was to determine whether various material and microstructural parameters of bone quality were altered in osteoporotic Caucasian females treated with various durations of bisphosphonate treatment compared to bone turnover matched untreated osteoporotic Caucasian females.

1.4.4 Bisphosphonate and Bone Intrinsic Mechanical Properties (Chapter 5)

The goal of this study was to determine if there are any relationships between the intrinsic mechanical properties of bone, as measured by nanoindentation, and the duration of bisphosphonate treatment.

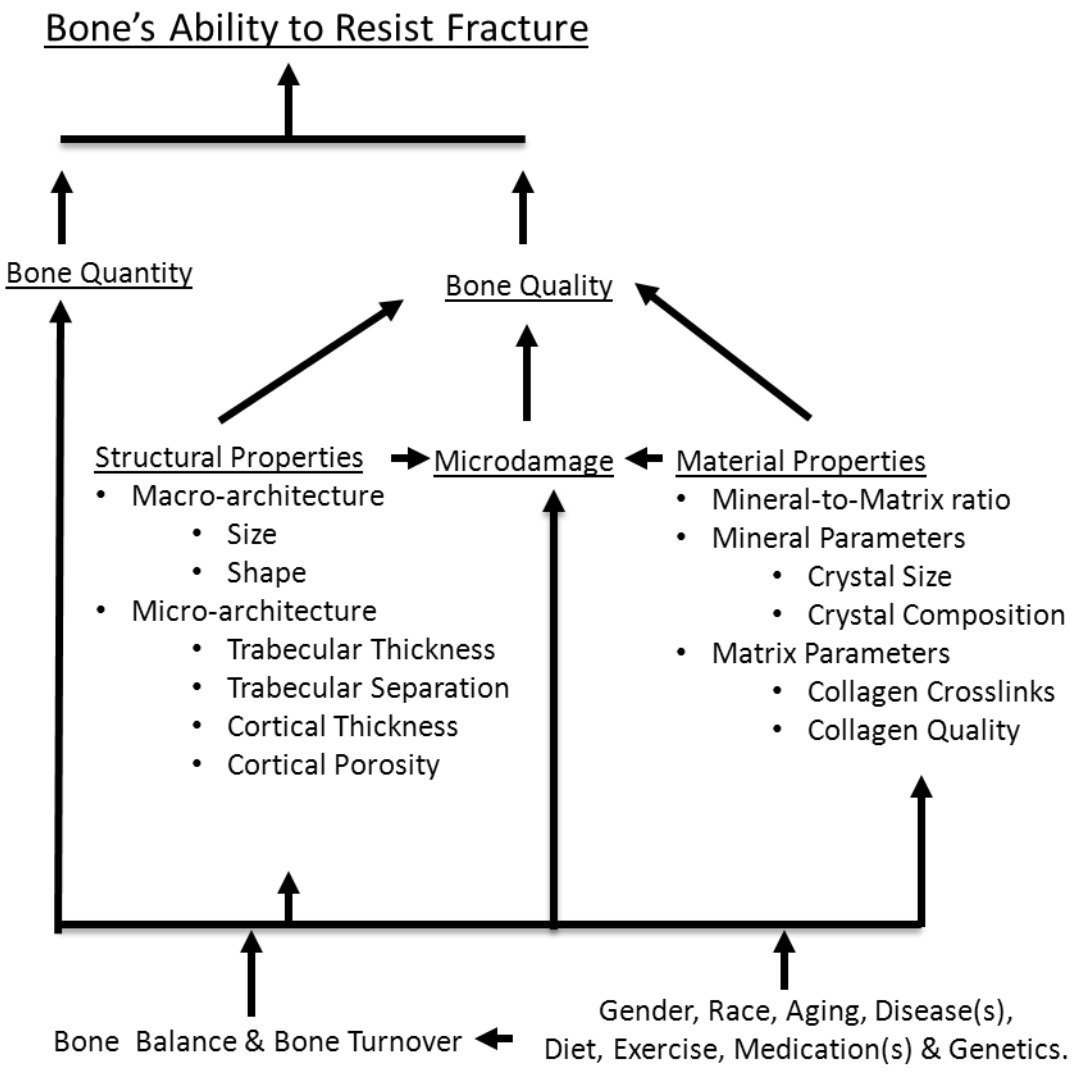


Figure 1.1: Bone's Ability to Resist Fracture

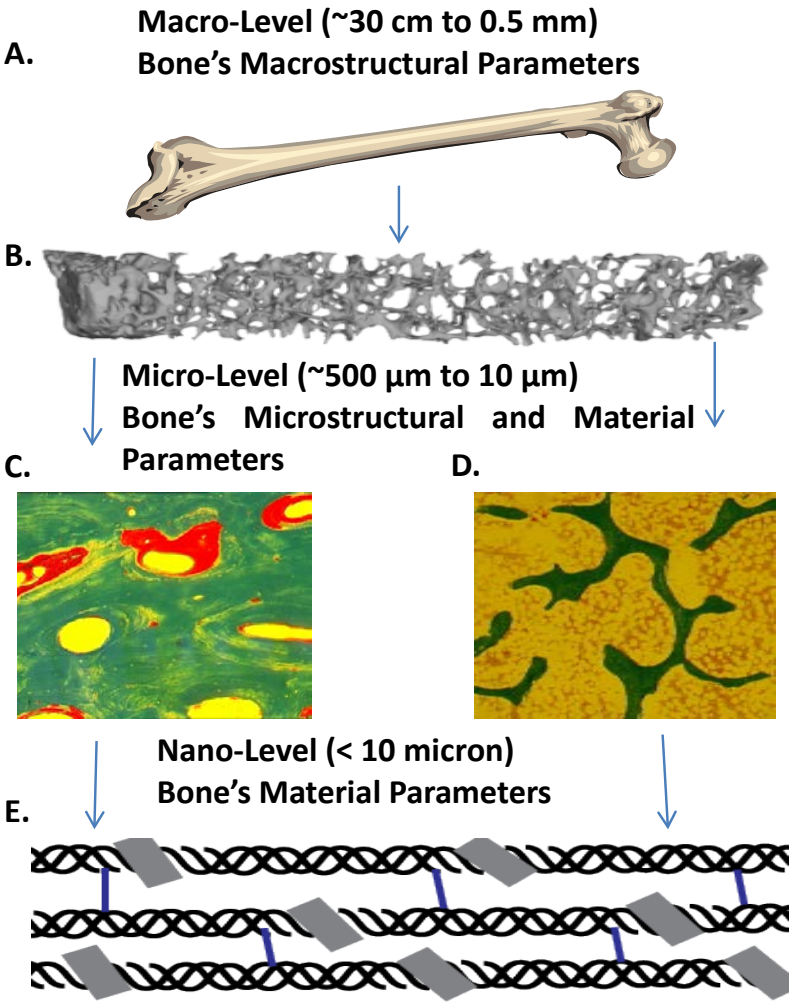


Figure 1.2: Bone structures on the macro-, micro-, and nano-level. A) Femoral bone B) Iliac crest bone specimen C) Masson-Goldner stained cortical bone D) Masson-Goldner stained cancellous bone E) Collagen (black triple helix), crystal (gray boxes), and crosslinks (blue lines) arrangement.

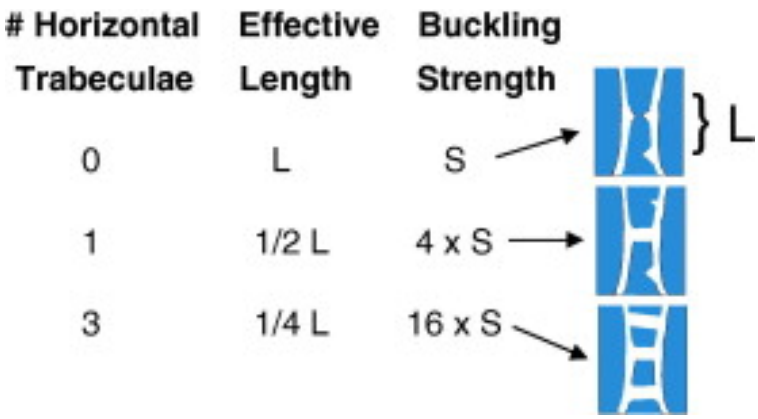


Figure 1.3: Effect of trabecular architecture on buckling strength. (Reprinted with Permission Figures A1.1 and A1.2 in Appendix)[46]

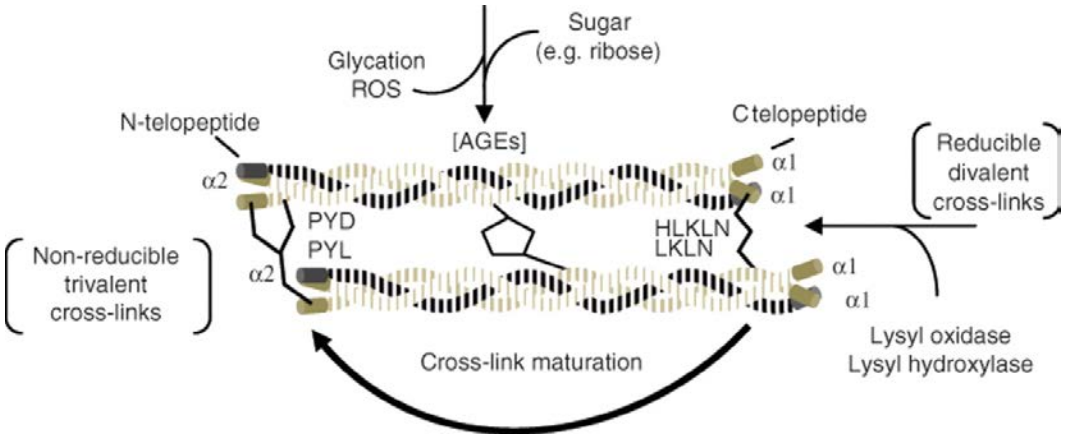


Figure 1.4: Types of Collagen Crosslinks (Reprinted with Permission Figure A1.3 in Appendix)[50]

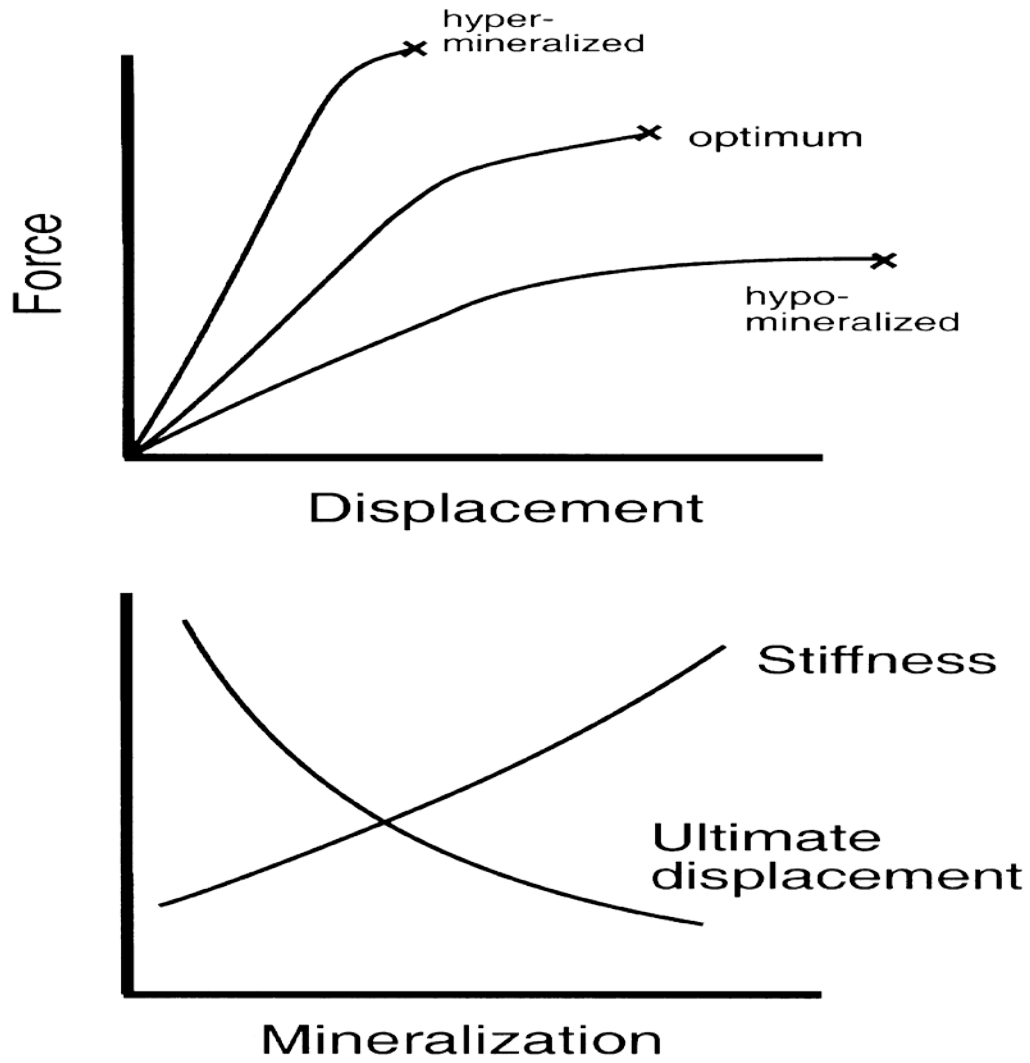


Figure 1.5: Stiffness of bone increases with increasing mineralization, but bone tissue also becomes more brittle (decreased ultimate displacement). Increased brittleness reduces work to failure as bone becomes more highly mineralized. (Reprinted with Permission Figure A1.4 in Appendix)[60]

CHAPTER 2 DIFFERENCES IN BONE QUALITY IN LOW- & HIGH-TURNOVER RENAL OSTEODYSTROPHY

Approval for this manuscript to be used in this dissertation was obtained via e-mail on 5/24/2013 from Bonnie O'Brien the current Managing Editor of Journal of the American Society of Nephrology (JASN).

The following text, data, and figures in this chapter are reproduced from the JASN publication cited below.*

Malluche H.H., Porter D.S., Monier-Faugere M.C., Mawad H., Pienkowski D., *Differences in Bone Quality in Low and High Turnover Renal Osteodystrophy*: JASN, 2012. 23(3): p. 525-32

*Please, note that in the JASN publication the methods section appears after the discussion section, but in this dissertation, the method section was repositioned to the conventional location.

2.1 Abstract

Abnormal bone turnover is common in CKD, but its effects on bone quality remain unclear. We qualitatively screened iliac crest bone specimens from patients on dialysis to identify those patients with low (*n*=18) or high (*n*=17) bone turnover. In addition, we obtained control bone specimens from 12 healthy volunteers with normal kidney function. In the patient and control specimens, Fourier transform infrared spectroscopy and nanoindentation quantified the material and mechanical properties of the specimens, and we used bone histomorphometry to assess parameters of bone microstructure and bone formation and resorption. Compared with high or normal turnover, bone with low turnover had microstructural abnormalities such as lower cancellous bone volume and reduced trabecular thickness. Compared with normal or low turnover, bone with high turnover had material and nanomechanical abnormalities such as reduced mineral to matrix ratio and lower stiffness. These data suggest that turnover-related alterations in bone quality may contribute to the diminished mechanical competence of bone in CKD, albeit through different mechanisms. Therapies tailored specifically to low- or high-turnover bone may treat renal osteodystrophy more effectively.

2.2 Introduction

Bone turnover abnormalities are well known in patients with chronic kidney disease (CKD).[93] These abnormalities encompass a spectrum from severely suppressed to markedly elevated bone turnover. Abnormal bone turnover occurs in approximately 85% of patients with CKD stage 5 on dialysis (CKD-5D),[80] and within this patient group, there is a greater risk of bone fracture than within the general population.[2, 12, 13] Although turnover abnormalities are well described,[93] little information is available on whether these abnormalities are associated with changes in bone quality. Bone quality is the contemporary term used to refer to the structural and material parameters that collectively enable bone to bear load and resist fracture or excessive deformation.[23, 25] The potential link between bone turnover and bone quality is an important question meriting study because of the relatively high incidence of fractures reported to occur with abnormal turnover.[14, 15, 94-98] Thus, the specific aim of this study was to advance the understanding of this potential link by quantifying how the microstructural parameters, material composition, and nanomechanical properties vary in bone with low- or high-turnover renal osteodystrophy (ROD) compared with bone with normal turnover from normal volunteers.

2.3 Methods

Subjects: Inclusion Criteria

Anterior iliac crest, double tetracycline-labeled bone biopsies received sequentially in the Bone Diagnostic and Research Laboratory at the University of Kentucky were screened to identify potential candidates for study enrollment. Inclusion criteria were signed informed consent from female Caucasian patients aged 40–70 years with CKD-5D on chronic maintenance dialysis and low or high bone turnover (see Qualitative and Quantitative Assessment of Bone). Twelve additional bone samples were obtained from healthy, consenting Caucasian female volunteers of the same age range. These subjects had normal kidney function and agreed to undergo baseline bone biopsy after double tetracycline labeling for an unrelated prospective research study. They had normal bone turnover. Design of this study conforms to the Declaration of Helsinki.

Subjects: Exclusion Criteria

Men and non-Caucasians were excluded to focus on the patient group with the highest fracture risk.[99, 100] Patients were also excluded if they had parathyroidectomy, osteomalacia, chronic alcoholism or drug addiction, kidney transplant(s), stainable aluminum in bone, past or present systemic illnesses, organ diseases, diabetes, or used medications within the past 6 months before biopsy that are known to alter bone metabolism, such as calcitriol, vitamin D analogs, and calcimimetics.

Biochemical Methods

Blood chemistry measurements for calcium and phosphorus were performed by using standard automated techniques. Total intact PTH level was measured by a

radioimmunometric assay (Scantibodies Inc., Santee, CA): normal range was 15–65 pg/ml, and intra- and interassay coefficients of variation were <5% and <7%, respectively. Calcidiol (25-OH vitamin D) was measured by liquid chromatography tandem mass spectrometry: normal range was 30–80 ng/ml, and intra- and interassay coefficients of variation were <13% and <14%, respectively. Blood samples for biochemical measurements were obtained immediately before biopsy.

