

**Unraveling Chemical Composition Heterogeneity of Type I Collagen in Trabecular
Bone as a Function of Disease and Treatment**

By

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For those who told me I did not have what it takes, and for those who never stopped believing in me for both influenced and shaped me in many ways.

This is for you.

Also, for those experiencing the physical and mental health tolls of graduate school, remember to breathe.

Just keep swimming.

You are worthy, you matter, you are enough.

Do not let a toxic environment or relationship keep you from reaching your true potential.

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LIST OF ABBREVIATIONS

Alendronate = ALN

Analysis of Variance = ANOVA

Area Under the Curve = AUC

Atomic Force Microscopy = AFM

Atomic Force Microscopy - Infrared Spectroscopy = AFM-IR

Bone Mineral Content = BMC

Bone Mineral Density = BMD

Bone Volume Fraction = BVF

Cathepsin K = CatK

Cathepsin K Inhibitor = CatKi

Cathepsin K Inhibitor Low Dose = CatKi-L

Cathepsin K Inhibitor High Dose = CatKi-H

Cranial = Cr

Denosumab = DMab

dual energy X-ray absorptiometry = DXA

Ethylenediaminetetraacetic acid = EDTA

Fourier Transform Infrared Spectroscopy = FTIR

Fracture Risk Assessment = FRAX

hydroxypropyl methyl cellulose acetate succinate polymer = HPMC-AS

intermittent Parathyroid Hormone = iPTH

Lumbar Vertebrae = LV

micro-Computed Tomography = μ CT

Non-Human Primate = NHP

Odanacatib = ODN

Odanacatib Research Rhesus Investigational Study = ORRIS

Ovariectomized = OVX

peripheral Quantitative Computed Tomography = pQCT

Photothermal Infrared Spectroscopy = PTIR

poly(methyl methacrylate) = PMMA

quantitative backscattered electron imaging = qBEI

RANK-Ligand = RANK-L

Receptor activator of nuclear factor κ B = RANK

Region of Interest = ROI

Standard Deviation = SD

Trabecular Number = Tb.N.

Trabecular Thickness = Tb.Th.

Vehicle = VEH

Ventral = V

Abstract

Nano- to microscale chemical heterogeneity plays a role in fracture prevention for osteoporotic bone. Once significant bone loss has occurred, the ability to restore biomechanical function may differ based on the drug chosen. In this study, we seek to understand changes in chemical composition linked to disease and treatment in lumbar vertebral trabecular bone using a *treatment* animal model. To the best of our knowledge, this is the first published *treatment* animal model to understand osteoporosis.

It should be noted that Merck, Inc. designed and performed all experiments relating to the *treatment animal model* including μ CT, mechanical testing and BMD determination. Study results and sliced LV samples were provided to our group at the University of Michigan for analysis and publication.

To assess this question, bisphosphonate (alendronate, ALN), cathepsin K inhibitor (MK-0674, CatKi), and denosumab (DMab) were employed in treatment mode to compare the relative changes to trabecular bone in ovariectomized (OVX) cynomolgus monkeys. Lumbar vertebrae (LV) bone mineral density (BMD) values taken two years post-surgery prior to drug treatment show a 10-15% decrease ($p < 0.05$) for all OVX animals. OVX animals were then treated with vehicle (VEH), ALN (0.03 mg/kg weekly), CatKi MK-0674 (0.6 or 2.5 mg/kg daily), and DMab (2.5 mg/kg weekly subcutaneously) for two years and compared to a Sham surgery group.

Ex-vivo microcomputed tomography (μ CT) of LV2 and compression testing of LV4-6 were used to measure trabecular bone microstructure and changes in bone mechanics,

respectively. After two years of treatment, ALN-treated animals showed no significant difference in μ CT or biomechanical parameters when compared to Veh. However, treatment with CatKi resulted in a 30% increase in yield and peak loads, and apparent peak and yield stress as compared to Veh ($p < 0.08$) and gave average mechanical values greater than the Sham. Intriguingly, these changes were realized despite no significant differences in mean values of trabecular bone morphologic parameters.

To understand changes in structure and composition, atomic force microscopy - infrared spectroscopy (AFM-IR) was used to obtain topographical information coupled to chemical composition/structure for cross-sections of lumbar vertebrae 4 (LV4). The average unraveled collagen content, average organic matrix, mineral content, and acid phosphate (PO_4^{3-}) substitution for each group was evaluated.

Average unraveled to intact ($1732 - 1748 \text{ cm}^{-1}/1656 - 1672 \text{ cm}^{-1}$) collagen integrated peak ratios were obtained for Sham, OVX, ALN, CatKi with DMab being significantly different from all other groups. From these results, CatKi is similar to Sham, ALN is similar to OVX and DMab is completely different from all other groups. Average mineral to matrix ($900-1200/1500-1800 \text{ cm}^{-1}$) ratios obtained for Sham, OVX, ALN, CatKi and DMab groups. No significant difference was detected. Average acid phosphate substitution ($1128/ 1096 \text{ cm}^{-1}$ intensity ratio) obtained for Sham, OVX, ALN, CatKi and DMab groups. CatKi was significantly different ($p < 0.05$) from OVX, ALN and DMab, yet no significant difference was detected for Sham. CatKi shows a preservation, or reversal, to normal levels of acid phosphate substitution. Unraveled collagen heterogeneity that appears as a function of OVX could be chemical biomarker of increased bone turnover.

With nano-scale resolution, we are capable of detecting compositional changes smaller than those detected with conventional spectroscopic techniques.

Chapter 1

Introduction

Unraveled Collagen as a Form of Compositional Heterogeneity in Bone: New Insights Offered by AFM-IR

1.1 Introduction

Bone is a composite material made up of Type I collagen, non-collagenous proteins, and carbonated hydroxyapatite mineral organized hierarchically at nano-, micro, and large length scales as illustrated in **Figure 1.1**.^{1–5}

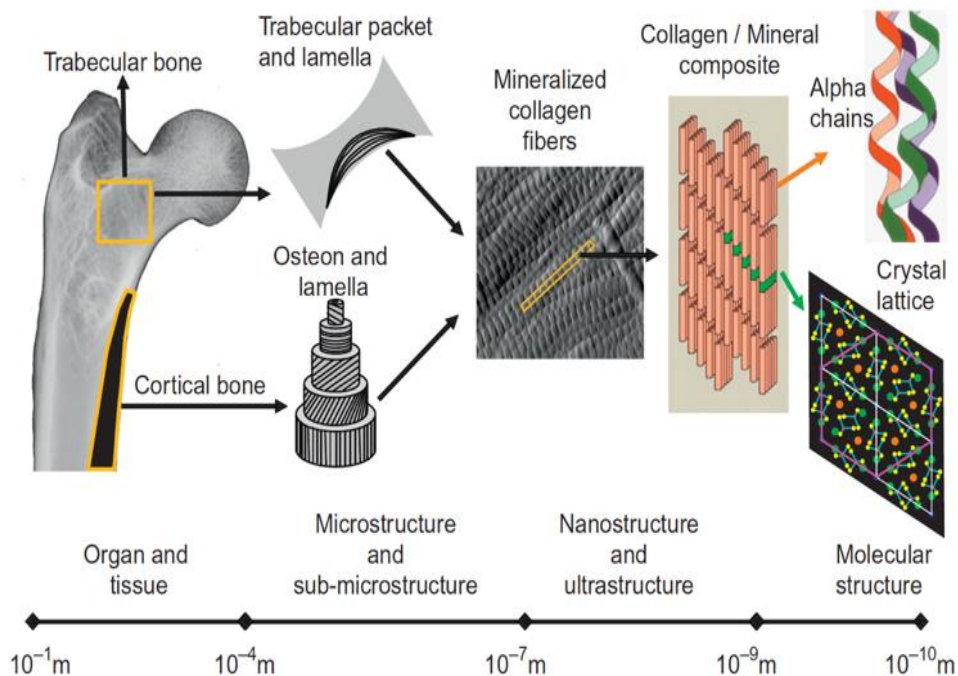


Figure 1.1 Bone Hierarchical Structure Organization. Adapted from *Basic and Applied Bone Biology*; Burr, D.B., Allen, M. R., Eds.; Elsevier, 2014.