Mineralized Bone Histology

The double tetracycline labeling schedule consisted of a 2-day oral administration of tetracycline hydrochloride (500 mg two times per day) followed by a tetracycline-free interval of 10 days and a subsequent oral administration of demeclocycline hydrochloride (300 mg two times per day) for 4 days. Bone biopsies were performed by using a one-step electrical drill technique (Straumann Medical, Waldenburg, Switzerland) as previously described.[101] Iliac crest bone samples were fixed with ethanol at room temperature, dehydrated, and embedded in methylmethacrylate.[93] Serial sections of 4 µm thickness were cut with a Microm microtome (model HM360; C. Zeiss, Thornwood, NY). Sections were stained with modified Masson–Goldner trichrome stain,[102] aurin tricarboxylic acid stain,[103] and solochrome azurine.[104] Unstained sections were prepared for fluorescent and polarized light microscopy.

Qualitative and Quantitative Assessment of Bone

Bone turnover was assessed qualitatively by examining bone slides under bright field, polarized, and fluorescent light microscopy. For inclusion in the low- or high-turnover group, the actively mineralizing bone surface and cellularity (hypo- versus hyper-) of bone cells had to be clearly different from normal. After enrollment in the study, histomorphometric analyses were done at standardized sites in cancellous bone to obtain quantitative static and dynamic parameters of bone structure, formation, and resorption. This process was done by using the semiautomatic method (Osteoplan II; Kontron, Munich, Germany).[105, 106] All measured parameters comply with the nomenclature of the Histomorphometry Committee of the American Society of Bone and Mineral Research.[107]

Spectroscopic Assessment of Bone Material

Cancellous bone mineral and matrix properties were quantified by using Fourier transform infrared spectroscopy (FTIR).[108-113] Briefly, a 4-µm-thick section was cut from each embedded bone sample and placed between two barium fluoride discs. Infrared spectra were collected from these sandwiched bone specimens using a microscope attached to a Nexus 670 FTIR spectrometer (Thermo Electron, Waltham, MA) operating in transmission mode for 200 scans at a 4-cm^{-1} resolution. Three randomly selected locations within the center of three randomly selected trabeculae were spectroscopically examined. Nine infrared spectral scans were obtained from each bone biopsy. All scans were directed at the center of each trabeculum to avoid the mineralization heterogeneity known to exist between the center of the trabeculum and

the edge (*i.e.*, between mature bone and recently formed bone).[114] The region subjected to FTIR analysis at each location was 40 × 40 µm. Background scans were performed to correct the resulting spectra from influences because of the environment, barium fluoride discs, and methylmethacrylate mount.

Bone mineralization (*i.e.*, relative mineral quantity) was calculated using the mineral to matrix ratio, a measure of the amount of bone mineral relative to the amount of collagen matrix. Greater values of the mineral to matrix ratio indicate a higher amount of bone mineralization. It has been shown that the mineral to matrix ratio correlates with ash weight and thus, is a reliable means of quantifying relative bone mineralization.[115] This ratio was calculated by dividing the area under the phosphate (mineral) peak (900–1200 cm^{-1}) by the area under the Amide I (matrix) peak (1590–1720 cm^{-1}) after both peaks were background and baseline shift corrected (Figure 2.1).[112] The purity of bone mineral was quantified using the carbonate to phosphate ratio, a measure of the amount of carbonate substituted (for PO_4^- or OH^- ions) within the mineral crystal structure. A low carbonate to phosphate ratio indicates a high degree of crystal purity. The carbonate to phosphate ratio was calculated by dividing the area under the carbonate peak (850–890 cm^{-1}) by the area under the phosphate peak. Crystallinity, a measurement of crystal size along the largest dimension, was calculated from the ratio of the areas under the peaks located at 1020 and 1030 cm^{-1}.[108, 110, 116] The relative amount of collagen crosslinking, also known as collagen maturation, was obtained by taking the ratio of the amount of mature enzymatic crosslinks (pyridinium) normalized by the amount of immature enzymatic crosslinks (reducible collagen crosslinks). Collagen crosslinking was calculated from the ratio of the areas under the peaks located at 1660 and 1690 cm^{-1}.[111] The coefficient of variation of the FTIR measurements was 4.3%.

Nanoindentation - Bone Preparation

The surface of each biopsy was polished and made uniplanar by sanding on a metallographic specimen preparation station holding abrasive silicon carbide papers of decreasing grit size (ending in 1200 grit). A final high polish was achieved by using a rotating microcloth wetted with deionized water in which diamond particles (0.3-µm grit size and then 0.05-µm grit size) were suspended. Finally, specimens were placed in an ultrasonic water bath for 10 minutes to remove surface debris.

Nanoindentation Testing Protocol

The hardness and Young's modulus of cancellous bone were quantified using established nanoindentation techniques.[117-120] This process was done by using a Nanoindenter XP (MTS Nano Instruments, Oak Ridge, TN) at Oak Ridge National Laboratories. The indenter was stationed on an anti-vibration table located within an isolation cabinet to reduce the potential for environmentally generated mechanical interference. A three-sided tip (Berkovich diamond indenter) was used for specimen indentation. The nanoindenter was calibrated by indenting fused silica of known modulus. All indentation sites were chosen based on microscopic visualization to ensure that, like the FTIR measurements, all indentation was done within the mineralized

center of each trabeculum. Twelve indentations were performed on each biopsy: three indentations within the center of four randomly chosen trabeculae.

Nanoindentation was performed by applying a peak load of 10 mN during each indentation at a constant strain rate of 0.05 second^{-1} (Figure 2.2). The maximum load was maintained for 10 seconds (hold time) to ensure that the subsequent unloading would be completely elastic.[117, 118] This load produced an indentation depth of approximately 700 nm. Based on the first 50% of the unloading curve, stiffness and hardness were quantified by using the Oliver and Pharr[121] method. The coefficient of variation of the nanoindentation measurements was 4.9%.

Statistical Analyses

Data were tested for normality by using the Kolmogorov–Smirnov test and equality of variances by using Levene's test. Normally distributed data were compared by using a one-way ANOVA with the Scheffe post hoc correction. Non-normally distributed data were compared by using the Kruskal–Wallis test; if the resulting *P* value was <0.05, then a Mann–Whitney test was used to identify which groups were significantly different. Microstructural and histomorphometric parameters were analyzed by using nonparametric methods; biochemical, material, and mechanical properties were analyzed by using parametric methods. Relationships among the histomorphometric parameters of bone turnover and the material and nanomechanical properties were evaluated by the Spearman test. All computations were done by using SPSS version 17 (SPSS, Inc, Chicago, IL).

2.4 Results

Among 163 iliac crest bone biopsies sequentially screened from patients with CKD-5D on dialysis, 35 patients met the stringent selection criteria (*Concise Methods*) and were included in the study; 17 of these 35 age-matched patients had high bone turnover (age: mean ± SD = 58.1 ± 8.1 years), and 18 patients had low bone turnover (age: mean ± SD = 56.6 ± 8.0 years). There was no significant difference in dialysis vintage between patients with low bone turnover (mean ± SD = 48.1 ± 35.4 months) and patients with high bone turnover (mean ± SD = 74.2 ± 71.0 months). Five of eighteen low-turnover patients and one of seventeen high-turnover patients had a history of bone pain. One clinically symptomatic fracture was documented in a patient with low bone turnover. Bone turnover in the 12 volunteers with normal kidney function (age: mean ± SD = 53.8 ± 4.7 years) was not significantly different from published data in normal individuals.[93, 122]

There were no significant differences in serum calcium, serum phosphorus, or calcidiol concentrations between patients with low or high bone turnover (Table 2.1). Serum phosphorus levels were significantly elevated in both low- and high-bone turnover groups compared with the normal bone turnover group (*P*<0.01). Serum parathyroid hormone (PTH) levels were approximately two times the upper normal range in patients with low bone turnover and approximately nine times the upper normal range in patients with high bone turnover (Table 2.1). There were no upward or

downward trends in serum PTH during the 6 months preceding the biopsy. Use of calcium-based phosphate binders was not different between patients who had bone with low or high turnover.

As expected, there were significant differences in histomorphometric cellular parameters of bone formation and resorption among patients with high, normal, and low turnover ($P<0.05$) (Figure 2.3).

Microstructural Parameters at Various Levels of Bone Turnover

No differences in microstructural parameters were observed between bone with high turnover and bone with normal turnover. In contrast, bone with low turnover had altered microstructural properties compared with bone with normal or high turnover (Figure 2.4). Specifically, cancellous bone volume in bone with low turnover was 16.9% ($P<0.05$) and 34.7% ($P<0.01$) less than in bone with normal and high turnover, respectively (Figure 2.4A). Trabecular thickness in bone with low turnover was 20.3% ($P<0.05$) and 33.1% ($P<0.01$) less than in bone with normal and high turnover, respectively (Figure 2.4B).

Material Composition at Various Levels of Bone Turnover

Less mineral (relative to matrix) was observed in bone with high turnover compared with bone with normal or low turnover (Figure 2.5A). Specifically, the mineral to matrix ratio of bone with high turnover was 9.7% less compared with bone with normal turnover and 9.1% less compared with bone with low turnover (both $P<0.01$).

The carbonate to phosphate ratio was 13.1% lower ($P<0.01$) in bone with low turnover compared with bone with normal turnover (Figure 2.5B). No significant differences were detected among the three turnover groups in crystallinity (inversely proportional to mineral crystal size) or collagen crosslinking (directly proportional to collagen maturation) (Table 2.2).

Bone Turnover and Nanomechanical Properties

Young's modulus (shape-independent material stiffness) was 11.9% ($p<0.05$) and 12.4% ($p<0.01$) less in bone with high turnover compared with bone with normal or low turnover, respectively (Figure 2.5C). Hardness (the ability to resist permanent shape change when a force is applied) of bone with high turnover was 13.1% less ($p<0.05$) compared with bone with low turnover (Figure 2.5D). No significant difference in hardness was observed between bone with high turnover and bone with normal turnover.

Correlation between Bone Turnover and Material & Nanomechanical Properties

Correlations were found between bone turnover parameters (when considered as continuum) and mineral to matrix ratio as well as Young's modulus. Specifically, osteoclast surface per bone surface and bone formation rate per bone surface correlated with mineral to matrix ratio and Young's modulus ($\rho = -0.33$ to -0.50, $p<0.01$) (Figure 6).

2.5 Discussion

The key finding of this study is that bone quality varies, albeit by different mechanisms, with different levels of bone turnover. Departures from normal bone quality were manifested in bone with low turnover by changes in microstructural parameters; in contrast, departures from normal bone quality were manifested in bone with high turnover by changes in material composition and nanomechanical properties.

Our data regarding high turnover are consistent with the findings of Ng *et al.*,[123] who reported that bone from patients with high-turnover renal osteodystrophy had lower mineralization and lower trabecular microhardness compared with bone from patients with low-turnover renal osteodystrophy.[123] The work by Ng *et al.*,[123] however, found no turnover-related differences in the microstructural parameters of bone. Bone samples for the retrospective study by Ng *et al*[123] were obtained between 1987 and 1989, a time period when aluminum and magnesium phosphate binders were commonly used. None of the patients in the present study were on aluminum- or magnesium-containing phosphate binders.

Isaksson *et al.*[124] measured the static histomorphometric parameters and material properties in bone from normal subjects and patients with high-turnover renal osteodystrophy. Relative mineralization, measured by the mineral to matrix ratio in the center of trabecular bone, was less in our study and in the study by Isaksson *et al.*[124] in renal osteodystrophy patients with high turnover compared with normal subjects. This difference did not reach significance in the study by Isaksson *et al.*[124] but was significant in the present study. Isaksson *et al.*[124] detected a significant turnover-related difference in the mineral to matrix ratio when this parameter was measured at the periphery of the trabeculae. This mineralization difference observed at the periphery may be explained by the high osteoid volume at the surface of bone with high turnover. For this reason, we did not measure the mineral to matrix ratio at the edge of the trabeculae.

Also, the study by Isaksson *et al.*[124] and the present study both showed that the carbonate to phosphate ratio was less in the center of trabecular bone with high turnover compared with the center of trabecular bone with normal turnover. This reduction (approximately 10%) reached statistical significance in the study by Isaksson *et al.*[124] *b*ut not in our study (the reduction was approximately 8%). In the present study, however, there was a significant difference in the carbonate to phosphate ratio between bone with low turnover and bone with normal turnover. Clinical relevance of the carbonate to phosphate ratio awaits additional study.[125]

Turnover-related differences in bone material properties between the present study and the study by Isaksson *et al*.[124] may be attributable to differences in patient characteristics including age, gender, treatment, and underlying kidney disease.

The observed reduction in mineral to matrix ratio and Young's modulus in bone with high turnover may be explained by the shorter duration between remodeling cycles. Specifically, the diminished remodeling duration may prevent full mineralization

and thus, cause reduced bone stiffness.[53] This explanation is supported by the negative relationship between bone turnover parameters and the mineral to matrix ratio or Young's modulus (Figure 6). It is consistent with the known increase in osteoid volume accompanying high turnover and should be not be interpreted as evidence of osteomalacia.[93] Studies of pediatric renal osteodystrophy find a greater prevalence of abnormal mineralization than in the adult skeleton.[126, 127] This discrepancy could be explained by the higher remodeling of bone in the growing skeleton in addition to increases in bone turnover because of secondary hyperparathyroidism. The present findings are also consistent with prior studies showing that a reduction in relative mineralization, a decreased mineral to matrix ratio, is associated with reduced stiffness in human[57] and animal bone.[58, 59] Reduced mineralization in bone with high turnover is clinically relevant, because other evidence shows that small decreases in mineral content are associated with disproportionately greater reductions in fracture toughness.[53]

The absence of changes in the mineral to matrix ratio of bone with low turnover suggests that mineral supersaturation may not accompany reduced remodeling activity. The accompanying lack of change in nanomechanical properties is expected, but the macromechanical properties of bone may be reduced because of the observed microstructural abnormalities.

The observed abnormal microstructural parameters (thinner trabeculae and less cancellous bone volume) in patients with low turnover are clinically relevant, because reducing support element size in any structure with unchanged material properties diminishes its mechanical competence.

Bone quality abnormalities accompanying different turnover states were studied by using the current gold standard sampling technique, which is bone biopsy. Of course, for routine clinical diagnostic purposes, a noninvasive approach is preferable. A recent study by Bhagat *et al*.[128] used noninvasive magnetic resonance imaging and finite element modeling of the distal tibial metaphysis to predict bone strength.[128] This promising approach awaits additional study.

This study was designed to detect differences in bone's microstructural and material properties but was not powered to assess overall fracture risk. The documented prevalence of bone pain and fractures in this study is in keeping with published studies in patients with renal osteodystrophy.[12] To prevent data confounding, this study was limited to Caucasian women with CKD-5D (40–70 years of age) with predefined selection criteria. Additional studies are needed to address the potential effects of gender, race, age, diabetes, and medications (including vitamin D) on bone quality.

Our data are clinically important, because they extend the studied spectrum of bone abnormalities in renal osteodystrophy to include bone with low turnover and measurement of bone's nanomechanical properties. This extension is clinically relevant, because bone strength and musculoskeletal competence are influenced by its microstructural parameters, material composition, and mechanical properties.[23, 25] The

information contributed by the present study provides substantial evidence linking bone quality and bone turnover in renal osteodystrophy.

In conclusion, abnormal bone turnover in renal osteodystrophy is associated with specific changes in bone quality as manifested on the microstructural, material, or mechanical levels. These abnormalities are dependent on the level of turnover. Specifically, bone with low turnover is associated with microstructural abnormalities, whereas bone with high turnover is associated with material and mechanical property abnormalities. Reduced bone quality of patients with either low- or high-turnover renal osteodystrophy may contribute to the known decreased mechanical competence in these patients[14, 15, 94-98] but for two different turnover-dependent reasons. These findings call for additional studies to evaluate modified treatment regimens for renal osteodystrophy by using tailored therapies for patients with low- or high-turnover bone.

2.6 Acknowledgments

We thank the Mechanical Properties and Mechanics Group at Oak Ridge National Laboratory for providing access to nanoindentation instrumentation, Dr. Guodong Wang for technical assistance in completing the bone histomorphometry, and Dr. K. Muse for help in recruiting normal volunteers.

This study was supported by National Institute of Health Grant R01 080770 and a grant from the Kentucky Nephrology Research Trust. Additional support was provided by the Division of Nephrology Bone and Mineral Metabolism, the Center for Biomedical Engineering, and the Department of Orthopaedic Surgery at the University of Kentucky.

2.7 Tables and Figures:

Table 2.1: Serum biochemical data from patients with high, normal, or low bone turnover

Bone Turnover	Calcium (mg/dl)	Phosphorus (mg/dl)	Calcidiol (ng/ml)	Total PTH (pg/ml)
High (n=17)	9.58 ± 1.05	6.01 ± 2.22*	39.0 ± 20.9	596 ± 469*†
Normal (n=12)	9.23 ± 0.33	3.53 ± 0.52	42.5 ± 9.78	30.8 ± 10.2
Low (n=18)	9.64 ± 1.09	6.31 ± 1.80*	43.8 ± 21.5	126 ± 168
Normal Range	9.00 - 10.5	3.40 - 4.50	30 – 80	15 – 65

(mean ± one standard deviation)

†$p < 0.01$ vs. low; *$p < 0.01$ vs. normal

Table 2.2: Crystallinity and collagen crosslinking from bone with high, normal, or low turnover

Bone Turnover	Crystallinity	Collagen Crosslinking
High (n=17)	0.93 ± 0.07	3.51 ± 0.75
Normal (n=12)	0.89 ± 0.04	3.53 ± 0.27
Low (n=18)	0.89 ± 0.04	3.62 ± 0.47

(mean ± one standard deviation)

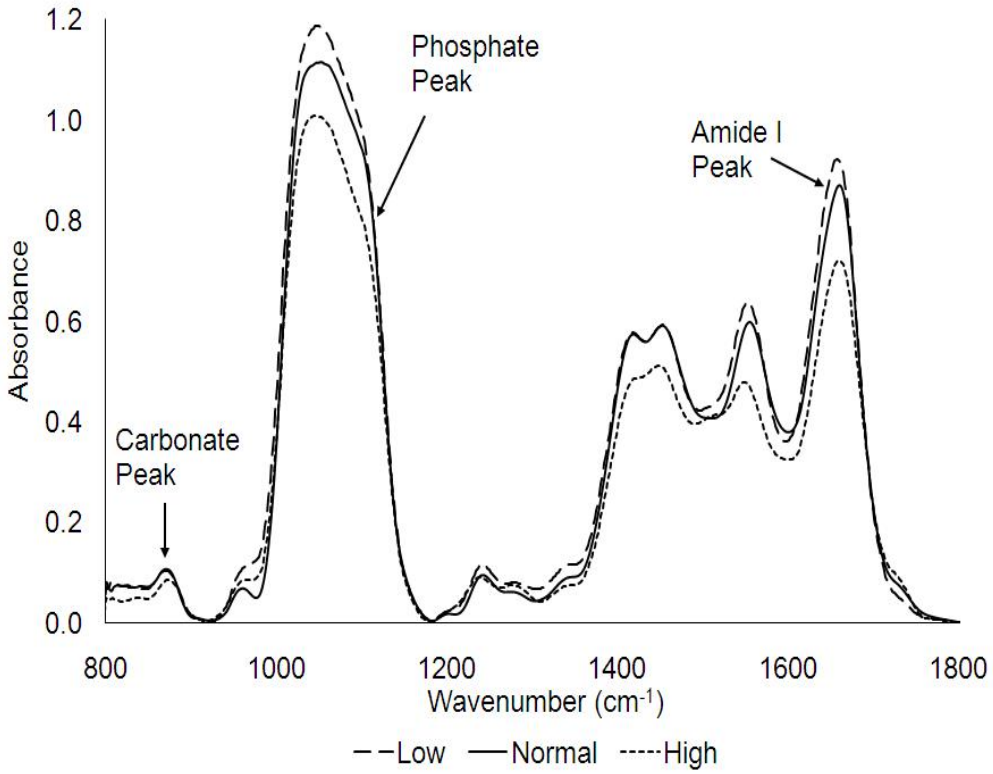


Figure 2.1: Typical FTIR spectra from bone with low, normal, and high turnover. The spectra were analyzed using the carbonate peak (carbonate substitution into hydroxyapatite) between 850 and 890 cm^{-1}, phosphate peak (mineral) between 900 and 1200 cm^{-1}, and Amide I peak (matrix) between 1590 and 1720 cm^{-1}.

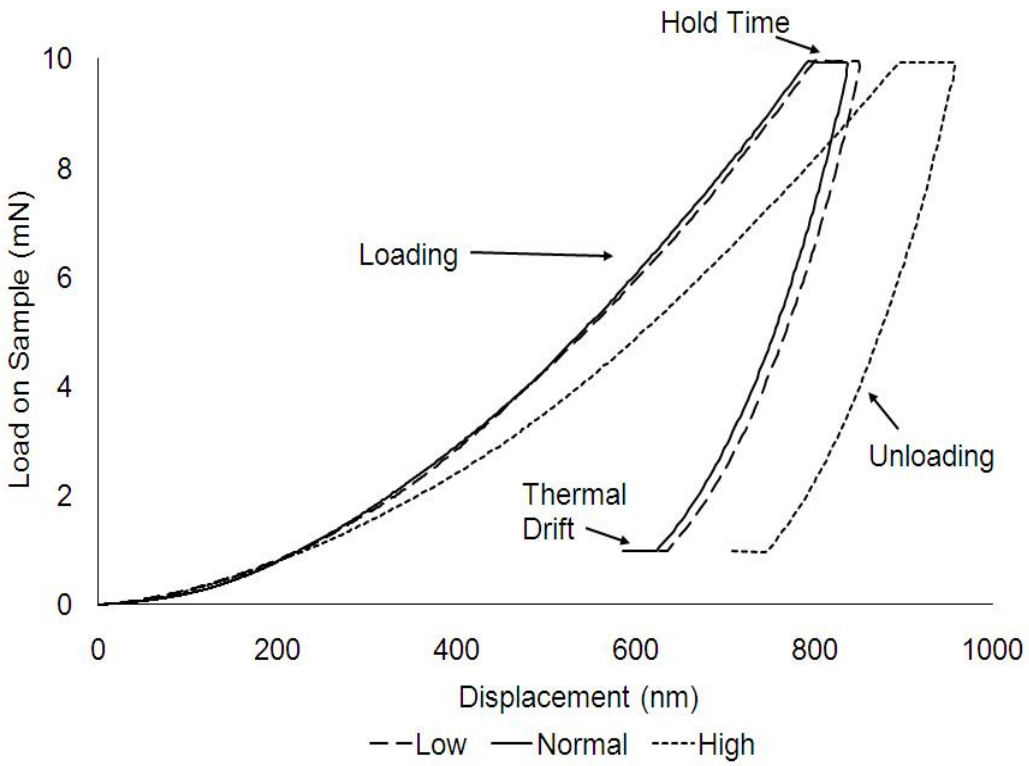
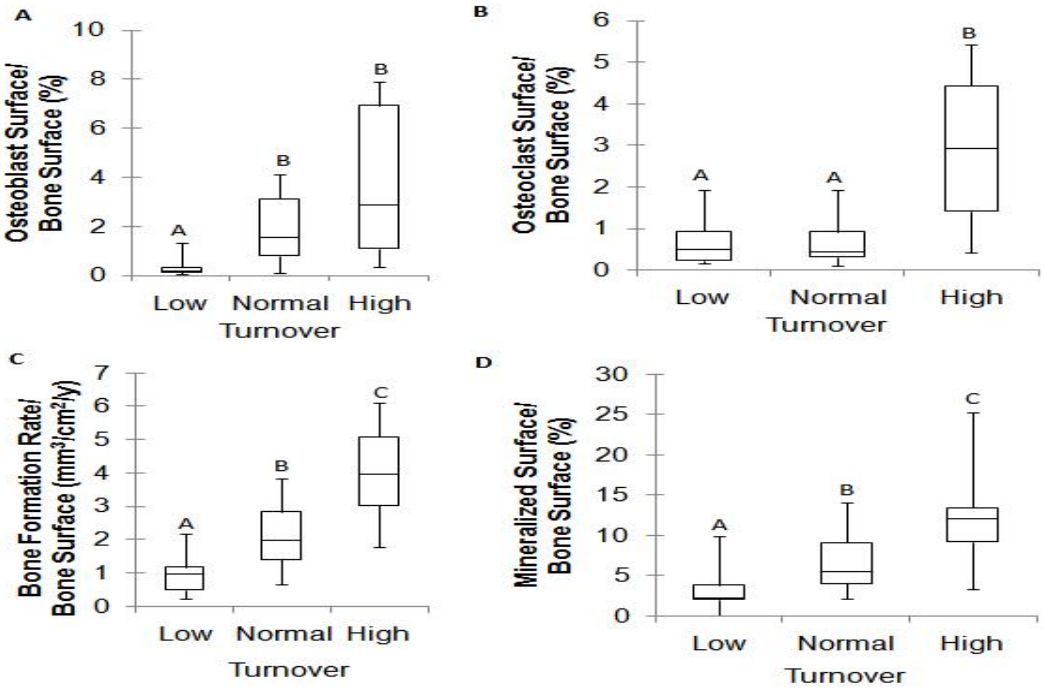
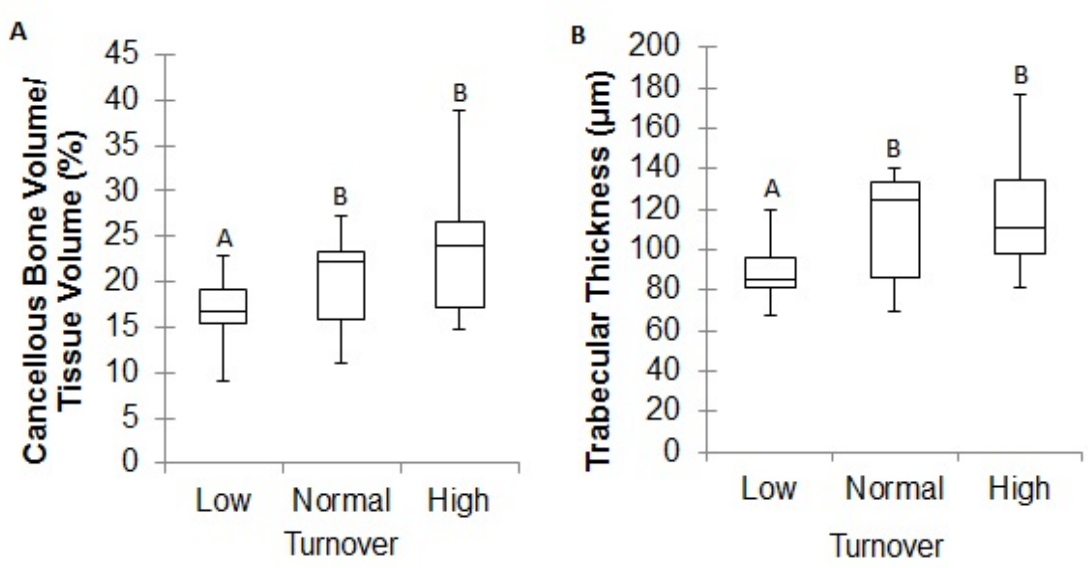


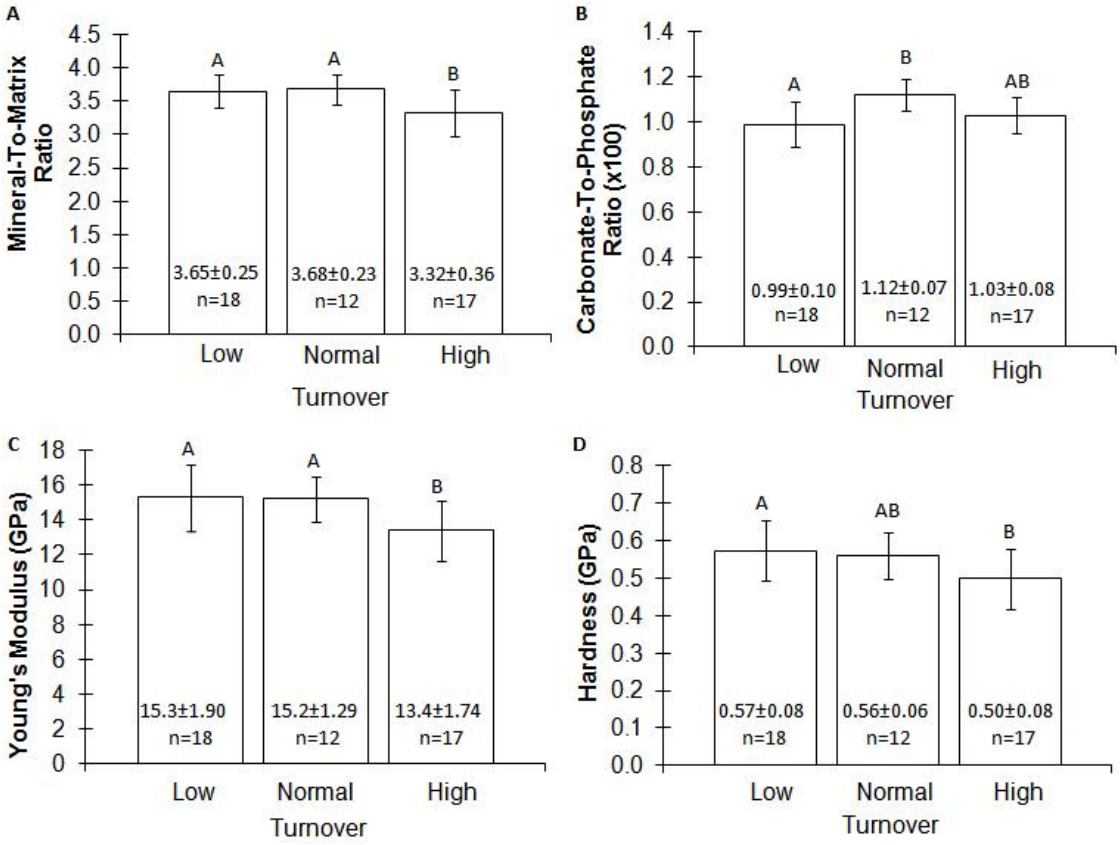
Figure 2.2: Typical load and unload cycle for nanoindentation of bone. Nanoindentation was performed by applying a maximum load of 10 mN at which a 10-s hold time was placed to ensure elastic unloading. The specimen was then unloaded to 90% of maximum load and held for 25 s to correct for thermal drift.



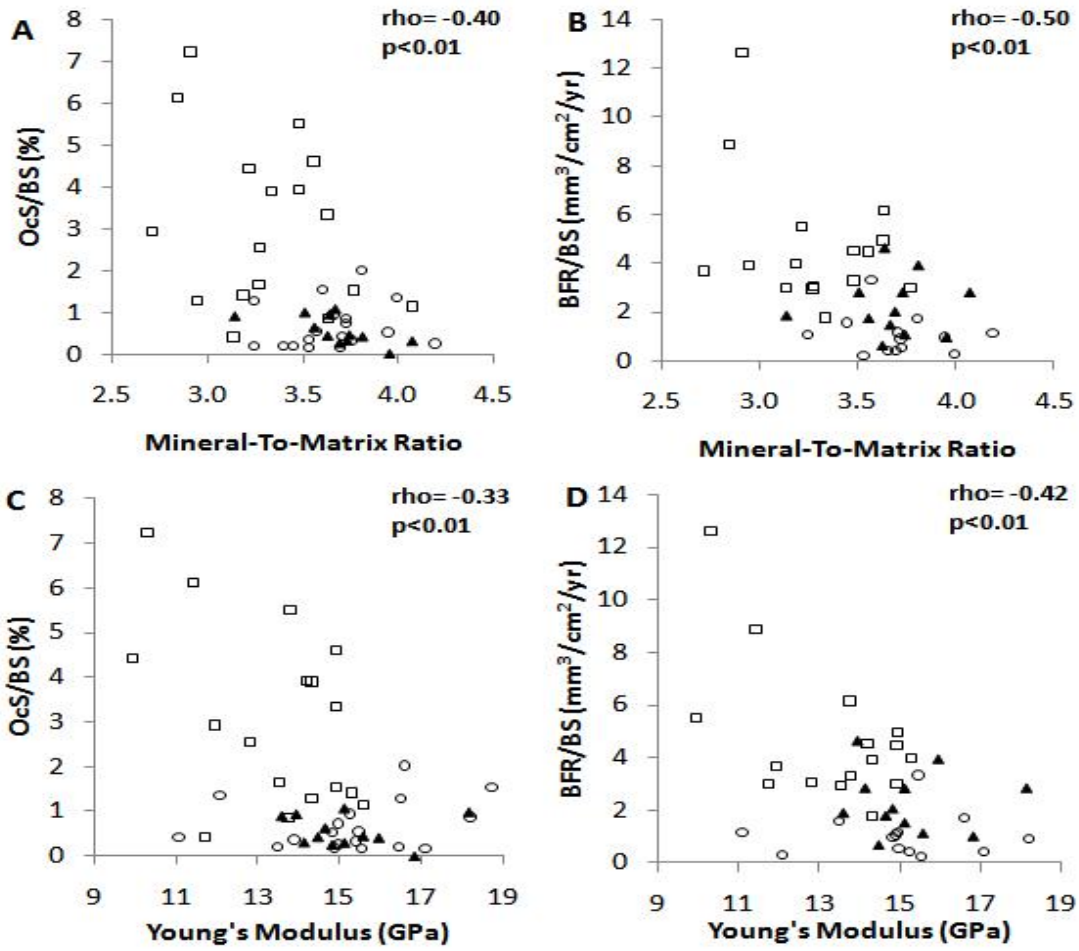
Figures 2.3: Box plots of various static and dynamic histomorphometric parameters of bone versus bone turnover. (A–D) The bottom and top of the box represent the lower (25%) and upper (75%) quartiles, respectively, and the middle line denotes the median (50%). The upper and lower bounds of the error bars denote the range. Values with the same letters are not significantly different.



Figures 2.4: Box plots of various microstructural parameters of bone versus turnover. The bottom and top of the box represent the lower (25%) and upper (75%) quartiles, respectively, and the middle line denotes the median (50%). The upper and lower bounds of the error bars denote the range. Values with the same letters are not significantly different.



Figures 2.5 A-D: Various material and mechanical properties of bone versus bone turnover. Mean (± SD) values of the mineral to matrix ratio, carbonate to phosphate ratio, Young's modulus, and hardness are shown versus bone turnover. Values with the same letter are not significantly different.



Figures 2.6 A-D: Relationships between bone material or mechanical properties and bone resorption or formation parameters. (A) Mineral to matrix ratio versus osteoclast surface/bone surface (OcS/BS), (B) mineral to matrix ratio versus bone formation rate/bone surface (BFR/BS), (C) Young's modulus versus OcS/BS, and (D) Young's modulus versus BFR/BS.

Legend: ○, Low turnover; ▲, normal turnover; □, high turnover.

CHAPTER 3 LOW-ENERGY FRACTURES WITHOUT LOW T-SCORES CHARACTERISTIC OF OSTEOPOROSIS: A POSSIBLE BONE MATRIX DISORDER

Approval for this manuscript to be used in this dissertation was obtained from Beth Ann Rocheleau Intellectual Property Manager at ©ROCKWATER, Inc. ROCKWATER, Inc. handles the intellectual property licensing, sales, and management for the Journal of Bone and Joint Surgery (JBJS). Please find the attached letter of approval in the appendix (Figure A1.5).