At the molecular level, each collagen molecule has a right-handed triple helix of three polypeptide chains with the repeating amino acid sequence Gly-X-Y where X is most likely proline and Y is most likely hydroxyproline. Type I collagen molecules are fibril-forming collagens that are the most prevalent in bone, ligament, skin and teeth, and are 1.5 nm in diameter and 300 nm in length.¹ Self-assembly of Type I collagen molecules with other types of collagens, proteins, and hydroxyapatite mineral into microfibrils, fibrils, and fibers spanning dimensions from a few nanometers to meters in size yields complex hierarchical materials that serve a wide role of structural and mechanical roles.¹

Fourier transform infrared spectroscopy (FTIR) has been widely used to characterize bone composition.^{3,6-14} Special attention has been paid to mineral-to-matrix ratio ($900\text{ cm}^{-1} - 1200\text{ cm}^{-1}/1550\text{ cm}^{-1} - 1800\text{ cm}^{-1}$)¹⁵, which is proportional to mechanical properties¹¹ and it is known to decrease with osteoporosis.¹³ More specifically, carbonate-to-phosphate ratio ($850\text{ cm}^{-1} - 890\text{ cm}^{-1}/900\text{ cm}^{-1} - 1200\text{ cm}^{-1}$)¹⁵ and hydroxyapatite crystal size are associated with fragility fractures.¹⁵

However, most studies found in scientific literature focus on intact collagen leaving a knowledge gap specific to unraveled, or denatured, collagen; little information is known about the role(s) of unraveled collagen in tissue and how unraveled collagen affects tissues' mechanical performance. Recent studies on unraveled collagen in tendon¹⁶, ligament¹⁷, and parchment¹⁸ demonstrate the importance of understanding the impact of unraveled collagen. Both IR studies were conducted using atomic force microscopy coupled to infrared spectroscopy (AFM-IR) to characterize unraveled collagen. The capability to discern the $\sim 1740\text{ cm}^{-1}$ band associated with triple helix unraveling or denaturation is one particular advantage of the recent development of commercial

photothermal infrared (PTIR) instruments capable of obtaining infrared spectra on fully hydrated collagen tissues. Conventional IR microscopes have relied on differential absorption-based measurements and have typically required that tissue samples be dehydrated prior to measurement. This treatment itself induces substantial or complete denaturation of collagen, and thus has masked this aspect of collagen structure within healthy and/or diseased tissue.

In this chapter, we will discuss how AFM-IR can be employed to characterize bone compositional heterogeneity and Type I collagen microstructure characterization with an emphasis on collagen structural changes as a function of osteoporosis and drug treatment. In addition, we will hypothesize how the disease may contribute to material fatigue.

1.2 Current Crisis in Osteoporosis Treatment

Osteoporosis, or porous bone, is a degenerative bone disease that causes significant bone density and bone strength loss. One of the primary causes is estrogen depletion, although the detailed mechanisms leading to bone loss and changes in bone material remain a field of active study.¹⁹ This disease affects more than 75 million people worldwide, and approximately 1.5 million fractures per year are attributed to osteoporosis causing patients and health insurance companies billions of dollars every year to tend and care for these fractures.³ In addition to this, seniors are more likely to die within a year of suffering a fracture due to complications, and their quality of life significantly decreases.³

One of the underlying problems of this disease is the lack of precise diagnostic tools, the concerns patients have about receiving treatment due to the serious associated negative effects of some of the available drugs, and that diagnosis most commonly occurs after the patient suffers a fracture.^{3,20} Therefore, better methods of diagnosing and more effective treatments need to be developed.²⁰

The current diagnostic tool used is bone mineral density, or BMD.³ Even though this is a well-established method, it only captures the influence of mineral matrix on the bone whereas Type I collagen has been observed to play a significant role in this disease, and it generates a two-dimensional value instead of a volumetric value which would be a better estimate to the actual shape of the bone.^{3,20} These two factors reduce the effectiveness of BMD as an accurate predictor of fracture risk.²¹

Type I collagen's role in osteoporosis has not been fully characterized yet, especially potential role of unraveled collagen in bone mechanical properties.^{2,3,20}

1.3 Decrease in Nanoscale compositional heterogeneity is related to fracture risk

Nano- to microscale bone heterogeneity likely plays a crucial role in understanding bone mechanical properties, their role in fracture-risk, and how they change as function of disease.^{8,14,15,22–28} To further understand the impact of compositional heterogeneity on mechanical properties such as elasticity and crack propagation, a computational study using finite element modelling was developed to understand fracture resistance in cortical bone.²⁴ This computational study demonstrated that the presence of younger bone, which contains small or imperfect mineral crystals and immature crosslinks, and more mature tissue in bone is important for fracture resistant bone.²⁴

Reduced mineral and protein matrix heterogeneity was observed in patients treated with bisphosphonates when compared to non-treated patients (both human and canine).^{22,25} This reduction in heterogeneity affects tissue-level toughening mechanisms.^{22,25,29,30} In addition, lack of heterogeneity in hardness and modulus have been observed to worsen bone quality at the nano-scale in cortical and trabecular bone.²⁶ Lack of heterogeneity in hardness and modulus have been observed to worsen bone quality at the nano-scale in cortical and trabecular bone.²⁶ Taking this into consideration, inelasticity variation plays a dominant role in energy dissipation in bone as explored experimentally and computationally.^{23,27}

Currently, there is a lack of nanoscale chemical composition information for bone in the literature. This is due to spatial resolution limitations for most common techniques of about 10 μm and 3 μm for IR and Raman spectroscopy, respectively; specifically in their application for bone characterization.³¹ It should be noted that the state of the art spatial resolution for both techniques is much smaller (4 μm for FTIR spectroscopy³² and ten of nanometers for tip enhanced Raman spectroscopy³³) than that achieved in bone characterization. This is a significant limitation given that the observed domain size for nano-scale bone compositional heterogeneity is at least as small as 200 nm.²⁶ A detection voxel on the order of 1000 μm^3 also makes it difficult to detect bone components within the hierarchical structure. For example, suppose one collagen fibril was fully unraveled within the voxel. Conventional IR or Raman spectroscopy would detect this fibril as just a percentage of the collagen signal and this would likely be undetectable in the signal to noise (S/N) of the measurement or be dismissed as insignificant. However, AFM-IR with detection voxels as small as $1 \times 10^{-6} \mu\text{m}^3$ (~10 nm resolution) to 1.3×10^{-4}

μm^3 (50 nm resolution) can easily detect a single unraveled fibril, and in fact the entire detection volume could be filled with just a single collagen fibril since typical sizes are roughly cylindrical with diameters of ~30-100 nm. In short, conventional IR and Raman microscopy do not have the ideal spatial resolution to fully characterize bone compositional heterogeneity.

1.4 Using AFM and AFM-IR to Characterize Bone

Past studies have used atomic force microscopy (AFM) to understand structural changes in Type I collagen that occur within healthy and osteoporotic bone that has been exposed to different treatments in *prevention mode*, or before the disease fully develops.^{20,21,34,35} In these past studies, significant differences were observed in nanomorphology of fibril between healthy and estrogen depleted animals.^{20,21,34} AFM provides detailed topographic information regarding fibril morphology for Type I collagen, but do not provide information on chemical composition.³⁴ Significant differences in axial D-periodic spacing distributions as a function of disease have been observed as well as significant differences between fibrils found in different bundles.^{21,34,36}

Other studies have evaluated the entropy, or the disorder in the alignment of collagen fibrils.²⁰ Ordered and disordered collagen fibrils have been observed to play a role in bone strength as a function of disease in cortical bone.²⁰ Healthy bone will have a higher percent of ordered collagen when compared to osteoporotic bone, and bone of an osteoporotic animal treated in *prevention mode*.²⁰ Past studies have been focused on cortical bone, which has a different organization than trabecular bone.

These studies were able to observe morphological differences and propose possible explanations to these observations. However, the chemical changes driving the nanomorphology and microarchitecture of Type I collagen fibril differences were not studied. With the emergence of the AFM-IR technique, it is now possible to answer the question of what chemical differences are driving those structural differences. AFM-IR is a powerful technique capable of providing topography, morphology and chemical information simultaneously.³⁷ The technique consists of a gold-coated probe that scans the sample as it is being irradiated with a tunable IR laser. A localized enhanced field is created by the gold coating of the tip creating a localized thermal expansion at the surface in contact with the probe. The probe serves as a detector as it detects the sample's localized thermal expansion which is then converted into a localized absorption spectra.³⁸

1.5 Recent Application of AFM-IR to Study Bone

A handful of AFM-IR studies performed on bone have been published in the last 5 years. Most recently, Imbert et al. evaluated the nanoscale chemical composition of bone tissue in sheep with the ultimate goal of validating this technique for use on bone tissue.³⁹ They validated the AFM-IR methods by comparing their results of bone and polymethyl methacrylate (PMMA), a polymer commonly used to embed bone tissue samples and as a standard for bone, to FTIR results.³⁹ They obtained patterns that suggested layers of composition or structures that were not seen in the FTIR results due to spatial resolution.³⁹

In a 2014 preliminary study, non-human primate (NHP) osteons were analyzed at 50-100 nm spatial resolution.⁴⁰ A single baboon bone was used in this study to

demonstrate the consistency of the spectra obtained and reproducibility of the patterns observed.⁴⁰ In this study, they observed higher mineralization adjacent to the osteon center suggesting that the matrix and mineral were not tightly associated.⁴⁰ These studies show a promising future for this technique for characterization of bone tissue including studies to explore differences with age and disease.

1.6 Summary and Outlook

The role of protein in bone, and the degree of mineralization as a function of osteoporosis and treatment has not been studied before at such a small scale. In the study presented in the following chapters, we seek to understand the role of nanoscale compositional heterogeneity as a function of disease and treatment. We present and discuss evidence for a new chemical marker of increased bone turnover, a characteristic of osteoporosis. With this study, we want to increase the visibility of AFM-IR in the bone community given how well it has been shown to work for bone characterization at a nanoscale.

In the following chapters of this thesis, the reader can expect in depth discussions on the chemical compositional heterogeneity, spatial distributions and variations observed as a function of disease and treatment, possible mechanisms that lead to these variations, and the biomechanical implications of these variations as outlined here.

In **Chapter 2**, we report that mechanical properties of lumbar vertebrae were preserved with CatKi treatment despite no detectable changes in bone microstructure by μ CT analysis. These results suggest that important changes in bone material composition contribute to the observed mechanical differences.