The following text, data, and figures in this chapter are reproduced from the JBJS publication cited below.*

Malluche H.H., Porter D.S., Monier-Faugere M.C., Mawad H., Pienkowski D., *Low-Energy Fractures Without Low T-Scores Characteristic of Osteoporosis: A Possible Bone Matrix Disorder:* JBJS, 2013. 95(19): p. e1391-6.

*Please note that the acknowledgements and source of funding were moved to after the discussion section. This was done for consistency with chapters 2 and 4. An appendix section was also added.

3.1 Abstract

Background:

Osteoporotic fractures commonly occur after low-energy trauma in postmenopausal women with reduced bone quantity documented by low bone mineral density (BMD). Low-energy fractures, however, have also been reported in premenopausal women with normal or near-normal BMD, suggesting the existence of a bone quality abnormality.

Methods:

Bone quality and quantity were evaluated in a cross-sectional study of three groups of premenopausal white females: (1) twenty-five subjects with low-energy fracture(s) and BMD in the normal range (t-scores > −2.0), (2) eighteen subjects with low-energy fracture(s) and BMD in the osteoporotic range (t-scores ≤ −2.5), and (3) fourteen healthy volunteers (controls). Bone quality was assessed by using Fourier transform infrared spectroscopy and histomorphometry in iliac crest bone samples obtained from all subjects; bone quantity was assessed by dual x-ray absorptiometry and histomorphometry.

Results:

The collagen crosslinking ratio in the non-low-BMD subjects with fractures was 13% greater than the ratio in the low-BMD subjects with fractures and 14% greater than the ratio in the controls ($p < 0.001$ for both). Cancellous bone volume was 29% greater ($p < 0.01$) and trabecular separation was 31% less ($p < 0.01$) in the non-low-BMD subjects with fractures than in the low-BMD subjects with fractures; the values in the non-low-BMD subjects did not differ from those in the controls. Bone turnover did not differ among the groups, and osteomalacia was not present in any subject. Thus, the non-low-BMD subjects with fractures maintained bone quantity, but the collagen crosslinking ratio, a parameter of bone quality, was abnormal. In contrast, the low-BMD subjects with fractures did not have this collagen crosslinking abnormality but did have abnormal bone quantity.

Conclusions:

This study highlights a collagen crosslinking abnormality in patients with low-energy fractures and nonosteoporotic t-scores. Reports have indicated that altered collagen crosslinking is associated with subnormal fracture resistance. A finding of nonosteoporotic bone mass in a patient with low-energy fractures would justify assessment of bone material quality, which currently requires a bone biopsy. Further studies are needed to search for possible noninvasive tests to diagnose abnormal crosslinking. Since no specific therapies for abnormal collagen crosslinking are currently available, studies are also needed to explore novel therapeutic modalities to reverse the underlying collagen crosslinking abnormality.

Level of Evidence:

Prognostic Level III. See Instructions for Authors for a complete description of levels of evidence.

3.2 Introduction

Before the advent of routine measurement of bone mineral density (BMD) by x-ray absorptiometry, osteoporosis was defined as a clinical syndrome in postmenopausal women with low-energy fracture(s) accompanied by low bone mass. Given the ease of use and widespread availability of dual x-ray absorptiometry (DXA), the World Health Organization subsequently defined osteoporosis as a reduction in BMD t-scores of $\geq$2.5 standard deviations from the mean value in young adults.[9] This definition is routinely used worldwide in clinical practice for the diagnosis of osteoporosis. Fractures may also occur, however, with low-energy trauma in premenopausal women who are nonosteoporotic as classified by their BMD t-scores.[9, 81, 82]

It is easy to understand the occurrence of low-energy fractures in patients with osteoporotic t-scores, but it remains unclear why low-energy fractures occur in premenopausal women with nonosteoporotic t-scores. Factors other than low bone quantity characteristic of osteoporosis must be considered, and chief among these is abnormal bone quality. Bone quality includes material properties and microarchitectural features, which are major contributors to the load-bearing capabilities of bone.[23, 25-27] Low-energy fractures associated with abnormal bone quality were reported in premenopausal women with idiopathic osteoporosis.[129-133] There is limited information evaluating bone quality in premenopausal women with fractures but without osteoporotic BMD or secondary osteoporosis while controlling for the potentially confounding effects of sex or race. The present study was designed to test the hypothesis that, in the absence of bone quantity abnormalities, abnormal bone quality in premenopausal women is associated with low-energy fracture.

3.3 Methods

Study Design

This cross-sectional study was designed to quantify bone quality and quantity in three groups of premenopausal women: (1) those with low-energy fractures and nonosteoporotic BMD t-scores (the non-low-BMD fracture group), (2) those with low-energy fractures and osteoporotic BMD t-scores (the low-BMD fracture group), and (3) healthy volunteers (the control group). Bone samples for the study were obtained from subjects undergoing iliac crest biopsy for work-up of low-energy fractures at our institution. Bone from the iliac crest serves as a useful model of the skeleton because histological and mechanical changes in this tissue are also associated with histological[134] and mechanical[135] changes in bone at other skeletal sites. The two primary study groups included premenopausal adult white women with one or more low-energy fractures; those in the non-low-BMD group had a nonosteoporotic BMD as indicated by a t-score of >−2.0 at both the hip and the lumbar spine, and those in the low-BMD group had an osteoporotic BMD as indicated by a t-score of ≤−2.5 at the hip or lumbar spine. Low-energy fractures were defined as those occurring without trauma during normal activities of daily living. Control bone samples were obtained from biopsies performed in healthy premenopausal white women volunteers with BMD t-scores of >−2.0 and no fractures.

Subjects were excluded if they had a diagnosis of osteogenesis imperfecta or other genetic bone disease, histologically proven osteomalacia (osteoid thickness of >20 μm and mineralization lag time of >100 days), hyperparathyroid bone disease or other disorders associated with secondary osteoporosis, chronic kidney disease, abnormal mineral metabolism, Marfan syndrome, endocrine abnormalities, celiac or other gastrointestinal disorders, bariatric procedures, diabetes, Paget disease of bone, amenorrhea, eating disorders, or malignancies. Subjects were also excluded if they had a history of drug or alcohol abuse or of prior use of bisphosphonates, teriparatide, selective estrogen receptor modulators, sex steroids, or any other medications known to alter bone metabolism. The protocol of this institutional review board-approved cross-sectional study adhered to the Declaration of Helsinki.

Bone Mineral Density

Bone mineral density was measured at the hip and at the lumbar spine (L2-L4) in all study subjects with use of DXA (Lunar iDXA; GE Healthcare, Madison, Wisconsin). The coefficient of variation of the BMD measurements was 1.2% at the spine and 0.9% at the hip.

Serum Biochemistry

A renal metabolic panel was obtained and serum alkaline phosphatase was measured by routine laboratory techniques. In addition, serum parathyroid hormone (PTH) levels were measured by radioimmunoassay (Total Intact PTH; Scantibodies, Santee, California), serum calcidiol was measured by liquid chromatography-tandem mass spectrometry (API 3200; AB SCIEX, Framingham, Massachusetts), serum bone-specific alkaline phosphatase was measured by immunocapture enzyme activity assay (Quidel, San Diego, California), serum N-terminal telopeptide was measured by ELISA (enzyme-linked immunosorbent assay) (Osteomark NTX; Inverness Medical Innovations, Waltham, Massachusetts), and serum osteocalcin was measured by ELISA (Quidel).

Mineralized Bone Histology and Bone Histomorphometry

Bone samples, obtained after tetracycline double-labeling,[136] were processed without mineral removal and were embedded in methylmethacrylate. Serial sections (thicknesses, 4 and 7 μm) were cut and were stained with modified Masson-Goldner trichrome stain. Unstained sections were prepared for fluorescent and polarized light microscopy.[93]

Histomorphometry was performed at standardized sites in cancellous bone to obtain quantitative static and dynamic parameters reflecting bone structure (cancellous bone volume/tissue volume), microarchitecture (trabecular separation, trabecular thickness), bone turnover (bone formation rate/bone surface area), and mineralization (osteoid thickness, mineralization lag time).[105, 106] Measurements were made at ×200 magnification (Osteoplan II System; Kontron, Munich, Germany). All measured parameters were defined in accordance with the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research.[107]

Bone Material (Mineral and Matrix) Properties

Bone material properties were measured with use of a Fourier transform infrared (FTIR) spectrometer (Nexus 670; Thermo Electron, Waltham, Massachusetts) on sections prepared from anterior iliac crest bone samples. A 4-µm-thick undecalcified section was cut from each bone sample and placed between two barium fluoride discs for FTIR analysis.[137] Infrared spectra were collected from these "sandwiched" bone sections with use of a microscope that was attached to the spectrometer and operated in transmission mode for 200 scans at 4 cm^{-1} resolution. Three trabeculae were chosen from each section. Trabeculae were evaluated beginning at a distance of five to seven optical fields (at ×200) below the cortex. Spectroscopic measurements were made in the center of each of these three trabeculae. Background scans were used to correct for the spectral contributions of the barium fluoride discs and the methylmethacrylate mount.

Established parameters reflecting bone quality were determined.[138] Specifically, the mineral-to-matrix ratio was obtained by dividing the area under the phosphate (mineral) peak (900 to 1200 cm^{-1}) by the area under the amide I (matrix) peak (1590 to 1720 cm^{-1}) after baseline correction of both peaks (see Appendix). The carbonate-to-phosphate ratio (i.e., the amount of carbonate substituted in the hydroxyapatite crystal) was obtained by dividing the area under the carbonate peak (850 to 890 cm^{-1}) by the area under the phosphate peak. Crystallinity, a measure of crystal size and perfection, was obtained by dividing the area under the 1020 cm^{-1} peak by the area under the 1030 cm^{-1} peak.[116] The collagen crosslinking ratio, a measure of collagen maturity, was the ratio of the areas under the 1660 cm^{-1} (mature crosslinks) and 1690 cm^{-1} (immature crosslinks) peaks.[111] The coefficient of variation was 4.3% for the mineral-to-matrix ratio, 2.0% for the carbonate-to-phosphate ratio, 1.7% for the crystallinity, and 4.1% for the crosslinking ratio.

Data Analyses

Data were tested for normality with use of the Kolmogorov-Smirnov test and for equality of variances with use of the Levene's test. Multiple-group comparisons were performed with use of analysis of variance (ANOVA) with Scheffe post-hoc correction. Two-group comparisons were made with use of the Student t test. Univariate analyses (Pearson tests) were used to determine whether BMD and age were correlated with the material and histomorphometric parameters of bone. A p-value of <0.05 was considered significant.

3.4 Results

Subject Characteristics and Biochemical Results

Fifty-seven premenopausal adult female white subjects met the selection criteria and were included in the study; twenty-five were in the non-low-BMD fracture group, eighteen were in the low-BMD fracture group, and fourteen were healthy volunteers (controls).

Subjects in the non-low-BMD group first presented with a mean of 3.6 low-energy fractures during adulthood compared with 1.4 low-energy fractures in the low-BMD group ($p < 0.05$). A hallmark of these low-energy fractures was that subjects were unable to identify a specific mechanical event associated with the fracture. The number of patients who sustained fractures in particular bones differed between the two fracture groups (Table 3.1). Nondisplaced metatarsal fractures (Fig. 3.1) were the most common fractures in the non-low-BMD group (experienced by 56% of the subjects), whereas spinal fractures were the most common fractures in the low-BMD group (experienced by 28% of the subjects) (Table 3.1). No atypical femoral fractures occurred in any of the study subjects.

The BMD values in the groups were consistent with those defined by the inclusion criteria. Subjects in the two fracture groups were younger than the controls, and no differences were detected among the three groups with respect to serum concentrations of calcium, phosphorus, creatinine, glucose, sodium, alkaline phosphatase, parathyroid hormone, calcidiol, bone-specific alkaline phosphatase, N-terminal telopeptide, or osteocalcin (Table 3.2).

Histomorphometric Parameters of Bone Structure, Microarchitecture, Turnover, and Mineralization

Cancellous bone volume was 29% greater ($p < 0.01$) and trabecular separation was 31% less ($p < 0.01$) in the non-low-BMD subjects with fractures than in the low-BMD subjects with fractures; the values in the non-low-BMD subjects did not differ from those in the controls (Figs. 3.2 and 3.3). Trabecular thickness did not differ significantly among the three groups (see Appendix).

Bone turnover and bone mineralization parameters did not different significantly among the three groups (see Appendix). None of the measured histomorphometric parameters were correlated with BMD or age.

Bone Material (Mineral and Matrix) Properties

The mean collagen crosslinking ratio in the non-low-BMD group was 13% greater ($p < 0.001$) that that in the low-BMD group and 14% greater ($p < 0.001$) than that in the controls (Fig. 3.4). The collagen crosslinking ratio did not differ significantly between the low-BMD group and the controls. No differences were observed among the three groups with respect to any other measured bone mineral parameter (see Appendix). None of the measured mineral or matrix properties were correlated with BMD, age, or any histomorphometric parameter.

3.5 Discussion

The novel result of this study is the greater collagen crosslinking ratio, a bone quality parameter, in non-low-BMD subjects with low-energy fractures. It is important to note that such fractures in the non-low-BMD subjects could not be attributed to abnormal bone structure or microarchitecture (including lower cancellous bone volume, thinner trabeculae, or greater trabecular separation). In contrast, such fractures in the

low-BMD group could be attributed to reduced bone quantity, and the subjects in this group did not have the material quality abnormality observed in the non-low-BMD group. These findings confirmed our hypothesis that low-energy fractures in premenopausal women with nonosteoporotic BMD are associated with an abnormality in bone quality evidenced by increased collagen crosslinking.

Clinically, patients who sustain low-energy fractures are considered osteoporotic regardless of their BMD t-score; however, the present findings of a different fracture distribution and of greater collagen crosslinking in the non-low-BMD group compared with the low-BMD group suggests that these two groups manifest different disease entities. One disease entity (seen in the low-BMD group) is attributable to abnormal bone quantity; the other (seen in the non-low-BMD group) is attributable to abnormal bone quality as manifested by abnormal collagen crosslinking. Reduced bone quantity is known to diminish bone fracture resistance, as demonstrated by the finding that spinal fractures, a typical manifestation of classic osteoporosis, were the most prevalent fractures in the low-BMD group. The most prevalent fracture site in the non-low-BMD group was the metatarsals, an uncommon site in classic osteoporosis.

Crosslinking is an important structural feature that affects mechanical performance. The types and extent of collagen crosslinking in bone have only recently been appreciated. Crosslinks alter the mechanical properties of bone.[65, 139] Collagen crosslinking abnormalities have been linked to altered bone biomechanics and diminished fracture resistance in both animal[65, 140] and clinical studies.[48, 67, 132, 133, 141] In Wistar rats, beta-aminopropionitrile administered to inhibit lysyl oxidase and thereby induce increased collagen crosslinking resulted in a 27% increase in the collagen crosslinking ratio and a 14% decrease in lumbar bone stiffness.[65] The present study, however, did not have the ability to establish a cause-and-effect relationship between changes in the collagen crosslinking ratio and reduced bone strength. Abnormally high collagen crosslinking has also been observed in diabetic Wistar Bonn/Kobori rats whose femora had diminished mechanical competence,[140] but the results of the present study cannot be explained by diabetes since diabetes was an exclusion criterion and morning blood glucose levels were normal. Misof et al. studied premenopausal women, regardless of BMD, who had fragility fractures and compared them with premenopausal women with low BMD and no fractures.[132] They found an increased collagen crosslinking ratio in subjects with fragility fractures and a significantly lower BMD when subjects with and without fractures were combined and compared with normal controls. The present study separated premenopausal women with fractures into two groups: those with osteoporotic BMD t-scores and those with nonosteoporotic t-scores. The findings showed that an increased collagen crosslinking ratio was associated with the occurrence of low-energy fractures in premenopausal women despite nonosteoporotic BMD that was not significantly different from that in normal controls. Thus, our design controlled for BMD and thereby isolated the effects of alteration in the collagen crosslinking ratio.

The greater collagen crosslinking ratio in the non-low-BMD group cannot be explained by lower bone turnover because turnover did not differ significantly between

the two fracture groups, nor can it be attributed to age because no associations between age and collagen crosslinking were found. A recent study showed an association between chronic hyponatremia and fractures in subjects with nonosteoporotic BMD.[142] Hyponatremia, however, was not observed in non-low-BMD subjects in the present study and there were no differences in serum sodium among the three study groups.

The present study was limited to white women; men and non-white women were excluded to focus on individuals at greatest risk for low-energy fracture. The external validity of these findings will be enhanced by additional data obtained from men and from women of other races.

In conclusion, the key finding of this study confirmed the hypothesis that, in the absence of osteoporotic t-scores, an abnormality in a particular bone quality (the collagen crosslinking ratio) is associated with low-energy fractures in premenopausal women. A finding of nonosteoporotic bone mass with low-energy fractures would justify assessment of bone material quality, which currently requires a bone biopsy. Further studies are needed to search for possible noninvasive tests to diagnose abnormal collagen crosslinking. Since no specific therapies for abnormal collagen crosslinking are available at this time, studies are also needed to explore novel therapeutic modalities to reverse the underlying collagen crosslinking abnormality.

3.6 Acknowledgments

The authors thank Lisa DeGnore, MD, of Kentucky Orthopaedic and Hand Surgeons and Veronica Vasicek, MD, of Bluegrass Orthopaedics and Hand Care for referring patients with low-energy fractures. The authors thank Guodong Wang, MD, for technical assistance in completing the bone histomorphometry. This study was supported by the National Institutes of Health (1RO1AR061578) and the Kentucky Nephrology Research Trust.

3.7 Appendix

Tables comparing properties of bone among the groups (Tables A1.1 and A1.2) and a figure (Figure A1.6) showing a typical FTIR spectrum of bone are available with the online version of this article as a data supplement at jbjs.org. Nanoindentation data were collected in 9 of 18 specimens in the non-low BMD group and in 12 of the controls. No indentations were made in the low-BMD group. The procedure used for nanoindentation is the same procedure used in chapter 2. No significant differences in Young's modulus or hardness were seen between the groups. These data are shown in Table A1.3. Please see the appendix at the end of this dissertation for these tables and the figure.