In **Chapter 3**, we describe the *animal treatment model* used in this study, and the complete data set obtained from all experiments conducted at the University of Michigan and Merck, Inc. In addition, a general summary of results and conclusions is provided to better summarize the changes in chemical compositional, structural and spatial heterogeneity differences of protein and mineral content as a function of OVX and treatment.

In **Chapter 4**, we discuss the hypothesis that unraveled collagen can be used as a biomarker for osteoporosis. We observe a prominent peak that has been reported in the bone IR literature before simply as a carbonyl stretch (C=O). This peak results from an unwinding, or denaturation, of the triple helix that exposes collagen's backbone's carbonyls generating a prominent 1740 cm^{-1} peak.⁴¹ We hypothesize this peak is a key marker of the increased bone turnover associated with osteoporosis as stated in **H1a**.

In **Chapter 5**, we report differences in the mineral-to-matrix ratio (900 cm^{-1} - 1200 cm^{-1} / 1550 cm^{-1} - 1800 cm^{-1}) and in acid phosphate substitution (1128 cm^{-1} / 1096 cm^{-1} intensity ratio).^{15,42} In bone, the mineral peak is a manifold of about a dozen vibrational groups that appear in similar IR ranges. Acid phosphate substitution indicates younger mineral, and mineral to matrix ratios indicate chemical heterogeneity in the bone.^{15,42} **Chapter 5** provides a discussion of **H1b** and **H2a**.

1.7 References

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Chapter 2

Cathepsin K Inhibition Preserves Compressive Load in Lumbar Vertebrae of Osteoporotic Monkeys

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2.1 Abstract

Anti-resorptive drugs treat bone loss by blocking osteoclast activity through a variety of mechanisms of action. Once significant bone loss has occurred, the ability to restore biomechanical function may differ based on the drug chosen. To assess this question, bisphosphonate (alendronate, ALN) and cathepsin K inhibitor (MK-0674, CatKi) were employed in *treatment mode* to compare the relative changes to cancellous bone microstructure and mechanical properties in ovariectomized (OVX) cynomolgus monkeys. Lumbar vertebrae (LV) bone mineral density (BMD) values taken two years post-surgery prior to drug treatment show a 10-15% decrease ($p < 0.05$) for all OVX animals. OVX animals were then treated with vehicle (VEH), ALN (0.03 mg/kg weekly), or CatKi MK-0674 (0.6 or 2.5 mg/kg daily, CatKi-L and H respectively) for two years and compared to a control Sham surgery group. Ex-vivo microcomputed tomography (μ CT) of LV2 and compression testing of LV4-6 were used to measure cancellous bone

microstructure and changes in bone mechanics, respectively. After two years of treatment, ALN-treated animals showed no significant difference in μ CT or biomechanical parameters when compared to Veh. However, treatment with CatKi-H resulted in a 30% increase in yield and peak loads, and apparent peak and yield stress as compared to Veh ($p < 0.05$) and gave average mechanical values greater than the Sham sample. Treatment with CatKi-L exhibited a similar trend of increase to CatKi-H ($p < 0.08$). Intriguingly, these changes were realized despite no significant differences in mean values of trabecular bone morphologic parameters. Together these data suggest matrix-level changes in bone composition that are unique to the CatK inhibition mechanism, resulting in the preservation of bone compressive load with treatment.

2.2 Introduction

Osteoporosis is characterized by a net bone loss due to an increase in bone resorption.¹ Once significant bone loss has occurred, restoring biomechanical function is a primary challenge for interventional therapies.^{2,3} Antiresorptive treatments are widely used in the clinic to prevent bone loss through a variety of mechanisms of action.^{1,3} The bisphosphonate Alendronate (ALN) adsorbs to the mineral in bone, and interferes with a key enzyme in the mevalonate pathway when taken up by the cell during bone resorption, leading to decreased osteoclast activity.^{4,5}

Despite the effectiveness of ALN, serious but rare associated adverse effects can occur such as atypical fractures, atrial fibrillation, and osteonecrosis of the jaw.^{6–9} While these associated adverse effects are extremely rare and occur more commonly when bisphosphonates are used to treat cancer instead of osteoporosis, statements released

by the Food and Drug Administration have raised concern regarding bisphosphonate use.⁹ This has led to a crisis in the treatment of osteoporosis due to high-risk patients denying treatment despite a beneficial clinical outlook.^{10,11} Therefore, new drugs, as well as a better understanding of antiresorptive mechanisms, are desired.

Recently, attention has been directed toward interventions that induce partial osteoclast inhibitory effects as promising alternatives to complete osteoclast inhibition. The inhibition of Cathepsin K (CatK), a lysosomal cysteine protease found in osteoclasts that is responsible for degrading Type I collagen,¹² is considered one of those promising alternatives. Cathepsin K-inhibition (CatKi) does not interrupt osteoclast to osteoblast communication, allowing bone formation to occur even though resorption is decreased through inhibition of protease activity.^{13–15} The effectiveness of pharmacological cathepsin K inhibition as a treatment for bone diseases such as osteoporosis and osteoarthritis has been demonstrated in the literature.^{14–19}

Previous studies of the effectiveness of CatKi in treating ovariectomized (OVX) skeletally mature rhesus monkeys focused on *prevention-model* studies where the drug is given immediately after the OVX surgery. Masarachia et al. noted an increase in lumbar vertebrae (LV) mineral density (BMD) upon treatment of skeletally mature rhesus monkeys with either 6 or 30 mg/kg Odanacatib (ODN).²⁰ A corresponding increase in the values of bone peak load and area under the curve were observed in mechanical testing, demonstrating functional significance to treatment-induced bone mass gains.²⁰ Jerome et al. also noted a partial prevention of bone loss for monkeys treated with 3 or 10 mg/kg Balicatib.²¹ In a comparative study with alendronate (ALN), Williams et al. noted that 2 mg/kg dose of ODN given to monkeys resulted in improvement in lumbar spine areal BMD

(aBMD) and trabecular volumetric BMD (vBMD); however, higher doses of 4 and 8 mg/kg did not further improve these properties.¹⁸ Treatment effects were not limited to structural gains, but also induced a shift in the calcium distribution to higher average values, as assessed using quantitative backscattered electron imaging (qBEI).²²

Importantly, none of these prior studies examined the effect of cathepsin K inhibition following a period of bone mass depletion, reflecting the clinical state of osteoporosis. In the present study, alendronate (ALN), and a cathepsin K inhibitor (CatKi, MK-0674) were employed in *treatment mode* to explore the impact of varying biological mechanisms of action on changes to trabecular bone microstructure and biomechanical properties after two years of estrogen depletion in OVX cynomolgus monkeys. This animal model is especially relevant for the study of osteoporosis.²³ To our knowledge, it is the first time that cathepsin K inhibition has been applied in a non-human primate (NHP) study of onset osteoporosis treatment. The findings from our present study demonstrate that treatment following a period of trabecular bone depletion induces changes in biomechanical indices that are not reflected by similar improvements in structural indices of trabecular architecture. Together, this data suggests that cathepsin K inhibition may induce matrix-level changes that partially correct for deficiencies in biomechanical predictors of fracture.

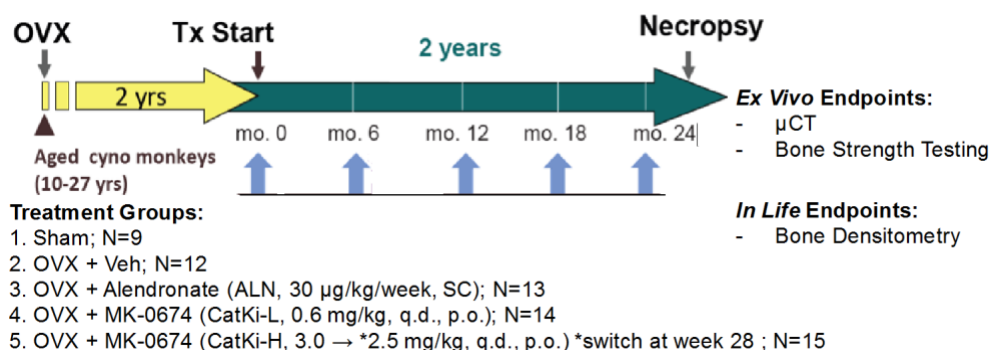
2.3 Methods

2.3.1 Cynomolgus monkeys, diets, surgery and dosing with CatKi

The in-life portion of this study was conducted at Charles River Laboratories (CRL, Montreal, Canada) with the approval of IACUCs from both CRL and Merck & Co., Inc.,

Kenilworth, NJ, USA. This study protocol were conducted essentially based on the study protocol of the Odanacatib Research Rhesus Investigational Study (ORRIS).¹⁸ Previously in the ORRIS study all drug dosing, including the CatKi odanacatib (ODN) were administered in Rhesus monkeys in *prevention mode* (right after OVX surgery). In contrast, in this study, all drug dosing including the CatKi MK-0674²⁴ were dosed on Cynomolgus monkeys in *treatment mode* (initiated at 2 years post-OVX). Female Cynomolgus monkeys (10-27 years of age) were subjected to ovariectomy (OVX) or sham surgery. All monkeys were subsequently randomized into groups by weight and lumbar spine (LV1-4) bone mineral density (LsBMD).