3.8 Tables and Figures

Table 3.1: Number of Patients with Low-Energy Fractures According to Bone Site

Bone Site	Non-Low BMD Group (N = 25)	Low-BMD Group (N = 18)
Metatarsal	14	3
Tibia	7	1
Femoral neck	6	3
Spine	3	5
Pelvis	3	1
Wrist	2	1
Calcaneus	2	0
Rib	1	3
Forearm	1	1
Talus	1	0
Metacarpal	1	0

Table 3.2: Subject Characteristics and Biochemical Results

	Non-Low BMD (Group 1, N = 25)	P Value, 1 vs. 2	Low-BMD (Group 2, N = 18)	P Value, 2 vs. 3	Controls (Group 3, N = 14)	P Value, 1 vs. 3
BMD, total hip (t-score)	−0.42 ± 0.97	0.001	−2.28 ± 0.79	0.001	−0.67 ± 0.98	>0.1
BMD, lumbar spine (t-score)	−0.53 ± 0.97	0.001	−2.79 ± 0.85	0.001	−0.53 ± 0.92	>0.1
Age (years)	37.2 ± 8.6	>0.1	40.7 ± 9.9	0.001	52.6 ± 3.5	0.001
Serum analysis						
Calcium (mg/dL)	9.43 ± 0.26	>0.1	9.37 ± 0.37	>0.1	9.27 ± 0.39	>0.1
Phosphorus (mg/dL)	3.45 ± 0.54	>0.1	3.54 ± 0.62	>0.1	3.64 ± 0.55	>0.1
Creatinine (mg/dL)	0.77 ± 0.08	>0.1	0.72 ± 0.13	0.074	0.85 ± 0.16	>0.1
Glucose (mg/dL)	88.7 ± 8.4	>0.1	93.7 ± 7.63	>0.1	92.8 ± 10.5	>0.1
Sodium (mmol/L)	139 ± 1.71	>0.1	138 ± 1.95	>0.1	137 ± 4.47	>0.1
Alkaline phosphatase (U/L)	65.3 ± 21.3	>0.1	70.8 ± 25.4	>0.1	91.1 ± 44.4	>0.1
Parathyroid hormone (pg/mL)	31.1 ± 18.5	>0.1	29.7 ± 15.7	>0.1	28.4 ± 10.5	>0.1
Calcidiol (ng/mL)	35.1 ± 11.8	>0.1	36.9 ± 13.7	>0.1	42.6 ± 10.7	>0.1
Bone-specific alkaline phosphatase (µg/L)	14.2 ± 6.61	>0.1	19.9 ± 8.12	0.068	12.1 ± 4.38	>0.1
N-terminal telopeptide (nM bone collagen equivalent)	12.6 ± 6.44	>0.1	14.8 ± 9.22	>0.1	10.8 ± 4.11	>0.1
Osteocalcin (ng/mL)	17.6 ± 8.85	>0.1	20.6 ± 6.17	>0.1	14.7 ± 7.06	>0.1

(mean ± one standard deviation)

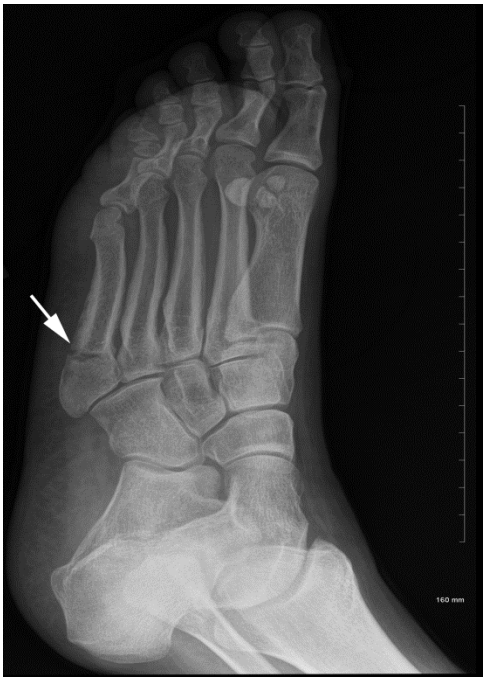


Figure 3.1: Oblique radiograph of a nondisplaced transverse fracture of the proximal fifth metatarsal (arrow) in a premenopausal subject with non-low BMD.

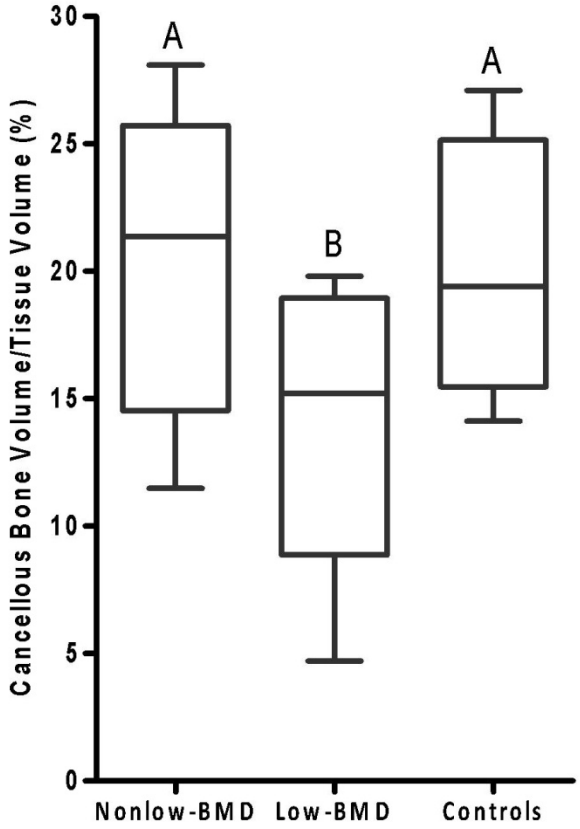


Figure 3.2: Box plots of cancellous bone volume/tissue volume in bone from subjects with non-low BMD (t-score > −2.0) and low-energy fractures, subjects with low-BMD (t-score ≤ −2.5) and low-energy fractures, and healthy volunteers (controls). The bottom and top of the box represent the interquartile range (25% to 75%), the line within the box denotes the median (50%), and the upper and lower bounds of the error bars denote the range. Box plots labeled with the same letters do not differ significantly.

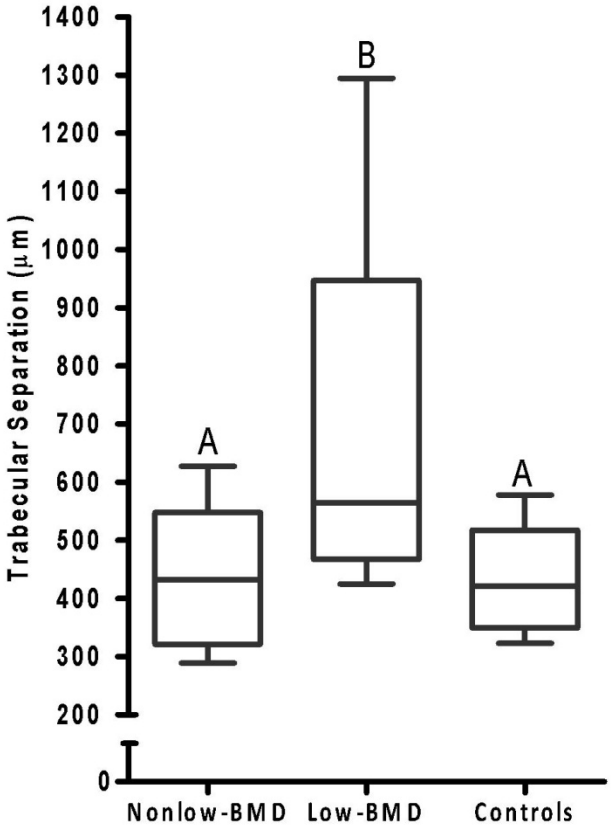


Figure 3.3: Box plots of trabecular separation in bone from subjects with non-low BMD (t-score > −2.0) and low-energy fractures, subjects with low-BMD (t-score ≤ −2.5) and low-energy fractures, and healthy volunteers (controls). The bottom and top of the box represent the interquartile range (25% to 75%), the line within the box denotes the median (50%), and the upper and lower bounds of the error bars denote the range. Box plots labeled with the same letters do not differ significantly.

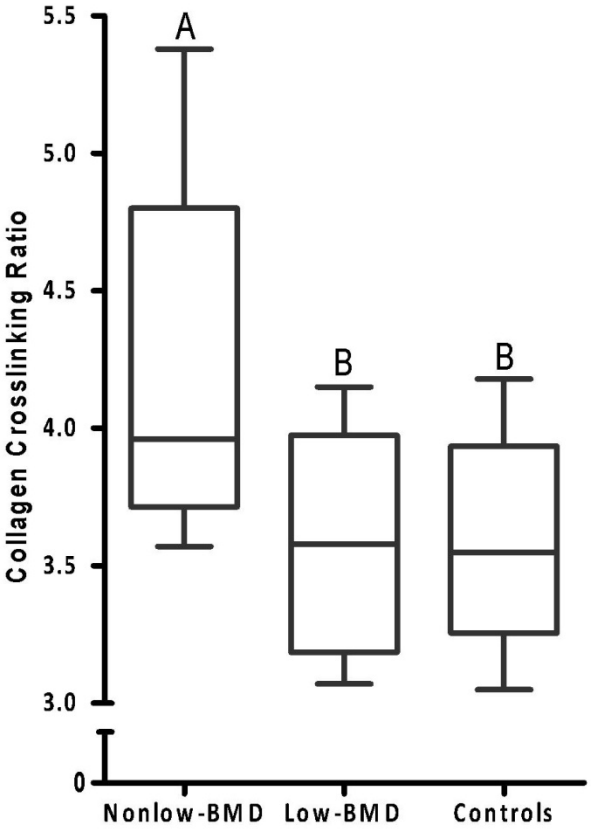


Figure 3.4: Box plots of the collagen crosslinking ratio in bone from subjects with non-low BMD (t-score > −2.0) and low-energy fractures, subjects with low-BMD (t-score ≤ −2.5) and low-energy fractures, and healthy volunteers (controls). The bottom and top of the box represent the interquartile range (25% to 75%), the line within the box denotes the median (50%), and the upper and lower bounds of the error bars denote the range. Box plots labeled with the same letters do not differ significantly.

CHAPTER 4 ALTERATIONS IN BONE MATERIAL QUALITY WITH BISPHOSPHONATE TREATMENT

This chapter was developed based upon an abstract presented at the 2012 Annual Meeting of the Orthopaedic Research Society: Pienkowski D., Porter D.S., Monier-Faugere M.C., Malluche H.H., *Is Bone Quality Altered with Alendronate Treatment in Osteoporotic Patients?* Orthopaedic Research Society Conference, San Francisco, CA, 2012

4.1 Abstract

Bisphosphonates are commonly prescribed in the treatment of osteoporosis; however, prolonged treatment with bisphosphonates may be associated with atypical femoral fractures. These fractures are believed to be due to alterations in bone quality accompanying bisphosphonate treatment. It is unclear whether these alterations in bone quality are due to the suppression of bone turnover associated with bisphosphonate treatment or to the bisphosphonate itself. The goal of this study was to evaluate various parameters of bone quality in iliac crest bone obtained from osteoporotic Caucasian females treated with bisphosphonates for less than five years (short-term, n=14) or greater than or equal to five years (long-term, n=15) compared to bone from turnover-matched untreated osteoporotic Caucasian females (No-BP "controls", n=17). Bone material quality was assessed by using Fourier transform infrared spectroscopy, while bone structural quality and parameters of bone turnover were evaluated by using histomorphometry. The key findings of this study were that the mineral-to-matrix ratio of bone was 11% and 15% higher in the short-term and long-term groups compared to the control group, respectively ($p<0.05$). This finding is important as small deviations in bone mineralization can result in reduced bone toughness. In conclusion, the greater mineral-to-matrix ratio, a parameter of bone material quality, is attributable to the effects of bisphosphonate drug treatment and not the suppression of bone turnover associated with treatment. Further studies are needed to determine when bisphosphonate treatment's positive effect (greater cancellous bone volume) on bone is outweighed by its negative effect (higher mineralization) on bone.

4.2 Introduction

Bisphosphonates are commonly used to treat osteoporosis and at least 4 million American women were prescribed these drugs in 2008.[83] Patients treated with bisphosphonates typically have reduced bone turnover, increase bone mineral density (BMD), and lower fracture risk. The benefit of lower fracture risk has been reported following three to five years of bisphosphonate treatment,[143, 144] while treatment longer than five years was shown to improve BMD, but no additional reduction in fracture risk was observed.[145, 146]

Recently, concerns have arisen that prolonged bisphosphonate treatment may be associated with atypical femoral fractures.[84-89] Long-term treatment with bisphosphonates has been defined as three to five years.[84] A recent report found that 94% of patients who suffered an atypical femoral fracture were treated with a bisphosphonate for five or more years.[85] These atypical femoral fractures are believed to be due to alterations in bone quality associated with long-term bisphosphonate treatment.[147-154] Bone quality consists of as various material and structural parameters that govern bone strength.[23, 25] It has been documented that bisphosphonates suppress bone turnover.[90-92] These alterations in bone quality are often attributed to the suppression of bone turnover associated with bisphosphonate treatment;[147, 149-152] however, it is unclear if these alterations are due to the suppression of bone turnover or to the bisphosphonates itself.[154] Thus, the goal of this study was to determine whether various material and structural parameters of bone quality were altered in osteoporotic Caucasian females treated with various durations of bisphosphonate compared to bone turnover-matched untreated osteoporotic Caucasian females.

4.3 Methods

Study Design and Inclusion/Exclusion Criteria

This study measured parameters of bone quality in iliac crest bone specimens obtained from Caucasian females between the ages of 40 to 80 who underwent a bone biopsy for workup of osteoporosis. Included in this study were bone specimens from Caucasian females that were: diagnosed with low-turnover osteoporosis (low turnover was defined as bone with an activation frequency (Acf.) less than 0.49 $yr.^{-1}$), and met any of the following criteria: a) bisphosphonate treatment less than five years (short-term), b) bisphosphonate treatment greater than or equal to five years (long-term), or c) no bisphosphonate treatment (No-BP).

Subjects were excluded from consideration if they had: osteogenesis imperfecta or other genetic bone disease, osteomalacia, hyperparathyroid bone disease, chronic kidney disease, endocrine abnormalities, Paget's disease of bone, history of drug or alcohol abuse, taken any other bisphosphonate besides alendronate, or prior use of teriparatide. The protocol of this IRB approved cross-sectional study adhered to the Declaration of Helsinki.

Bone Mineral Density

BMD was measured at the lumbar spine (L2-L4) and hip by using a Lunar iDXA (General Electric Inc., Madison, WI). The coefficients of variation of BMD measurements were 1.2% at the spine and 0.9% at the hip.

Biochemistry Parameters

Serum calcium and phosphorus were measured in all subjects by using routine laboratory techniques. In addition, the following laboratory tests were also performed: serum parathyroid hormone (PTH) levels by Total PTH™ radioimmunoassay (Scantibodies, Santee, CA), and serum calcidiol by API 3200 liquid chromatography-tandem mass spectrometry (AB Sciex, Framingham, MA).

Mineralized Bone Histology

Anterior iliac crest bone specimens, obtained after tetracycline double labeling, were processed without mineral removal and embedded in methylmethacrylate. Serial sections of four micron thickness were stained with the modified Masson-Golden Trichrome stain, and seven micron thick unstained sections were prepared for fluorescent and polarized light microscopy.[93]

Histomorphometric Parameters of Bone Structure, Formation, resorption, and Turnover

Histomorphometry was done at standardized sites in cancellous bone to obtain quantitative static parameters of bone structure (cancellous bone volume/tissue volume, trabecular separation, and trabecular thickness), formation (osteoblast surface/bone surface), resorption (osteoclast surface/bone surface) and dynamic parameters of bone turnover (mineralizing surface/bone surface and Acf).[105, 106]

All measurements were performed by using the Osteoplan II System (Kontron, Munich, Germany) at 200x magnification. All measured parameters comply with the nomenclature of the Histomorphometry Committee of the American Society of Bone and Mineral Research.[107]

Parameters of Bone Material Quality

Fourier Transform Infrared spectroscopy (FTIR) was used to measure established parameters of bone material quality.[137, 138] A 4 micron-thick section was cut from each embedded bone sample and placed between two barium fluoride discs. Infrared spectra were collected from these bone specimens by using a microscope attached to a Nexus 670 FTIR spectrometer (Thermo Electron, Waltham, MA, USA) operating in transmission mode for 200 scans at a 4 cm^{-1} resolution. Three randomly selected locations within the center of three randomly selected trabeculae were spectroscopically examined. Trabeculae were evaluated 5 to 7 optical fields (at 200X) below the cortex. Background scans were performed to correct the resulting spectra from influences due to the barium fluoride discs and methylmethacrylate mount.

The following parameters of bone material properties were measured: mineral-to-matrix ratio, carbonate-to-phosphate ratio, crystallinity, and collagen crosslinks. The

mineral-to-matrix ratio was calculated by dividing the area under the phosphate (mineral) peak (900-1200 cm^{-1}) by the area under the amide I (matrix) peak (1590-1720 cm^{-1}) after both peaks were baseline corrected. The carbonate-to-phosphate ratio, a measure of the amount of carbonate substituted within the mineral structure, was calculated from the quotient of the area under the carbonate peak (850-890 cm^{-1}) by the area under the phosphate peak after both peaks were baseline corrected. Crystallinity, a measurement of crystal size and perfection, was calculated from the ratio of the areas under the peaks located at 1020 cm^{-1} and 1030 cm^{-1}.[116] The collagen crosslinking, a measurement of collagen maturation, was calculated from the ratio of the areas under the peaks located at 1660 cm^{-1} (mature crosslinks) and 1690 cm^{-1} (immature crosslinks).[111] Coefficients of variation of these parameters were 4.3% for mineral-to-matrix ratio, 2.0% for carbonate-to-phosphate ratio, 1.7% for crystallinity, and 4.1% for crosslinking ratio.

Data Analyses

Data were tested for normality by using the Kolmogorov-Smirnov test and for equality of variances by using Levene's test. The data were compared by using ANOVA with Scheffe post-hoc correction. Pearson's correlation was used to determine if BMD, age or duration of bisphosphonate treatment correlated with the material or structural parameters of bone. All computations were done by using SPSS version 20 (IBM SPSS Inc., Chicago, IL).

4.4 Results

Patient's Characteristics and Biochemical Parameters

Forty-six osteoporotic subjects met the inclusion criteria but not the exclusion criteria and were categorized into one of the following groups: short-term (n=14), long-term (n=15), and No-BP (n=17) treatment. The mean (± SD) duration of bisphosphonate treatment for the short-term group was 3.4 ± 1.0 years; the mean (+/- SD) duration of bisphosphonate treatment for the long-term group was 8.6 ± 3.0 years. These two groups were different ($p<0.05$). No differences in BMD at the lumbar spine or hip were observed among any of the groups (Table 4.1). Mean patient age in the long-term group was greater than the mean patient age in the No-BP group ($p<0.05$). Biochemical parameters did not differ among the three groups.

Histomorphometric Parameters of Bone Structure, Formation, Resorption, and Turnover

Cancellous bone volume was greater in the short-term group compared to the No-BP group ($p<0.05$, Table 4.2). No other differences were detected in cancellous bone volume between the groups. Trabecular thickness and trabecular separation did not differ among the three groups. No differences in bone formation, resorption, or turnover parameters were observed among the three groups. Duration of treatment, BMD, and age did not correlate with any of these parameters.

Parameters of Bone Material Quality

The short and long-term groups had an 11% and 15% higher mineral-to-matrix ratio compared to the No-BP group, respectively ($p<0.05$, Table 4.3). The mineral-to-matrix ratio was not different between the short- and long-term groups. No other differences in bone material parameters were observed. Duration of treatment, BMD, and age did not correlate with any bone material quality parameters.

4.5 Discussion

The key finding of this study is that bisphosphonate treatment is associated with altered bone quality independent of bone turnover. Specifically, mineral-to-matrix ratio was higher in the bisphosphonate treated groups compared to the No-BP group. This finding also cannot be attributed to age or duration of treatment as no correlations were observed.

Relative bone mineralization has an important role in determining bone strength given that small deviations from ideal bone mineralization have been associated with a reduction in bone toughness[53-55, 60] Thus, the observed greater mineral-to-matrix ratio may help explain the increased susceptibility of atypical femoral fractures in the patients treated with bisphosphonates.

The higher mineral-to-matrix ratio has previously been reported after one year of bisphosphonate treatment in canines,[147, 151] and in patients taking alendronate for three years.[150] Another study reported higher bone mineralization, as measured by BMD, in patients treated with bisphosphonates between 3 to 10 years compared to untreated postmenopausal women.[152] These studies, however, conclude that the higher bone mineralization is due to the suppression of bone turnover, while the current study concludes that the altered bone mineralization is due to the bisphosphonates itself. The discrepancy between conclusions may be explained by differences in study goals and designs; the current study matched for bone turnover in the untreated osteoporotic group.

No alterations in enzymatic collagen crosslinks due to bisphosphonates were observed in this study. This finding agrees with a prior study of bone from patients that were taking alendronate for three years.[150] This is further supported by a study, which found that osteoporotic women treated with bisphosphonates maintained, but did not increase, collagen crosslinks.[155] In contrast, a few studies have found higher collagen crosslinks in canines and humans treated with bisphosphonates.[151, 153] Like collagen crosslinks, there is conflicting evidence regarding the role of bisphosphonates on crystallinity. The current study found no difference in crystallinity, which also agrees with the prior study where patients were taking Alendronate for three years.[150] Greater crystallinity, however, has been reported in canines that were administered bisphosphonates.[147] One study found that crystallinity was lower in patients treated with alendronate for 8 years.[156] The exact role bisphosphonates have on collagen crosslinks and crystallinity are unclear, and this issue warrants further study as both of these material quality parameters influence bone strength.[61-64, 67]

This study is limited to patients who only took alendronate. Further studies are needed to determine if the higher mineral-to-matrix ratio observation in patients taking alendronate treatment will occur in patients taking bisphosphonates other than alendronate.

In conclusion, the higher mineral-to-matrix ratio, a parameter of bone material quality, was due to the bisphosphonate itself and not due to the suppression of bone turnover associated with treatment. The key finding from this study adds more evidence to the idea that bisphosphonate treatment has both a positive (greater cancellous bone volume) and negative (higher mineralization) effect on bone and may lead to changes in the current practice paradigms regarding bisphosphonate treatment with osteoporosis.

4.6 Acknowledgments

The authors thank Guodong Wang, MD, for technical assistance in completing the bone histomorphometry.

4.7 Appendix

Nanoindentation data were collected in 9 of 17 specimens in the No-BP group, 8 of 14 specimens in the short-term group, and 8 of the 15 specimens in the long-term group. The procedure used for nanoindentation is the same as that used in chapter 2. No significant differences in Young's modulus or hardness were seen between these groups. These data are shown in Table A1.4. Please see the appendix at the end of this dissertation for this table.

4.8 Tables:

Table 4.1: Patient's Characteristics and Biochemical Parameters

	No-BP (n=17)	Short-Term (n=14)	Long-Term (n=15)
Patient's Characteristics			
BMD Total Hip (t-score)	-1.72 ± 1.24	-1.98 ± 0.91	-1.58 ± 0.79
BMD Lumbar Spine (t-score)	-2.30 ± 1.31	-2.16 ± 1.19	-1.89 ± 1.34
Age (years)	56.2 ± 9.4	62.4 ± 6.8	63.3 ± 3.8[a]
Biochemical Parameters			
Serum Calcium (mg/dL)	9.56 ± 0.49	9.52 ± 0.37	9.55 ± 0.44
Serum Phosphorus (mg/dL)	3.53 ± 0.59	3.96 ± 0.48	3.50 ± 0.56
Serum Parathyroid Hormone (pg/mL)	32.8 ± 17.9	37.7 ± 9.44	34.9 ± 7.33
Serum Calcidiol (ng/mL)	40.6 ± 12.5	37.9 ± 20.0	27.1 ± 13.0

(mean ± one standard deviation)

a = $p<0.05$ vs. No-BP

Table 4.2: Histomorphometric Parameters of Bone Structure, Formation, Resorption, & Turnover

	No-BP (n=17)	Short-Term (n=14)	Long-Term (n=15)
<u>Bone Structure</u>			
Cancellous Bone Volume/ Tissue Volume (%)	15.3 ± 3.25	20.0 ± 2.99[a]	18.3 ± 7.75
Trabecular Thickness (µm)	102 ± 28.5	111 ± 25.8	110 ± 38.0
Trabecular Separation (µm)	551 ± 116	453 ± 113	564 ± 314
<u>Bone Formation and Resorption</u>			
Osteoblast Surface/ Bone Surface (%)	0.66 ± 0.73	0.51 ± 0.66	0.22 ± 0.21
Osteoclast Surface/ Bone Surface (%)	0.41 ± 0.24	0.75 ± 0.52	0.76 ± 0.78
<u>Bone Turnover</u>			
Mineralizing Surface/ Bone Surface (%)	2.00 ± 1.29	1.82 ± 1.61	2.62 ± 1.50
Activation Frequency (yr^{-1})	0.13 ± 0.10	0.13 ± 0.14	0.18 ± 0.14

(mean ± one standard deviation)

a = $p<0.05$ vs. No-BP

Table 4.3: Parameters of Bone Material Quality

	No-BP (n=17)	Short-Term (n=14)	Long-Term (n=15)
Mineral-to-Matrix Ratio	3.63 ± 0.40	4.03 ± 0.47[a]	4.16 ± 0.38[a]
Carbonate-to-Phosphate Ratio (x100)	1.07 ± 0.14	1.09 ± 0.14	1.10 ± 0.13
Crystallinity	0.90 ± 0.07	0.94 ± 0.05	0.94 ± 0.08
Collagen Crosslinking	3.29 ± 0.36	3.46 ± 0.40	3.31 ± 0.31

(mean ± one standard deviation)

a = $p<0.05$ vs. No-BP

CHAPTER 5 ALTERATIONS IN THE INTRINSC MECHANICAL PROPERTIES OF BONE WITH BISPHOSPHONATE TREATMENT

This chapter will be revised and submitted for publication.

5.1 Abstract

Bisphosphonates are commonly prescribed for the treatment of osteoporosis; however, prolonged bisphosphonate treatment may be associated with atypical femoral fractures. These fractures may be due to alterations in bone quality. The goal of this study was to determine if the duration of bisphosphonate treatment was associated with changes in the intrinsic mechanical properties (Young's modulus and hardness) of bone as measured by nanoindentation. Ninety-two iliac crest bone specimens from Caucasian females treated with bisphosphonates were included. Mean patient age was 60.5 ± 8.8 years (±SD) and mean duration of bisphosphonate treatment was 6.0 ± 2.9 years (±SD). Bisphosphonate treatment type and patient age were unrelated to Young's modulus or hardness of cortical or trabecular bone. Significant positive linear relationships were observed between the intrinsic mechanical properties of bone and duration of treatment in trabecular and cortical bone ($p<0.05$). Based upon an animal study showing the relationship between modulus and fracture toughness, these results may provide insight regarding why patients with prolonged bisphosphonate treatment suffer atypical femoral fractures.

5.2 Introduction

Bisphosphonates are commonly used in the treatment of osteoporosis. In 2008, approximately 4 million American women were prescribed bisphosphonates to treat osteoporosis.[83] Patients treated with bisphosphonates typically have reduced bone turnover, increased bone mineral density (BMD), and lower fracture risk. The benefit of lower fracture risk has been reported after three to five years of bisphosphonate treatment,[143, 144] while treatment longer than five years was shown to improve BMD, but no additional reduction in fracture risk was observed.[145, 146]

Recently, concerns have arisen that long-term bisphosphonate treatment may be associated with an increased fracture risk as manifested by "atypical" femoral fractures.[84-89] An ASBMR task force report found that 94% of patients who suffered an atypical femoral fracture were treated with a bisphosphonates for five years or longer.[85]

These atypical femoral fractures are believed to be due to alterations in bone quality associated with bisphosphonate treatment.[147-154] Bone quality is defined by various material, microdamage, and structural parameters that collectively result in bone's ability to resist fracture.[23, 25] These alterations in material, microdamage, or structural parameters may result in changes to the extrinsic and intrinsic mechanical properties of bone. Nanoindentation has been used to measure the intrinsic mechanical properties of bone (Young's modulus and hardness) that comprise a portion of the material parameters of bone quality.[119] The goal of this study was to determine if Young's modulus and hardness of bone varied with the duration of bisphosphonate treatment.

5.3 Methods

Ninety-two iliac crest bone specimens from Caucasian females treated with bisphosphonates were included in this study. Specimens were excluded if they had osteogenesis imperfecta, osteomalacia, Paget's disease of bone, history of drug or alcohol abuse, or prior use of teriparatide. The protocol of this IRB approved cross-sectional study adhered to the Declaration of Helsinki.

Nanoindentation

The surface of each biopsy was polished and made uniplanar by sanding on a metallographic specimen preparation station holding abrasive silicon carbide papers of decreasing grit size (ending in 1200 grit). A final high polish was achieved by using a rotating microcloth wetted with deionized water in which diamond particles (0.3-µm grit size and then 0.05-µm grit size) were suspended. Finally, specimens were placed in an ultrasonic water bath for 10 minutes to remove surface debris.

The Young's modulus and hardness of cortical and trabecular bone were quantified using established nanoindentation techniques.[44–47] This process was done by using a microscope-equipped Nanoindenter G200 (Agilent, Oak Ridge, TN). The indenter was stationed on an antivibration table located within an isolation cabinet to reduce the

potential for environmentally generated mechanical interference. A three-sided tip (Berkovich diamond indenter) was used for specimen indentation. The nanoindenter was calibrated by indenting fused silica of known modulus. Young's modulus and hardness were measured at 6 standardized cortical and 6 standardized trabecular sites on each sample. Five indents were done at each site resulting in 60 measurements per sample.

Nanoindentation was performed by applying a peak load of 8 mN during each indentation at a constant loading rate of 0.4 mN/second^{-1}. The maximum load achieved during each indent was maintained for 10 s (hold time) to ensure that the subsequent unloading would be completely elastic.[44,45] Based on the first 50% of the unloading curve, stiffness and hardness were quantified by using the Oliver and Pharr method.[48]

Data Analyses

The data were analyzed by using PROC general linear model in SAS (version 5.1) using linear mixed models that adjusted for the covariates of age and treatment group. The linear fit was tested for the lack of fit. Residuals were tested for non-normality by using the Kolmogorov-Smirnov test.

5.4 Results

The mean age of the patients was 60.5 ± 8.8 years (±SD) and the mean duration of bisphosphonate treatment was 6.0 ± 2.9 years (±SD). The range of treatment duration was 1.1 to 14 years. Although various bisphosphonates used were used by these patients (Table 1), the type of bisphosphonate and patient age were unrelated to Young's modulus or hardness of cortical or cancellous bone.

Overall, Young's modulus and hardness increased with increasing bisphosphonate treatment duration. Specifically, in trabecular bone, significant positive linear relationships were observed between Young's modulus ($p<0.01$, $r^2=0.09$, figure 1) and hardness ($p<0.01$, $r^2=0.13$, figure 2) with the duration of treatment. Similarly, significant positive linear relationships between Young's modulus ($p<0.05$, $r^2=0.09$, figure 3) and hardness ($p<0.01$, $r^2=0.18$, figure 4) with duration of treatment were observed in cortical bone.

5.5 Discussion

The key findings from this study are the significant positive relationships between the duration of bisphosphonate treatment and the nano-scale intrinsic mechanical properties of cortical and trabecular bone. It has been documented that bisphosphonates suppress bone turnover.[90-92] The current finding is relevant because reduced bone turnover is associated with greater bone mineralization.[157-159] Higher bone mineralization was previously reported after one year of bisphosphonate treatment in canines,[147, 151] and in patients taking alendronate for three years.[150] Moreover, greater bone mineralization is associated with increases in Young's modulus.[59, 158, 160] Mineralization was positively correlated to Young's modulus of

various animal bones; it was also observed in this model that small deviations in mineralization had a negative effect on bone toughness.[53]

A few studies have looked at bisphosphonate treatment and bone's intrinsic mechanical properties as measured by nanoindentation. A study done by Tjhia *et al*., found higher mean values for Young's modulus and hardness of trabecular bone in twelve patients treated with bisphosphonates who suffered an atypical femoral fracture compared to eleven age-matched untreated osteoporosis patients.[154] The patients in this study were taking bisphosphonates for at least 3 years, but the exact duration was not given. Another study found that patients treated with alendronate had a lower Young's modulus than untreated age-matched osteoporosis women.[153] These patients were treated for an average of 8 ± 2 years. This study had only five treated patients versus six untreated patients. These studies have conflicting results with regard to bisphosphonate treatment and its effect on intrinsic mechanical properties of bone. The conflicting results may be due to the potential difference in the duration of treatment between the studies. Additionally, these studies could not determine if the duration of treatment influenced the intrinsic properties of bone since it had a limited number of samples in each group.

The cross-sectional design of the present study and consequent lack of baseline information limits the information obtainable from the data.

In conclusion, increases in nanoscale-derived mechanical bone quality parameters (Young's modulus and hardness) are associated with increasing duration of bisphosphonate treatment. Given the relationship between Young's modulus and bone toughness shown in a previously published animal study, the current findings may provide more information regarding atypical femoral fractures that occur with prolonged bisphosphonate use.

5.6 Acknowledgments

The authors thank Y.T Cheng PhD, for access to Agilent G200 nanoindenter. This study was supported by the National Institutes of Health (RO1AR061578).

5.7 Tables:

Table 5.1: Number of patients versus bisphosphonate type

Bisphosphonate	# of patients
Actonel	10
Boniva	2
Fosamax	56
Multiple	23
Pamidronate	1
Total	92

5.8 Figures

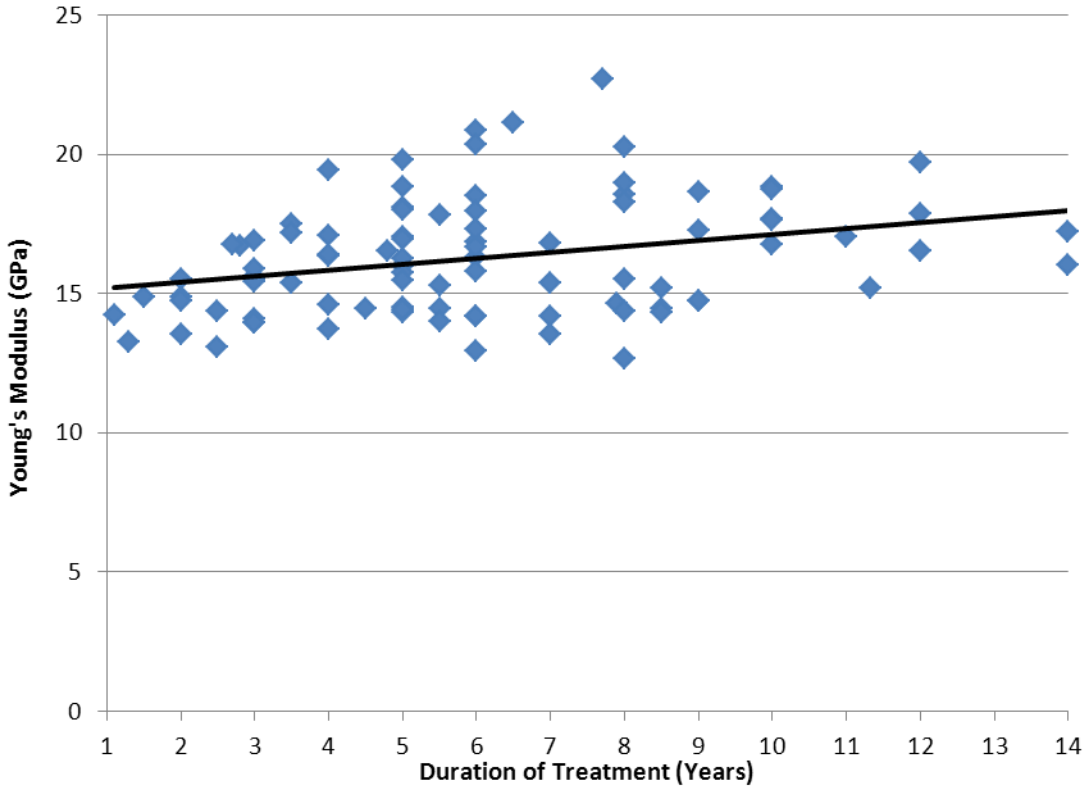


Figure 5.1: Linear relationship between duration of treatment and Young's modulus of trabecular bone ($p<0.05$, $r^2= 0.09$).