Sham animals (n=16) were dosed daily with vehicle (hydroxypropyl methyl cellulose acetate succinate (HPMC-AS) polymer), per orem (p.o., orally). OVX animals were randomized into 4 groups, vehicle (n=16), MK-0674 at 0.6 mg/kg p.o., quaque die (q.d., daily) (AUC=10.9±4.5; n=16), MK-0674 at 2.5 mg/kg p.o., q.d (AUC=69.1±28.7; n=16), or Alendronate (ALN) at 30mg/kg/wk subcutaneously (SC) once-weekly (n=16). Drug administration were continued for 24-months. Prior to necropsy, bone mineral density of the lumbar spine was monitored with dual energy X-ray absorptiometry (DXA) and peripheral Quantitative Computed Tomography (pQCT) at baseline (before randomization and before dosing started), and approximately at 3, 6, 9, 12, 16, and 24-months after drug administration initiated. For a visual summary see **Scheme 2.1**.



Scheme 2.1 Treatment Animal Study Timeline

Cynomolgus monkeys were treated with CatKi, and ALN for 24 months in *treatment mode* two years post-surgery. Intact (Sham), and a disease (OVX+Veh) groups were included. *In-life* bone densitometry was evaluated every six months as shown above with the blue arrows. *Ex vivo* µCT, and bone compression testing were performed on LVs 4 and 5.

2.3.2 Dual energy X-ray absorptiometry (DXA) and Computed Tomography (CT) imaging

The animals were anesthetized by ketamine and maintained under isoflurane as for OVX. *In vivo* areal BMD, bone mineral content (BMC) and bone area of the lumbar vertebrae LV1 to LV4 were measured by DXA using a Hologic QDR Discovery A bone densitometer (Hologic, Inc., Waltham, MA). *Ex vivo* Peripheral QCT scans were acquired as single slice using a XCT Research SA or SA+bone scanner with software version 5.50D (Stratec Medizintechnik; Pforzheim, Germany).^{20,25}

Ex vivo volumetric measures of total and trabecular BMD and BMC of vertebral bodies (LV4 and LV5) were obtained from a 0.8 mm-thick slice taken from the middle of each vertebral body excised for biomechanical testing.³ Another vertebral site (LV2) was further subjected to µCT scanning at an isotropic 20 µm resolution using a Scanco µCT-40 scanner (Scanco Medical AG, Brütisellen, Switzerland) as previously described.²⁶

µCT scans were analyzed using MicroView. Original scans had to be cropped using MicroView Parallax (Ilderton, ON) due to file size limitations, and a median filter was applied to reduce noise. A region of interest (ROI) was defined from superior growth

plate to inferior growth plate for each sample. Trabecular bone was contoured every 10 slices, and a 3D ROI was defined within the contoured area. The ROI was analyzed to obtain trabecular morphologic parameters, see **Table 2.1**.

Table 2.1. Bone Densitometry, Microstructure, and Bone Strength Results and Statistical Analysis

Parameter	Sham	OVX			
		Veh	CatKi-L	CatKi-H	ALN
	N = 9	N = 12	N = 14	N = 15	N = 13
<i>In-Life Pre-Treatment</i>					
Bone Mineral Density (g/cm ²)	0.781 (0.068)***	0.695 (0.072)	0.698 (0.091)	0.662 (0.090)	0.674 (0.066)
<i>Post-Treatment</i>					
Trabecular Number (1/mm)	2.30 (0.23)	2.15 (0.24)	2.24 (0.18)	2.15 (0.35)	2.18 (0.23)
Trabecular Thickness (mm)	0.185 (0.055)	0.168 (0.037)	0.194 (0.055)	0.175 (0.027)	0.177 (0.036)
Connectivity Density (1/mm ³)	8.6 (4.2)	7.9 (4.2)	6.0 (3.9)	6.8 (2.9)	7.8 (1.8)
Vertebral Height (mm)	7.43 (0.11)	7.35 (0.22)	7.35 (0.11)	7.39 (0.12)	7.355 (0.14)
Peak Load (N)	3097 (550)	2620 (580)	3460 (1100)*	3507 (860)**	3053 (490)
Apparent Peak Stress (MPa)	27.0 (3.6)	24.3 (4.0)	32.9 (11)**	32.5 (7.5)*	26.6 (4.8)
Yield Load (N)	2660 (450)	2280 (490)	2980 (940)*	2974 (690)*	2678 (400)
Yield Stress (MPa)	23.1 (2.8)	21.2 (3.3)	28.3 (9.3)**	27.5 (5.6)*	23.3 (3.6)
Stiffness (N/mm)	18750 (3800)	16630 (4300)	20280 (4300)	20530 (4100)	19480 (2600)
Modulus (MPa)	1214 (220)	1122 (210)	1420 (370)*	1400 (250)	1250 (190)
Area Under the Curve (N/mm)	907 (330)	673 (240)	1224 (960)	1214 (780)	628 (190)
Toughness (F)	1.08 (0.41)	0.85 (0.28)	1.6 (1.4)	1.5 (1.0)	0.76 (0.28)

Data are presented as means ± (SD), and rounded to represent significant figures based on SD; BMD presented was collected after two years of estrogen depletion and prior to treatment

* $p < 0.1$ vs Veh; ** $p < 0.05$ vs. Veh; *** $p < 0.05$ vs. Veh and other treatments

2.3.3 Biomechanical Testing

Bones were thawed overnight in a refrigerator at 4°C prior to μ CT and bone strength testing. Vertebrae were trimmed, removing dorsal elements, spinal processes, and the cartilaginous endplates with a diamond saw leaving only the cartilage-free

vertebral body with plano-parallel ends. Biomechanical testing of the vertebral bodies (LV4 and LV5) was performed using the 858 Mini Bionix Servohydraulic Test System, Model 242.03.³ All data were collected in an Excel Spreadsheet which was used to manually derive the required biomechanics parameters. The height of the vertebral body test specimen was measured using digital calipers before compression testing with a load cell of 15 kN and a loading rate of 20 mm/minute. Peak load was recorded as the maximum of the load–displacement curve, and stiffness were defined as the slope of the linear portion.²⁷ Work, the energy required to break the bone, was calculated as the area under the curve to peak load for compression tests. The apparent yield and peak stress were calculated using the yield load and peak load divided by the cross-sectional area respectively. The area was determined by peripheral quantitative tomography (data not presented here). Modulus was calculated using stiffness, thickness and area, see **Equation 2.1**.^{3,27} Toughness was calculated using work-to-failure, thickness and area, see **Equation 2.2**.³

$$\text{Equation 2.1} \quad \text{Modulus (MPa)} = \frac{\text{Stiffness (N/mm)} * \text{thickness of specimen (mm)}}{\text{Area (mm}^2\text{)}}$$

$$\text{Equation 2.2} \quad \text{Toughness (MPa)} = \frac{\text{Area Under the Curve (N/mm)}}{(\text{Area (mm}^2\text{)} * \text{thickness of specimen (mm)})}$$

2.3.4 Statistical Analysis

Microstructure and biomechanical results are reported as means ± (SD), as seen in **Table 2.1**. Statistical analysis was performed using R. A one-way analysis of variance (ANOVA) was used to determine treatment group differences for each specific variable. Tukey's Test was used to detect differences in means between specific treatment group.

2.4 Results

2.4.1 OVX induces trabecular bone loss over a 2-year depletion period

Two years after initial surgery, but prior to drug treatment, all experimental groups demonstrated similar loss (10-15%) of bone mineral density (BMD) at LV when compared to Sham-operated controls (**Table 2.1** and **Figure 2.1**) with no difference between individual groups, indicating a well-balanced study allocation. Bone loss was consistent with previous work demonstrating an 11% decrease in BMD for OVX animals vs. sham controls over 21 months.¹⁷

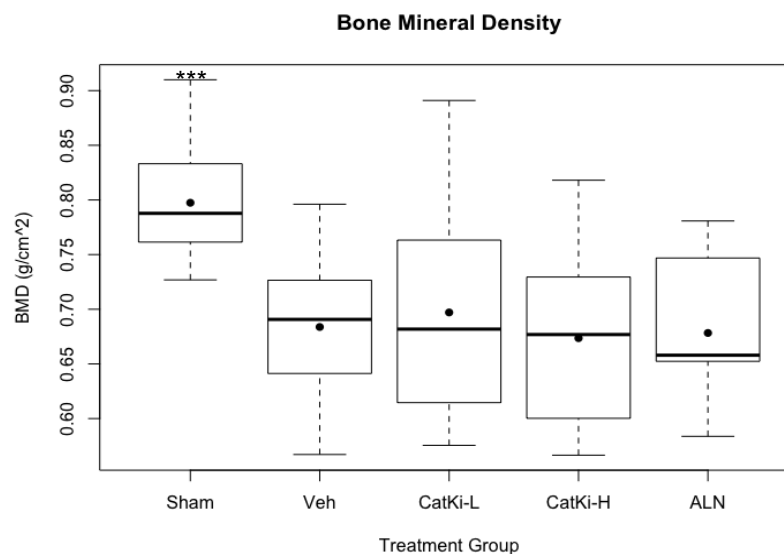


Figure 2.1

In Life DXA assessment of Bone Mineral Density 2 years after surgery, before start of treatment, shows 10-15% reduction in BMD at the lumbar vertebrae for all OVX groups when compared to Sham group.

*** p < 0.05 vs. Veh, CatKi-L and -H, ALN.

Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

2.4.2 Two-year treatment following trabecular depletion shows no effect on trabecular microarchitecture

Ex vivo μ CT scans performed on LV2 after 2 years of treatment demonstrated no significant differences in trabecular bone architectural parameters between Sham

animals and any treatment group for bone volume fraction (BVF), trabecular number (Tb.N.), trabecular thickness (Tb.Th.), or connectivity density (**Table 2.1**).

2.4.3 Cathepsin K inhibition preserves biomechanical indices

Compression testing was performed on LV4-5. Values presented in **Table 2.1** are an average of LV4 and LV5. OVX induces modest decreases in yield and peak loads vs. sham controls as observed in **Table 2.1**. While ALN-treated vertebrae showed no differences vs. Sham or OVX-Veh, CatKi-L and CatKi-H increased yield and peak loads, and apparent yield and peak stress to levels significantly greater than OVX-Veh (**Figure 2.2**). All intervention biomechanical parameters were statistically indistinguishable from Sham controls (**Figure 2.2**), suggesting a preservation of effects of OVX itself.

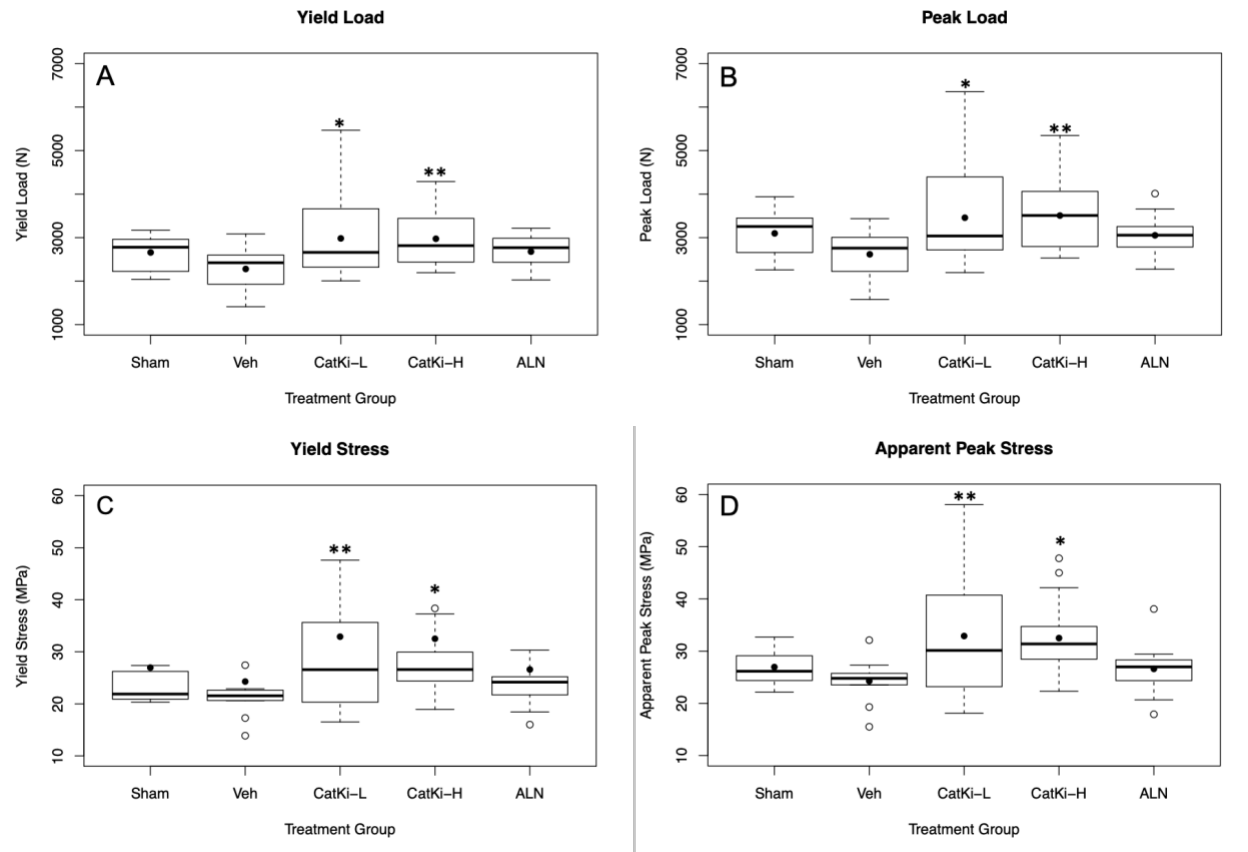


Figure 2.2

Biomechanical testing shows significant (~30%) increase in yield load (**Figure 2.2A**), peak load (**Figure 2.2B**), yield stress (**Figure 2.2C**), and apparent peak stress (**Figure 2.2D**) after 2 years of CatKi treatment vs. Veh animals. No significant difference was observed in ALN animals vs. Veh.

* $p < 0.1$ vs Veh; ** $p < 0.05$ vs Veh.

Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

2.5 Discussion

Treatment with CatKi has been able to preserve biomechanical properties in NHP osteoporosis/bone loss *prevention models* through a unique mechanism of action.³ In the present study, biomechanical properties of lumbar vertebrae were increased with CatKi treatment administered to NHP in *treatment mode*. Specifically, yield and peak loads as well as apparent yield and peak stress were significantly increased between CatKi treated groups and OVX-Veh treatments. However, no detectable changes in bone

microstructure by μ CT analysis were observed. These improvements in biomechanical properties are similar to results observed in *prevention mode* studies done on rhesus monkeys.

Masarachia et al. assessed the long-term (21-months) effects of ODN on newly OVX rhesus monkeys.²⁰ In that study, the effects of ODN on reduction of trabecular bone turnover, prevention of estrogen deficiency–induced bone loss, and a significant increase in lumbar spine BMD were observed.²⁰ This increase highly correlated to higher bone peak loads indicating a preservation of biomechanical properties after treatment.²⁰ These results were reproduced in radius and tibia²⁸, hip²⁹, and femurs²⁵ demonstrating that CatKi preserves biomechanical properties of bone while inhibiting resorption. Our results are consistent with Masarachia et al.'s results of biomechanical properties preservation after treatment with CatKi in lumbar vertebrae of rhesus monkeys. Increase in BMD, inhibition of trabecular bone turnover, while stimulation of periosteal bone formation in femurs was observed in cynomolgus monkeys treated with Balicatib, another CatK inhibitor.²¹

In the ODN study in rhesus monkeys, Williams et al. demonstrated ODN dosing at approximated clinical exposure for 21 months in *prevention mode* increased lumbar spine aBMD (areal BMD), and trabecular vBMD (volumetric BMD) with comparable efficacy to ALN.¹⁸ Higher dose of ODN showed no additional efficacy. In that same study, Duong et al. evaluated the long-term effects of ODN versus ALN in the prevention of bone microstructure deterioration.³ At the end of the study, no significant differences in BMD (both cortical and trabecular), trabecular microstructure, and biomechanical properties including peak load and stiffness were observed in lumbar vertebrae from the treatment

groups, unlike this study.³ However, our results of no detectable trabecular microstructure change or stiffness is consistent with Duong et al.'s study.

In the present study, we compared CatKi to ALN dosed in *treatment mode*. CatKi-L and CatKi-H treatments differentiated from ALN by increased yield and peak loads over Veh. ALN treatment was no different than Sham and Veh groups. The changes in mechanical properties, without concomitant changes in bone microstructure, suggest that important changes in bone material composition may contribute to the observed mechanical difference, further supported by the observed increase in calculated apparent yield and peak stress. Compositional changes in bone have been associated with osteoporosis, and heterogeneity of these measurements is also emerging as an important contributor to bone mechanical properties, and fragility fracture risk.^{30–38} Nano- to microscale bone heterogeneity likely plays a crucial role in understanding bone mechanical properties and their role in fracture-risk.^{30–38} The present findings suggest that inhibition of Cathepsin K may preserve, or reverse compositional changes and improve vertebral biomechanics with treatment.

Studies have shown how reductions in compositional heterogeneity can affect tissue-level toughening mechanisms.^{32,36} In addition, lack of heterogeneity in hardness and modulus have been observed to worsen bone quality at the nano-scale in cortical and trabecular bone.³⁷ Taking this into consideration, inelasticity variation was also explored experimentally and computationally.^{33,38} These studies found that inelasticity plays a dominant role in energy dissipation in bone.^{33,38} Our study suggests a potential for multiple regulators of bone mechanical function beyond bone quantity and structural organization alone.

This study has several limitations. First, the ovariectomy may have not produced a strong biomechanical phenotype because of genetic variability in our samples. Our animal model was designed to mimic the treatment of osteoporosis in the clinic. Therefore, the subjects were not genetically identical contrary to most rodent models. However, that did not observe a difference between OVX and Sham controls was consistent with previous *prevention mode* studies in rhesus monkeys³ and rabbits³⁹ that were treated with ODN. Second, an intermittent parathyroid hormone (iPTH) control group would have added value to this study as a positive control, as well as providing a deeper understanding of how CatKi compares to an anabolic drug when administered after a significant period of estrogen depletion. In an analogous study, iPTH was administered to rhesus monkeys for 16 months following 9 months of estrogen depletion.⁴⁰ Similar to our present findings, Fox et al. reported that treatment with iPTH for 16 months resulted in a dose-dependent increase in lumbar spine yield load and stiffness compared to Veh animals to values that were statistically no different than sham controls.⁴⁰ The magnitude of response appears greater with iPTH, and this may reflect the mechanism of action of the drug, along with the difference in magnitude of bone loss between the two studies in the depletion period (Fox et al. 5% BMD depletion, present study 10-15% BMD depletion). Third, cortical bone was not evaluated in the present study. Recently, it was proposed that CatK degrades periostin thereby modulating Wnt-b-catenin signaling and generating higher osteoblast activity in cortical versus trabecular bone.¹³ This mechanism suggests that cortical bone volume can increase in response to CatK inhibition and several prevention mode studies have observed that CatKi promotes new periosteal surface bone

formation in tibia²⁸, hip²⁹, and femurs.²⁵ The potential role of such effects for vertebrae is an important area for future study.