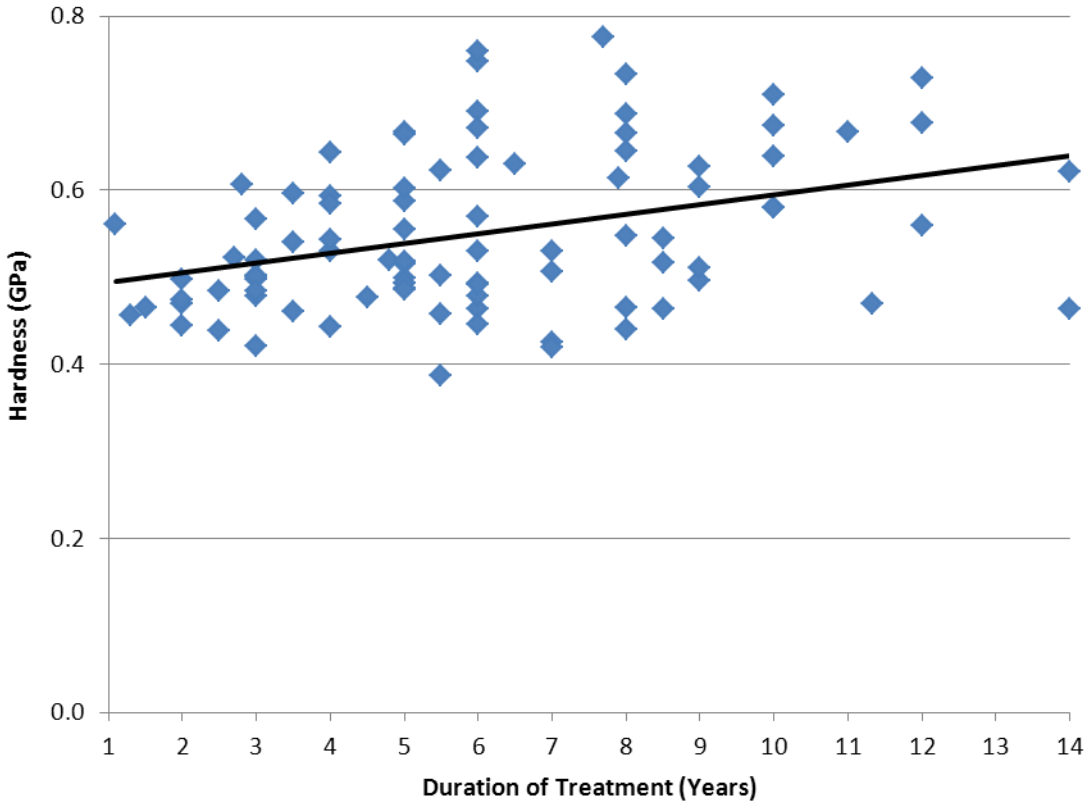


Figure 5.2: Linear relationship between duration of treatment and hardness of trabecular bone ($p<0.05$, $r^2= 0.13$).

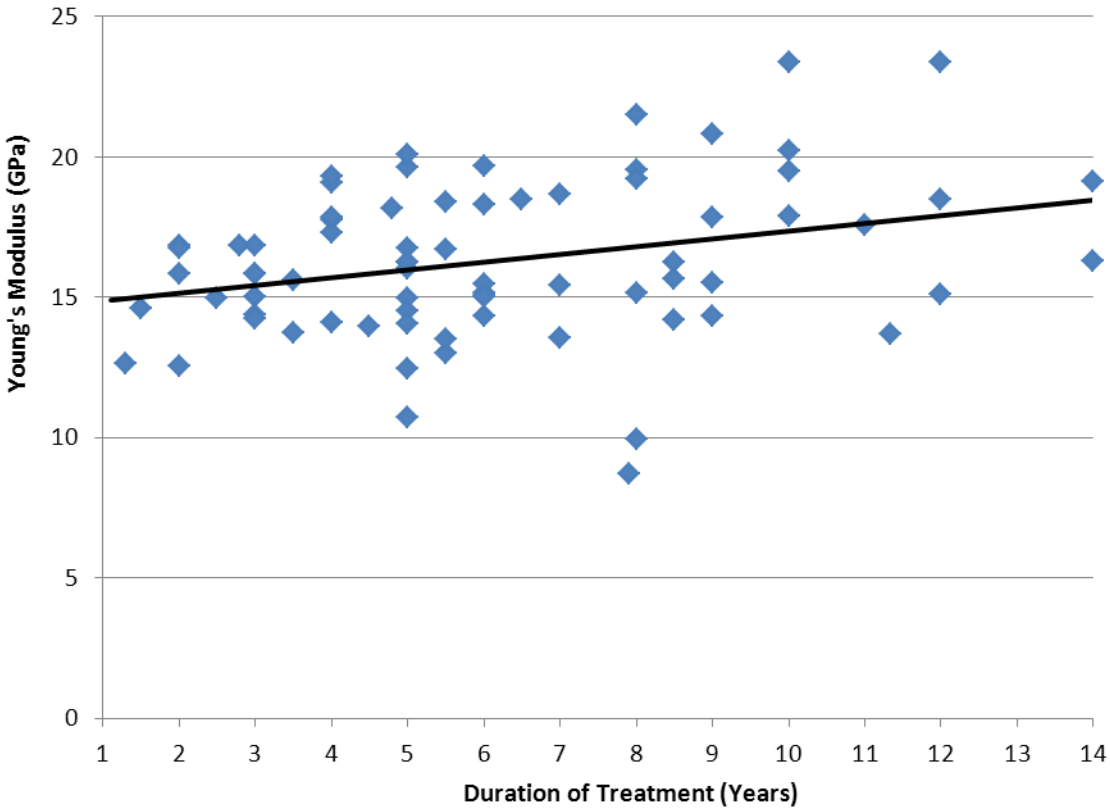


Figure 5.3: Linear relationship between duration of treatment and Young's modulus of cortical bone ($p<0.05$, $r^2= 0.09$).

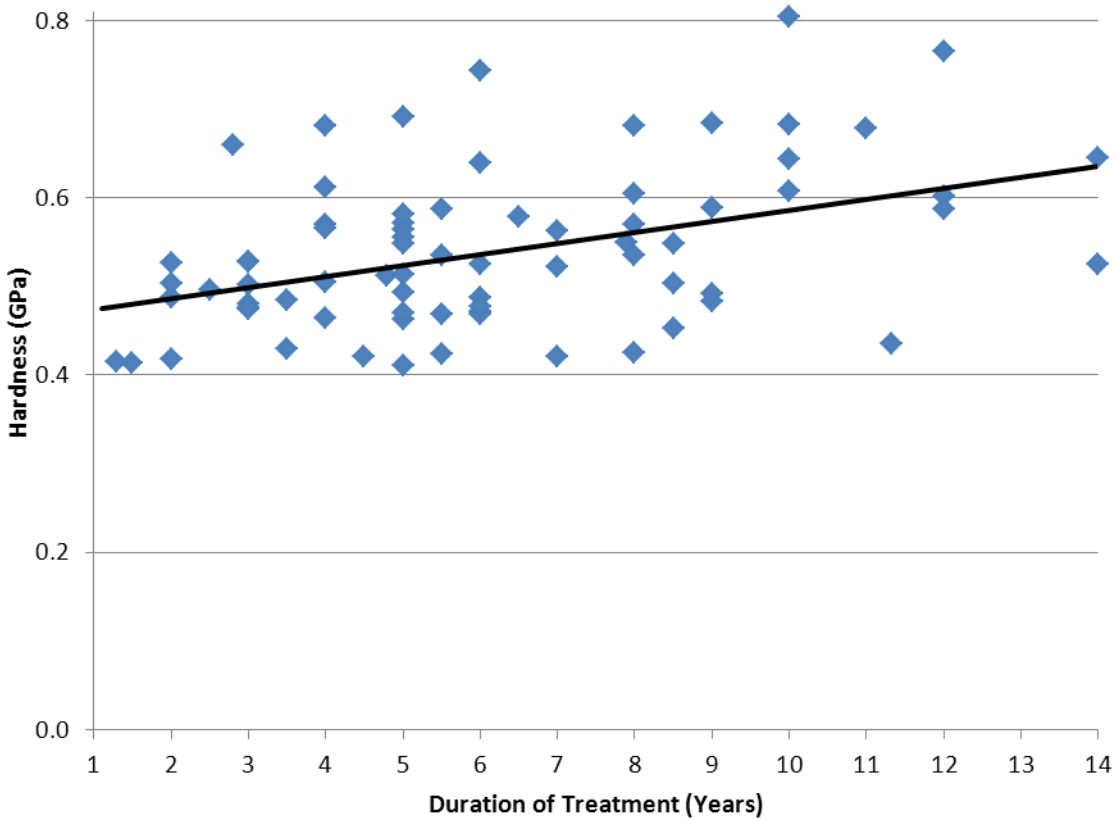


Figure 5.4: Linear relationship between duration of treatment and hardness of cortical bone ($p<0.05$, $r^2= 0.18$).

CHAPTER 6 CONCLUDING REMARKS

6.1: Summarized Key Findings

The objective of this dissertation research was to examine whether various bone quality parameters were altered in iliac crest bone specimens obtained from patients who: 1) had abnormal bone turnover due to CKD, 2) suffered a low-energy fracture despite normal BMD, or 3) had osteoporosis and were treated with bisphosphonates. The key finding from the bone turnover study (Chapter 2) was that high and low bone turnover due to CKD altered bone quality. These alterations, however, are turnover specific. High turnover had a lower mineral-to-matrix ratio and Young's modulus compared to normal and low turnover, while low turnover had a lower cancellous bone volume and trabecular thickness compared to normal or high turnover. In the fracture study (Chapter 3) the key finding was that patients with non-low-BMD and low-energy fractures had a greater collagen crosslinking ratio compared to patients who had low-BMD with low-energy fractures and controls. The main result from the bisphosphonate study (Chapter 4) was that bisphosphonate treatment resulted in a greater mineral-to-matrix ratio compared to untreated turnover matched osteoporotic patients. Last, the main results in nanoindentation bisphosphonate study (Chapter 5) were the multiple significant positive linear relationships between bisphosphonate treatment and the intrinsic mechanical properties of bone.

6.2 Importance of the Key Findings

The alterations in bone quality documented in chapters 2 through 4 have been linked to a reduction in bone's mechanical properties; specifically deviations from ideal bone mineralization have been associated with reduced load to failure as shown in Figure 1.5.[60] The observed alteration in the collagen crosslinks is important because the crosslinks are believed to play a key role in bone's tensile strength and the post-yield mechanical properties.[67-69, 75] In rats, greater collagen crosslinking is associated with lower mechanical properties in bone.[65, 140] The lower cancellous bone volume correlates to lower yield stress and bone strength,[43, 45] while the loss of structural integrity (trabecular thickness and separation) is associated with a reduced bone strength.[40, 41, 43] In chapter 5, the duration of bisphosphonate treatment was associated with changes to the intrinsic mechanical properties of bone. These changes in bone's mechanical properties may help explain the increased fracture risk seen in patients with abnormal bone turnover due to CKD, patients with normal BMD and fracture, and the atypical fracture risks seen in patients taking bisphosphonates for prolonged periods.

6.3 Discussion

Currently, bone quantity (as measured by DXA) is most commonly used to determine the effectiveness of various treatment plans for osteoporosis and to predict a patients' lifetime risk of fracture; however, bone quantity does not fully explain bone's ability to resist fracture. Thus, it is important to measure both the quantity and quality of bone, as it is possible that a BMD scan will miss potential changes in bone quality.[161] For example, figure 6.1 shows that despite no changes in bone quantity there were

increases in the outer diameter of bone (macro-architecture parameter of bone quality) resulting in greater bending strength (section modulus).[162]

6.3.1 Bone Turnover

Knowing the rate of bone turnover is important as the results from the turnover study showed that abnormalities in bone quality were turnover dependent. This finding suggests the need for different treatments based on the type of bone turnover. In the case of high turnover bisphosphonates (as long as the glomerular filtration rate is above 35 ml/min/1.73m^2), denosumab, calcimimetics (drugs which mimic the action of calcium on tissue), 1,25-dihydroxyvitamin D, or Vitamin D receptor analogs can be used to reduce turnover. In the case of low bone turnover, teriparatide can be used to elevate turnover. Like CKD, bone turnover in osteoporosis can be classified as either high or low bone turnover, and different treatments based on the type of bone turnover should be considered. Currently, most patients with osteoporosis are treated with bisphosphonates regardless of the rate of bone turnover. It is possible that giving a bisphosphonate to a patient with low turnover will not stop bone loss or restore bone quantity. This is because bisphosphonates reduce osteoclast activity; in low turnover bone, the osteoclast activity is already reduced. In addition, giving bisphosphonates to patients with low turnover bone may result in other bone quality issues such as a greater accumulation of microdamage. An alternative to bisphosphonates for patients with low turnover bone is teriparatide treatment as it increases osteoblast activity and can lead to new bone formation. Studies are needed to determine if these different treatment paradigms will alleviate the bone quality abnormalities associated with high and low turnover and to make sure that these treatment plans do not result in other complications that reduce bone's ability to resist fracture.

6.3.2 Collagen Crosslinking

In the fracture study, a collagen crosslinking abnormality was not observed by a DXA scan. This crosslinking abnormality may help explain the lower mechanical competency of the bones in these patients. A possible explanation for the greater enzymatic collagen crosslinking in the non-low-BMD group is the inhibition of lysyl oxidase (LOX). Inhibition of LOX can be due to copper deficiency, Vitamin B6 deficiency, hyperhomocysteinemia (HHCY) and the chemical compound β-aminopropionitrile (β-APN). This inhibition LOX can lead to osteolathyrism, which is a collagen crosslinking deficiency. These LOX inhibitors have all been associated with a reduction in enzymatic collagen crosslinks or a greater mature to immature crosslinks ratio. Specifically administration of β-APN in rats resulted in higher mature to immature collagen crosslinking ratio and lower bone strength without affecting the mineral component.[65] Another study found that rats given β-APN for 4 weeks had a 45% reduction of mature crosslinks and 26% and 30% reduction in bending strength and Young's modulus respectively compared to control rats.[163] Copper deficiency in chickens resulted in lower amount of enzymatic collagen crosslinks[164] and lower torsional strength with a lack of plastic deformation.[66, 165] Vitamin B6 deficient rats had a 25% decrease in immature collagen crosslinking formation compared to rats feed a regular diet.[166] One

study found that elevated homocysteinemia in individuals resulted in lower concentrations of enzymatic collagen crosslinks and a greater amount of non-enzymatic crosslinks.[167] The main reasons for HHCY are deficiencies in Vitamin B6, Vitamin B12, or folic acid along with aging and kidney failure.[168] It must be noted while inhibition of lysyl oxidase is associated with lathyrism and collagen abnormalities, overexpression is linked to metastasis.[169] Future studies should investigate whether eliminating any of the previous discussed LOX inhibitors will restore the collagen crosslinking ratio to normal. Additionally, future studies should also examine non-enzymatic crosslinks, as increases in these crosslinks have been associated with alterations in bones mechanical properties. Greater amounts of non-enzymatic collagen crosslinking have been associated with aging, CKD and diabetes.[67, 170]

6.3.3 Bisphosphonates and Bone Quality

Finally, in the bisphosphonate studies the initial gains in bone quantity might be offset by changes in bone quality that may have greater negative effect on bone's ability to resist fracture. Initially, with bisphosphonate treatment there is an increase in bone quantity (as measured by DXA), which has a positive benefit on bone's ability to resist fracture; however, this benefit has been shown to stabilize after about five years. [145, 146] The results from chapters 4 and 5 showed that bisphosphonate treatment reduces bone quality (via an abnormal mineral-to-matrix ratio) and thus diminishes the intrinsic mechanical properties of bone. Thus, it is possible to theorize that with longer duration of bisphosphonate treatments that bone quality abnormalities will have a greater negative effect on bone quality than the initial positive gains in bone quantity. This theory is illustrated in figure 6.2. This greater negative effect on bone quality might help explain the atypical fractures associated with prolonged bisphosphonate treatment.

6.3.4 Beyond A BMD Scan

To make bone quality a part of the everyday clinical workup, other methods beyond BMD scan are needed. In this dissertation, iliac crest bone biopsies were analyzed by histomorphometric analysis, FTIR, and nanoindentation. These methods allow for measurements of bone turnover and bone quality's microstructural, material, and mechanical parameters. Recently, a few other studies have used finite element analysis (FEA) to measure bone's mechanical properties[171] and histomorphometric analysis to measure the microdamage in bone.[172] Applications of these methods in the everyday clinical work-up will allow for a more complete assessment of bone quality.

Future studies, however, should continue to look for non-invasive methods to measure bone quality. One such method is high-resolution peripheral quantitative computer tomography (HR-pQCT), which has been used to measure macro and micro-structural bone quality parameters in patients with CKD and osteoporosis.[173, 174] FEA has also been applied to the 3-D images obtained from the HR-pQCT to measure bone's mechanical properties.[174] There is, however, a drawback to HR-pQCT as it tells us nothing about the rate of bone turnover.