2.6 Conclusion

In conclusion, increases in yield and peak load were observed for CatKi treatment when compared to OVX-Veh despite no significant differences in trabecular architectural parameters as measured by μ CT. Treatment with CatKi resulted in a 30% increase in yield and peak loads, and apparent yield and peak stress when compared to Veh. This preservation of biomechanical properties appears to be unique to cathepsin K inhibition, as compared to alendronate. This is the first time a preservation in mechanical properties has been observed for an antiresorptive osteoporosis drug administered in *treatment mode*. Improvement of biomechanical properties is one of the current challenges in the field of osteoporosis.

Considering the results presented and discussed in this study, we pursued the question of matrix-level changes in bone composition that resulted in a preservation of bone compressive load with treatment. To answer this question, we used atomic force microscopy coupled to infrared spectroscopy (AFM-IR) to understand what chemical composition changes arise as a function of OVX and treatment. Samples used in the AFM-IR portion of this study were taken from the animals used in this study. Following **Chapters (3-5)** in this dissertation will go in depth into compositional changes regarding Type I collagen disorder, mineral-to-matrix ratio, mineralization, and a detailed account of the treatment animal model and all the data collected in the study.

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2.8 Author Roles

In vivo study designed and conducted by LD and BP. DEXA, biomechanical testing, and microCT imaging performed by LD and BP. MicroCT analysis performed by IDCB. Data analyzed and interpreted by IDCB, JH, KMK, MMBH. Manuscript written by IDCB and revised and approved by all authors.

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Chapter 3

Combined AFM-IR for Preclinical Studies of Lumbar Vertebrae Trabecular Bone Structure as a Function of Estrogen Depletion and Drug Treatment

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3.1 Abstract

Cathepsin K inhibition has been proven to be effective at preventing changes in bone mineral density (BMD) upon induction of estrogen depletion, and has prevented microstructural changes in Type I collagen. Both results have been observed in *prevention-based* animal models, which do not reflect the clinical state for human disease. In this study, we seek to understand changes in chemical composition linked to disease and drug treatment in lumbar vertebral trabecular bone using a *treatment-based* animal model to address this clinical discrepancy. To assess this question, bisphosphonate (alendronate, ALN), cathepsin K inhibitor (MK-0674, CatKi), and denosumab (DMab) were employed in treatment mode to compare the relative changes to trabecular bone microstructure and mechanical properties in ovariectomized (OVX) cynomolgus monkeys. Lumbar vertebrae (LV) bone mineral density (BMD) values taken two years post-surgery prior to drug treatment show a 10-15% decrease ($p < 0.05$) for all OVX

animals. OVX animals were then treated with vehicle (VEH), ALN (0.03 mg/kg weekly), CatKi MK-0674 (0.6 or 2.5 mg/kg daily, CatKi-L and H respectively), denosumab (DMab; 2.5 mg/kg weekly subcutaneously) for two years and compared to a control Sham surgery group.

To understand changes in microstructure, LV 2 was submitted to Micro-computed tomography (μ CT) imaging. To understand changes in mechanical properties, LVs 4-6 were submitted to compression load testing, and their averaged values were obtained. After two years of treatment, ALN-treated animals showed no significant difference in μ CT or biomechanical parameters when compared to Veh. However, treatment with CatKi resulted in an increase in yield and peak loads, and apparent peak and yield stress as compared to Veh ($p < 0.08$) and gave average mechanical values greater than the Sham sample. Intriguingly, these changes were realized despite no significant differences in mean values of trabecular bone morphologic parameters.

To understand changes in structure and composition, atomic force microscopy - infrared spectroscopy (AFM-IR) was used to obtain topographical information coupled to chemical composition/structure for cross-sections of lumbar vertebrae 4 (LV4). The average collagen disorder content, average organic matrix, mineral content, and acid phosphate (PO_4^{3-}) substitution for each group was evaluated. In conclusion, with this data set we are able to better understand the chemical composition, structural and spatial heterogeneity differences of protein and mineral content as a function of OVX and treatment, and their effects on mechanical properties and bone microstructure.

3.2 Introduction

A series of publications from the Bone Biology group at Merck, Inc. demonstrated that Cathepsin K inhibitors (CatKi) are effective at preventing changes in bone mineral density (BMD) upon induction of estrogen depletion in ovariectomized rabbits¹ and monkeys.²⁻⁴ We have shown using atomic force microscopy (AFM) analysis that the Type I collagen microstructure changes upon OVX treatment of rabbits.^{5,6} Treatment with CatK inhibitor (odanacatib, ODN) prevents these microstructural changes and treatment with alendronate (ALN) partially prevents these changes.⁵ This is particularly interesting because formation of the Type I collagen protein scaffold occurs before mineralization. Our pre-clinical animal data suggests drug treatment can prevent changes in protein structure induced by OVX; however, the animal studies were performed in *prevention mode*¹⁻⁴ and thus are limited in their ability to predict the impact of the drug on a fully evolved disease state. Although the effectiveness of pharmacological cathepsin K inhibition as a treatment for bone diseases has been demonstrated in the literature,^{4,7-11} a substantial gap in understanding the structural and compositional changes associated with protein and mineral remains.

Masarachia et al. noted an increase in lumbar vertebrae (LV) mineral density (BMD) and mechanical properties upon treatment of skeletally mature rhesus monkeys with either 6 or 30 mg/kg Odanacatib (ODN) demonstrating functional significance to treatment-induced bone mass gains.⁸ In a comparative study with alendronate (ALN), Williams et al. noted that 2 mg/kg dose of ODN given to monkeys resulted in improvement in lumbar spine areal BMD (aBMD) and trabecular volumetric BMD (vBMD).⁴ Treatment effects also induced a shift in the calcium distribution to higher average values, as

assessed using quantitative backscattered electron imaging (qBEI).¹² In that same study, Duong et al. evaluated the long-term effects of ODN versus ALN in the prevention of bone microstructure deterioration; however, no significant differences in BMD trabecular microstructure, and biomechanical properties were observed in lumbar vertebrae from the treatment groups.^{13,14} From the point of view of clinical application, it is critical to know if treatment with drug can restore the changes in OVX bone to the native Sham condition.

Table 3.1. Summary of Bone Densitometry, Microstructure, and Bone Strength Results and Statistical Analysis¹⁴

Parameter	Sham	OVX			
		Veh	CatKi-L	CatKi-H	ALN
	N = 9	N = 12	N = 14	N = 15	N = 13
<i>In-Life Pre-Treatment</i>					
Bone Mineral Density (g/cm ²)	0.781 (0.068)***	0.695 (0.072)	0.698 (0.091)	0.662 (0.090)	0.674 (0.066)
<i>Post-Treatment</i>					
Trabecular Number (1/mm)	2.30 (0.23)	2.15 (0.24)	2.24 (0.18)	2.15 (0.35)	2.18 (0.23)
Trabecular Thickness (mm)	0.185 (0.055)	0.168 (0.037)	0.194 (0.055)	0.175 (0.027)	0.177 (0.036)
Peak Load (N)	3097 (550)	2620 (580)	3460 (1100)*	3507 (860)**	3053 (490)
Apparent Peak Stress (MPa)	27.0 (3.6)	24.3 (4.0)	32.9 (11)**	32.5 (7.5)*	26.6 (4.8)
Yield Load (N)	2660 (450)	2280 (490)	2980 (940)*	2974 (690)*	2678 (400)
Yield Stress (MPa)	23.1 (2.8)	21.2 (3.3)	28.3 (9.3)**	27.5 (5.6)*	23.3 (3.6)
Modulus (MPa)	1214 (220)	1122 (210)	1420 (370)*	1400 (250)	1250 (190)

Data are presented as means ± (SD), and rounded to represent significant figures based on SD; BMD presented was collected after two years of estrogen depletion and prior to treatment

* $p < 0.1$ vs Veh; ** $p < 0.05$ vs. Veh; *** $p < 0.05$ vs. Veh and other treatments

DMab treatment group was not considered for publication in **Chapter 2** because of neutralizing anti-drug anti-body development during study.

Importantly, all prior studies have used *prevention mode* models, which do not reflect the clinical state of osteoporosis. In the present study, the drugs were administered in *treatment mode* to address the clinical state discrepancy. To our knowledge, **Chapter 2** is the first, published study in which cathepsin K inhibition has been applied in a non-

human primate (NHP) study of onset osteoporosis treatment.¹⁴ The findings of **Chapter 2** are summarized in **Table 3.1**.¹⁴ Briefly, in **Chapter 2**, we demonstrated that treatment following a period of trabecular bone depletion induces changes in biomechanical indices that are not reflected by similar improvements in structural indices of trabecular architecture in lumbar vertebrae.¹⁴ Together, this data suggests that cathepsin K inhibition induces matrix- and/or mineral level changes that partially correct for deficiencies in biomechanical predictors of fractures.

To further assess this matrix- and/or mineral level changes question, samples from the same animals discussed in **Chapter 2** were analyzed using atomic force microscopy coupled to infrared spectroscopy (AFM-IR). The *treatment* animal study employed 11-25 year old cynomolgus monkeys with 9 Sham and 79 OVX animals. Ovariectomy's were performed in Oct 2008 and drug treatment was initiated in Sept 2010 and completed in Sept/Oct of 2012. Drug treatments included a clinical exposure of CatKi MK-0674 (0.6 mg/kg/d),¹⁵ a high exposure of MK-0674 (2.5 mg/kg/d), a clinical exposure of ALN (30 µg/kg/d/wk), and a ~10x clinical level of the anti-RANKL antibody denosumab (DMab; aka Prolia). MK-0674 differs from odanacatib by replacement of the methyl sulfone by a dimethylcarbinol. MK-0674 has been shown to be potent and selective with good pharmacokinetic and metabolic properties.¹⁵ As shown in **Figure 3.1**, both CatKi and ALN treatment exhibited substantial improvement in whole body, lumbar spine, and total hip (not shown) area bone mineral density (aBMD) with little improvement seen for DMab, potentially due to the development of neutralizing anti-drug antibodies.

Treatment-related Increases in Whole Body and Lumbar Spine aBMD

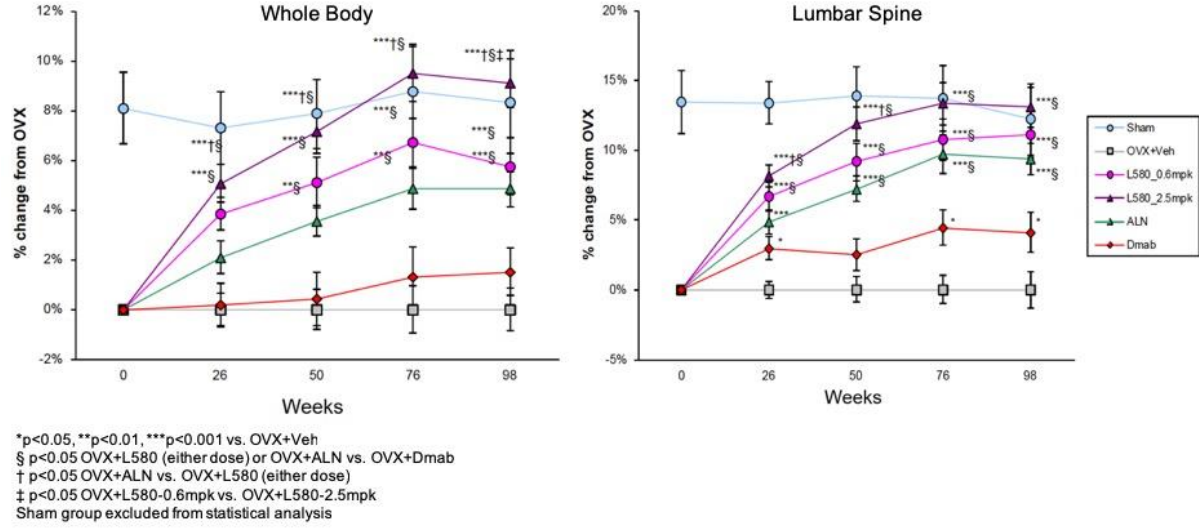


Figure 3.1 Treatment Related Increases in whole body and lumbar spine aBMD
 Graphs provided by L. Duong

In this study, AFM-IR data were collected on a cynomolgus monkey (MK-0674, ALN, Dmab) lumbar vertebrae set to meet the following study objectives. Collection of infrared spectral imaging and AFM-IR spectra of Type I collagen fibril microstructure for sham and OVX cynomolgus monkeys dosed with either vehicle, cathepsin-K (MK-0674), ALN, or denosumab in *treatment mode* (see **Table 3.2**). AFM images, local IR spectra (~50-100 nm area resolution; AFM-IR), and IR images were obtained for trabecular bone section of lumbar vertebrae. For every LV section, we obtained a minimum of 16 10 μ m AFM images and 32 3.5 μ m AFM images from 16 independent trabecular locations distributed in a representative fashion across the LV quarter. IR spectra were obtained in all locations. Additional IR imaging was done to further characterize collagen disorder (1740 cm^{-1}), mineral-to-matrix ratio (900 cm^{-1} – 1200 cm^{-1} /1550 cm^{-1} – 1800 cm^{-1}) spatial variation, mineralization (900 cm^{-1} – 1200 cm^{-1}) and mineral age (1128/1096 cm^{-1}) changes that will be discussed in depth in **Chapters 4 and 5**.

These data were employed to test the following hypotheses:

H1) Healthy lumbar vertebrae exhibit heterogeneity in protein structure including less disordered collagen, mineral-to-matrix ratio, and mineral composition at the level of individual trabeculae and within a single trabecula than OVX disease state.

H1a) Collagen disorder (1740 cm^{-1} peak)^{16,17} present in osteoporotic bone is a key marker of the increased bone turnover associated with osteoporosis.

H1b) Mineral-to-matrix ratio and mineral composition decreases as a function of OVX disease state and drug treatment partially recovers bone towards healthy state as observed for sham animals.

H2) Structure, compositional and spatial heterogeneity decreases in the OVX disease state and drug treatment at least partially recovers the bone towards the original, healthy state observed for sham animals.

H2a) These compositional changes occur independent of mineral age.

We use atomic force microscopy coupled to infrared spectroscopy (AFM-IR) to address the question of what specific protein and mineralization variation drives material changes as a function of osteoporosis and treatment.

Table 3.2. Summary of cynomolgus monkeys and drug treatments

Treatment Group	Sample Number	Treatment Administered
Sham	9	Vehicle-only
OVX	14	Vehicle-only
CatKi	14	MK-0674; 0.6 mg/kg/d
CatKi	15	MK-0674; 2.5 mg/kg/d
ALN	14	30 $\mu\text{g}/\text{kd}/\text{wk}$
DMab	13	2.5 mg/kg/mo

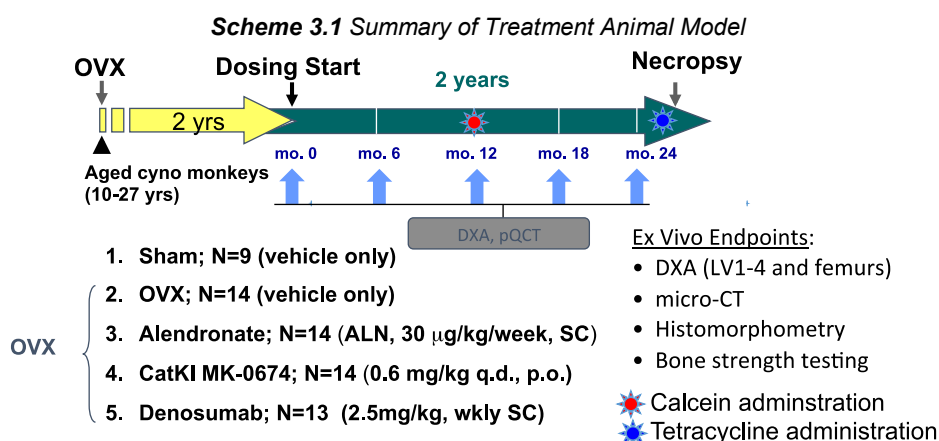
3.3 Methods and Biological Samples

3.3.1 Animal Treatment Model

The in-life portion of this study was conducted at Charles River Laboratories (CRL, Montreal, Canada) with the approval of both CRL and Merck IACUCs. The study protocol were based on those used for the Odanacatib Research Rhesus Investigational Study (ORRIS).⁴ Previously in the ORRIS study, all drug dosing, including the CatKi odanacatib (ODN), was administered in Rhesus monkeys in *prevention mode* (right after OVX surgery). In contrast, for this study, all drug dosing including the CatKi MK-0674¹⁵ was in *treatment mode* (initiated at 2 years post-OVX). Female Cynomolgus monkeys (10-27 years of age) were subjected to ovariectomy (OVX) or sham surgery. All monkeys were subsequently randomized into groups by weight and lumbar spine (LV1-4) bone mineral density (LsBMD). Baseline BMD values taken prior to drug treatment show a significant decrease in BMD for all OVX animals (10-15% decrease in BMD) indicating the animals became osteoporotic.¹⁴

Sham animals (n=9) were dosed daily with vehicle (hydroxypropyl methyl cellulose acetate succinate (HPMC-AS) polymer), per oreum (p.o., orally). OVX animals were randomized into 4 groups, vehicle (n=14), MK-0674 at 0.6 mg/kg p.o., quaque die (q.d., daily) (AUC=10.9±4.5; n=14), Alendronate (ALN) at 30 µg/kg/wk subcutaneous (SC) once-weekly (n=14), or Denosumab (DMab) at 2.5mg/kg, subcutaneous (SC) once-weekly (n=13). Drug administration was continued for 24-months. Prior to necropsy, bone mineral density of the lumbar spine was monitored with dual energy X-ray absorptiometry (DXA) and peripheral Quantitative Computed Tomography (pQCT) at baseline (before randomization and before dosing started) at 3, 6, 9, 12, 16, and 24-months after drug

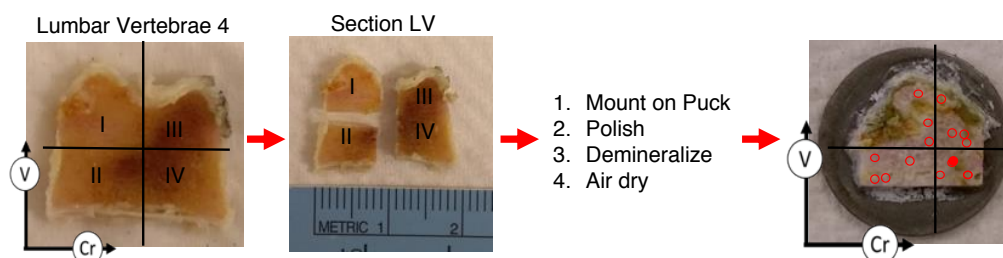
administration initiated (**Scheme 3.1**). Samples sent to the University of Michigan did not include the high exposure CatKi (MK-0674; 2.5 mg/kg/d) treatment group nor samples previously excluded by Merck, Inc., explaining the discrepancy in sample quantities between **Table 3.2** and **Scheme 3.1**.