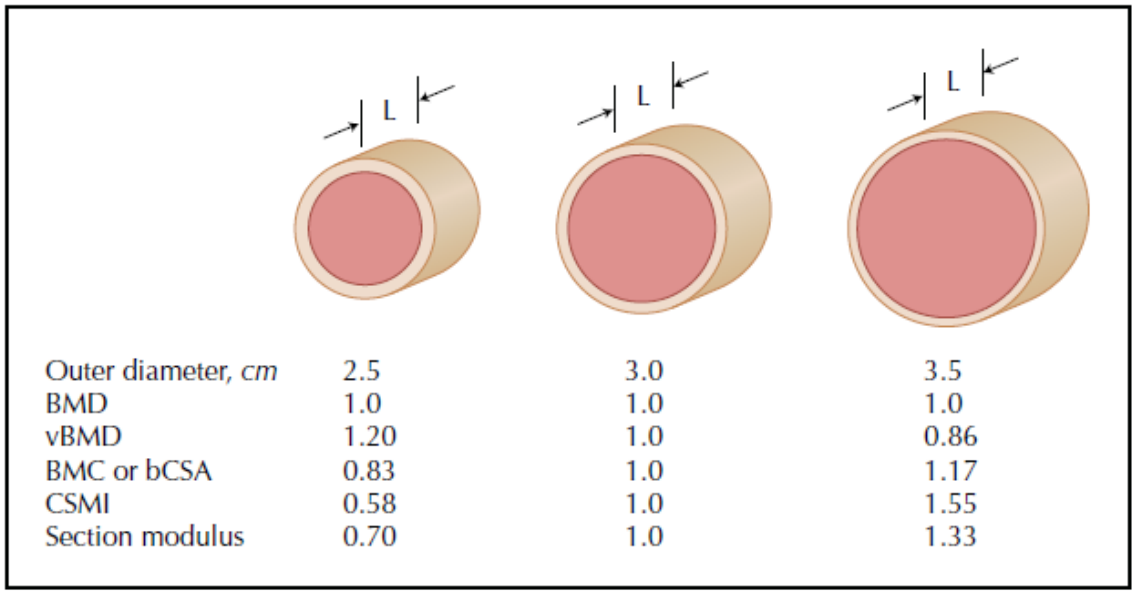
6.3.5 The Goldilocks Effect

As stated earlier, the Goldilocks effect is when something must fall within a certain range, as opposed to reaching extremes. The results from this study suggest that bone needs an ideal amount of bone mineral, collagen crosslinks, and microarchitecture parameters to ensure that bone has the optimal ability to resist fracture. This effect is seen with bone mineralization as described earlier in section 1.3.2 (bone's material parameters) and is shown in figure 1.5.[60]

Ideal microarchitecture and bone quantity parameters are thought to be important as too little bone, excess separation, and insufficient trabecular thickness have all been associated with lower mechanical properties. Thus, it may seem optimal to have bone with extremely thick trabecular bone, or very little trabecular separation, which will result in a greater trabecular bone volume. This, however, would not be ideal as too much bone may result in alterations in serum calcium and phosphorus, as more calcium and phosphorus will be stored in the bone. Additionally, too much bone as seen in Sclerosing bone dysplasias can result in severe functional limitation; extensive pain; malformed or immobilized muscles, tendons or ligaments; and limb, and hand or foot deformity.[175]

6.4 Conclusions

In conclusion, both bone turnover and bisphosphonates altered bone quality; and the fractures seen in patients with normal BMD may be explained by a bone quality abnormality. The findings in this dissertation research highlight how alterations in the material and microstructural parameters of bone quality may help explain the overall decrease in bone's ability to resist fracture in these pathologies beyond bone quantity.



Outer diameter, *cm*	2.5	3.0	3.5
BMD	1.0	1.0	1.0
vBMD	1.20	1.0	0.86
BMC or bCSA	0.83	1.0	1.17
CSMI	0.58	1.0	1.55
Section modulus	0.70	1.0	1.33

Figure 6.1: Scale drawing of three cylindrical cross-sections with different outer diameters, fixed region length (L), but equivalent areal bone mineral density (BMD). Also shown are the corresponding (relative) values of volumetric BMD (vBMD), bone mineral content (BMC), the cross-sectional moment of inertia (CSMI), and the section modulus. BMC is not equivalent to bCSA (cross-sectional area excluding spaces occupied by soft tissue), but in a cross-section they scale linearly.[162]

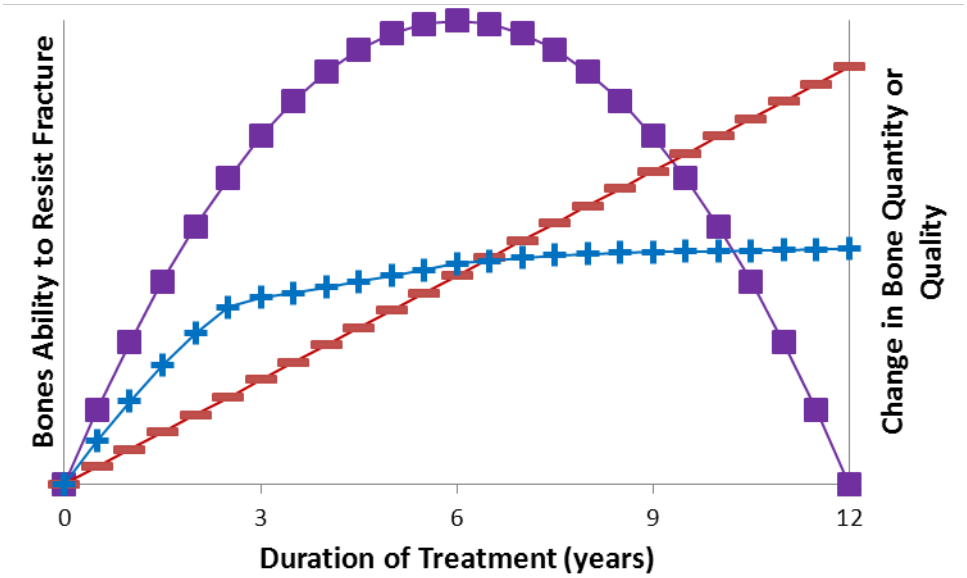


Figure 6.2: Postulated ability of bone to resist fracture as a function of the duration of bisphosphonate treatment and changes in bone quality.

Legend: + = Bone Quantity; - = Bone Quality (Mineral-to-Matrix Ratio);

■ = Bone's Ability to Resist Fracture.

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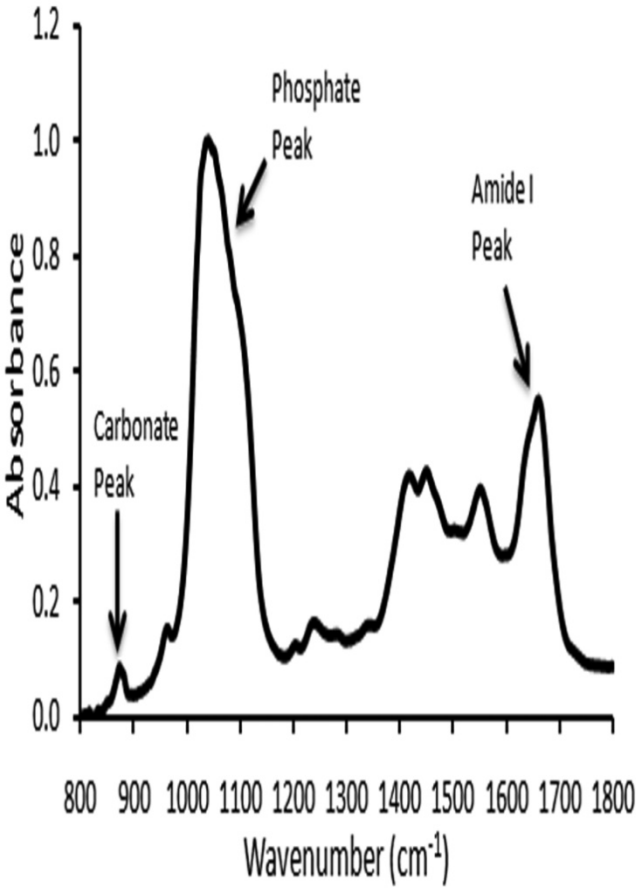

Figure A1.6: Typical FTIR spectrum of bone. Spectra were analyzed with use of the carbonate peak (indicating carbonate substitution into hydroxyapatite) between 850 and 890 cm^{-1}, the phosphate peak (mineral) between 900 and 1200 cm^{-1}, and the amide I peak (matrix) between 1590 and 1720 cm^{-1}.

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Table A1.1: Parameters of Bone Structure, Microarchitecture, Turnover, and Mineralization

	Non-Low-BMD (Group 1, N = 25)	P Value, 1 vs. 2	Low-BMD (Group 2, N = 18)	P Value, 2 vs. 3	Controls (Group 3, N = 14)	P Value, 1 vs. 3
Cancellous bone volume/tissue volume (%)	20.9 ± 4.41	0.001	14.9 ± 4.13	0.01	20.1 ± 4.12	>0.1
Trabecular separation (µm)	429 ± 86.3	0.001	620 ± 242	0.01	428 ± 69.1	>0.1
Trabecular thickness (µm)	114 ± 23.4	>0.1	100 ± 17.8	>0.1	107 ± 23.9	>0.1
Bone formation rate/bone surface area ($mm^3/cm^2/yr$)	1.34 ± 0.98	>0.1	1.41 ± 1.33	>0.1	1.97 ± 0.99	>0.1
Osteoid thickness (µm)	10.3 ± 4.23	>0.1	9.73 ± 3.87	>0.1	9.08 ± 3.49	>0.1
Mineralization lag time (d)	40.3 ± 39.3	>0.1	31.2 ± 16.1	>0.1	47.0 ± 29.8	>0.1

(mean ± one standard deviation)

Table A1.2: Parameters of Bone Mineral Quality

	Non-Low-BMD (Group 1, N = 25)*	P Value, 1 vs. 2	Low-BMD (Group 2, N = 18)*	P Value, 2 vs. 3	Controls (Group 3, N = 14)*	P Value, 1 vs. 3
Collagen crosslinking ratio	4.12 ± 0.46	<0.001	3.58 ± 0.33	>0.1	3.60 ± 0.30	<0.001
Mineral-to-matrix ratio	4.16 ± 0.39	>0.1	3.93 ± 0.61	>0.1	3.83 ± 0.44	>0.1
Carbonate-to-phosphate ratio × 100	1.04 ± 0.08	>0.1	1.03 ± 0.13	>0.1	1.09 ± 0.08	>0.1
Crystallinity	0.88 ± 0.04	>0.1	0.88 ± 0.08	>0.1	0.89 ± 0.03	>0.1

(mean ± one standard deviation)

Table A1.3: Bone's mechanical properties in the non-low BMD group versus controls.

	Non-Low BMD (n=9)	Controls (n=12)
Young's modulus (GPa)	16.0 ± 1.94	15.2 ±1.30
Hardness (GPa)	0.62 ± 0.08	0.56 ± 0.06

(mean ± one standard deviation)

Table A1.4: Bone's mechanical properties as function of bisphosphonate treatment

	No BP (n=9)	Short-Term (n=8)	Long-Term (n=8)
Young's modulus (GPa)	13.3 ± 1.4	14.5 ± 1.4	14.1 ± 1.4
Hardness (GPa)	0.57 ± 0.04	0.59 ± 0.06	0.58 ± 0.02

(mean ± one standard deviation)

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VITA

Daniel Shaw Porter

Education:

University of Kentucky (UK) Lexington, KY

- PhD Biomedical Engineering GPA 3.5/4.0
  PhD Candidate: Post qualifying exams completed on 5/2/2006
  Dissertation: The Effect of Various Pathologies on Bone Quality
  Advisor: Dr. David Pienkowski

West Virginia University (WVU) Morgantown, WV

- Master of Science Mechanical Engineering June 2004 GPA 3.1/4.0
  Thesis: Smoking and Dose Dependent Early Effects of Nicotine on Bone Mechanical Properties and Histology. Advisor: Dr. Timothy Norman
- Bachelor of Science Mechanical Engineering December 2002 GPA 3.3/4.0

Research Experience:

- 09/08-Present Research Assistant UK Division of Nephrology, Lexington, KY
- 12/08-04/09 Research Assistant UK Department of Orthopaedics, Lexington, KY
- 09/08-09/10 Research Consultant Shriners Hospitals, Lexington, KY
- 01/03-06/04 Research Assistant WVU Department of Orthopaedics, Morgantown, WV

Industrial Work Experience:

- 1/00-8/01 Co-op student, North Anna nuclear power plant, Component engineering department, Dominion Generation, Mineral, VA

Other Work Experience:

- 09/08-05/11 Substitute Teacher Woodford County Board of Education, Versailles, KY
- 08/07-08/10 Quality Control Inspector Aktrion, Nicholasville, KY
- 04/07-05/08 Night Organic Lab Personnel UK Department of Chemistry, Lexington, KY
- 9/05-05/07 Student Worker/Supervisor UK Dining Services, Lexington, KY

Seminars

- "Bone Turnover and Mineral Quality in Patients with Chronic Kidney Disease" November 10th 2006 at the Center for Biomedical Engineering, University of Kentucky, Lexington, KY
- "Bone Mineralization and Nanomechanical Properties are Dependent on Turnover" April 24th 2009 at the Center for Biomedical Engineering, University of Kentucky, Lexington, KY

- "The Effect of Various Pathologies on Bone Quality" March 15th 2013 at the Center for Biomedical Engineering, University of Kentucky, Lexington, KY

Awards and Honors:

- Dissertation Enhancement Award: Summer 2007, UK
- Max Stickler Fellowship: Fall 2006 & 2007, UK
- Harold M. Cather Scholarship: Spring 2001, WVU
- Eagle Scout: 10/07/96

Publications:

- Malluche H, Porter DS, Pienkowski D: "Evaluating Bone Quality in Patients with Chronic Kidney Disease" Nature Review Nephrology 2013. 9(11):671-80
- Malluche H, Porter DS, Monier-Faugere MC, Mawad H, Pienkowski D: "Low-Energy Fractures without Low T-Scores Characteristic of Osteoporosis: A Possible Bone Matrix Disorder" JBJS 2013. 95(19): p. e1391-6.
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Abstracts:

Conference Presentations

- Pienkowski D, Porter DS, Faugere MC, Malluche H: Bone Quality and Total Joint Replacement. International Society for Technology in Arthroplasty 26th Congress, Palm Beach, FL 2013
- Malluche H, Porter DS, Faugere MC, Pienkowski D: Bone Turnover and Abnormal Collagen Crosslinking in Women with Atraumatic Fracture and Near-Normal BMD. American Society of Bone Mineral Research Conference 33nd San Diego, CA 2011
- Malluche, H, Porter DS, Wright R, Faugere MC, Pienkowski D: Abnormal Bone Stiffness in Patients with Low and High Turnover Renal Osteodystrophy. American Society of Nephrology 14th Conference, San Diego, CA, 2009
- Porter DS, Faugere MC, Wang G, Rosenblum W, Pienkowski D and Malluche H: Bone Turnover and Mineral Quality in Renal Bone Disease. International Society of Bone Morphometry 10th Congress, Philadelphia, PA, 2006

Posters

- Porter DS, Pienkowski D, Wood C, Mawad H, Malluche M: Bone Quality Changes in Osteoporotic Patients with Long-Term Bisphosphonate Treatment American Society of Bone Mineral Research Conference 2014, submitted

- Ing S, Malluche H, Faugere MC, Porter DS, Pienkowski D: Bone Mineral and Material Properties in a Patient with Alendronate Associated Atypical Fracture before and After Two Years Treatment with Teriparatide. American Society of Bone Mineral Research Conference, Minneapolis, MN 2012
- Pienkowski D, Porter DS, Faugere MC, Malluche H: Is Bone Quality Altered with Alendronate Treatment in Osteoporotic Patients? Orthopaedic Research Society Conference, San Francisco, CA, 2012
- Porter DS, Wright R, Pienkowski D, Faugere MC, Malluche H: Differences in Structural and Material Properties of Low and High Turnover Bone. American Society of Bone Mineral Research Conference 32nd Toronto, Canada 2010
- Porter DS, Wright R, Pienkowski D, Faugere MC, Malluche H: Bone Mineralization and Stiffness are Gender Dependent. American Society of Bone Mineral Research Conference 31th, Denver, CO, 2009
- Pienkowski D, Porter DS, Faugere MC, Malluche H: Difference in Bone Mineral Quality between Caucasians and African Americans. Orthopaedic Research Society 55th Conference, Las Vegas, NV, 2009
- Norman TL, Chen X, Noble G, Porter DS, Kish V, Pechey C, Les C, Yeni Y: Fracture Toughness is More Sensitive to Bone Remodeling Parameters than Strength and Toughness. Orthopaedic Research Society 54th Conference, San Francisco, CA, 2008
- Malluche H, Porter DS, Faugere MC, Pienkowski D: Bone Mineral Quality and its relationship to Race, Gender and Bone Turnover. The Importance of Bone Quality in Patients with Renal Osteodystrophy. American Society Nephrology 12th Conference, San Francisco, CA, 2007
- Porter DS, Faugere MC, Pienkowski D, Malluche H: Gender Related Differences in Bone Mineral Quality. American Society of Bone and Mineral Research 29th Conference, Honolulu, HI, 2007
- Porter DS, Wang G, Faugere MC, Rosenblum W, Pienkowski D, Malluche H: Bone Turnover and Mineral Quality in Chronic Kidney Disease (CKD). Orthopaedic Research Society 53th Conference, San Diego, CA, 2007
- Porter DS, Norman T, Kish, V: Smoking and Dose Dependent Early Effects of Nicotine on Bone Mechanical Properties and Histology. Orthopaedic Research Society 51th Conference, Washington D.C., 2005