3.3.2 Specimen Preparation and Mounting for AFM-IR Imaging

Lumbar Vertebrae 4 samples were extracted, sliced and provided to the Banaszak Holl research group at the University of Michigan. All samples were stored in 70% ethanol and kept at 4° C until the samples were prepared for AFM-IR imaging. Each vertebrae slice was sectioned into 4 quadrants arranged as shown in **Scheme 3.2** where V is ventral, and Cr is cranial. Quadrant 1 was mounted on a magnetic AFM puck, polished and demineralized for 30 minutes in 20% EDTA (0.5 M, pH = 8), and stored at 4° C when not imaging. Each sample was imaged at ambient atmospheric conditions in 16 different locations on the exposed trabecular surface using a nanoIR2 AFM-IR instrument (Anasys Instruments, California, USA) as detailed in the next section.

Scheme 3.2 Sample preparation for AFM-IR imaging and spectra collection

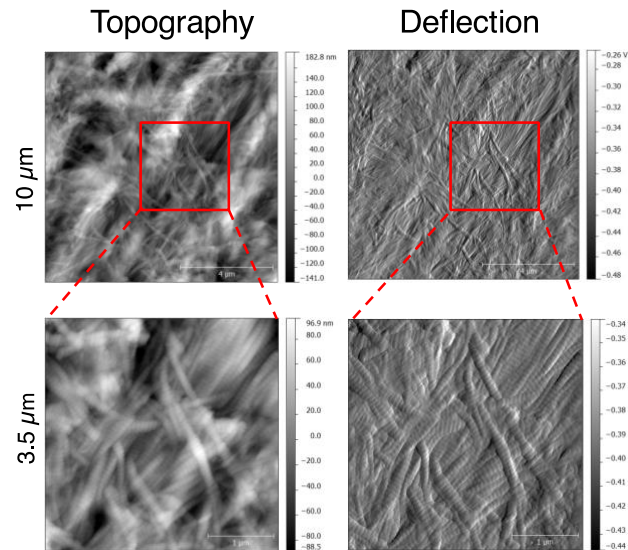


3.3.3 AFM Imaging and IR Spectra Collection

AFM-IR (nanoIR2 System, Anasys Instruments, California, USA) was used to obtain topographical information coupled to chemical composition for cross-sections of lumbar vertebrae 4 (LV4). AFM imaging was carried out using nanoIR2 Contact Mode nIR2 probes (gold coated silicon cantilever, nominal radius 25 nm, force constant 0.07-0.4 N/m, resonance frequency 13 ± 4 kHz). Line scan rates were 1 Hz, and the resolution was 512 pixels/line. A total of 16 locations were imaged per sample. Each location consisted of one 10 by 10 μm image and two 3.5 by 3.5 μm images collected within that 10 by 10 μm area, as illustrated in **Figure 3.2**. For each 3.5 by 3.5 μm location, an average localized IR spectrum consisting of 4 raw spectra was collected and smoothed using the Savitzky-Golay feature in Analysis Studio (Anasys Instruments, California, USA). IR spectra were taken at a resolution of $4 \text{ cm}^{-1}/\text{pt}$ using gold-coated contact mode nIR2 probes (Anasys Instruments, California, USA). See **Table 3.3** for a summary of all images and spectra collected for the entire sample set. Data acquisition was completed while blinded to sample identities.

Table 3.3. Summary of Data Collected with AFM-IR on all LV4 Samples

Total Number of Samples (All groups)	Number of Locations per Sample	Number of 10x10 μm Images	Number of 3.5x3.5 μm Images	Number of Averaged Localized IR Spectra	Number of Raw Localized IR Spectra
63	16	1008	2016	2016	8064

**Figure 3.2** Exemplar Topography and Deflection Images of Trabecular Bone

3.4 Results

For each location, four individual IR spectra were collected and averaged. In order to compare spectra between location, all spectra were normalized to the Amide I peak (1672 cm^{-1}). **Figure 3.3** provides an overall summary by providing an average of every spectrum collected for all locations per sample in a given treatment group. The mineral band intensities are similar for all treatment groups in the 1030 cm^{-1} region; however, both Sham and CatKi exhibit greater band intensity in the 1096 cm^{-1} region as compared to OXV, ALN, and DMac treatment groups.

When individual vertebrae averages are shown (**Figure 3.4**), compositional differences are observed for both protein ($1550\text{ cm}^{-1} - 1800\text{ cm}^{-1}$) and mineral bands (900

cm^{-1} – 1200 cm^{-1}) as a function of disease and treatment. Both protein and mineral band assignments are based on established literature assignments for bone.¹⁸ In terms of mineral content, averaged spectra from the Sham, OVX, ALN and DMar groups had similar numbers of samples with higher mineral content as well as samples with no mineral content. However, the CatKi treatment group had more vertebrae with low mineral content as well as samples with much higher mineral content than the other treatment groups. Mineral content heterogeneity will be further discussed in **Chapter 5**.

In addition, we observe variations in protein content (1550 cm^{-1} – 1800 cm^{-1})¹⁸ as a function of disease. This difference is particularly pronounced for unraveled collagen (1740 cm^{-1} peak)^{16,17} as further discussed in **Chapter 4**. The Sham group only had 1 vertebral sample with a prominent collagen disorder peak. All other treatment groups have at least 3 samples with OVX and ALN having 4 or more samples with prominent collagen disorder peaks. In addition, Amide II ($\sim 1550 \text{ cm}^{-1}$)¹⁹ variations are observed across and within groups.

Significant levels of both protein and mineral heterogeneity are observed as a function of location for each vertebra as illustrated in **Figure 3.5**. Each circle in panel A identifies the spectral location and spectra is color coded along with the AFM deflection image and IR spectrum. The size of the circles on each deflection image are representative of the estimated spatial region, based on probe used and imaging mode, from which the IR data was obtained.²⁰

Figure 3.5 highlights variations in total mineral content as a function of location, which is the area under the curve from 900 cm^{-1} to 1200 cm^{-1} . For further discussion see **Chapter 5**. In addition, we observe variation in the intensity of unraveled collagen in

vertebral bone (1740 cm^{-1} peak). All data acquisition for study objectives was completed as shown in **Table 3.4**.

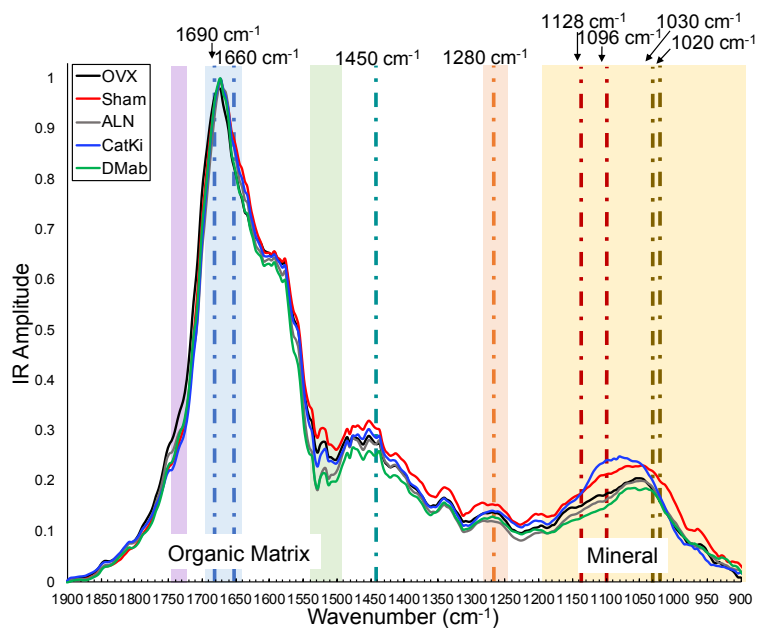


Figure 3.3 Averaged IR Spectra for each Treatment Group. Spectra are Normalized to Amide I peak; Wavenumbers of interest are based on the bone IR spectroscopy field's assignments summarized in **Table 5.1**.

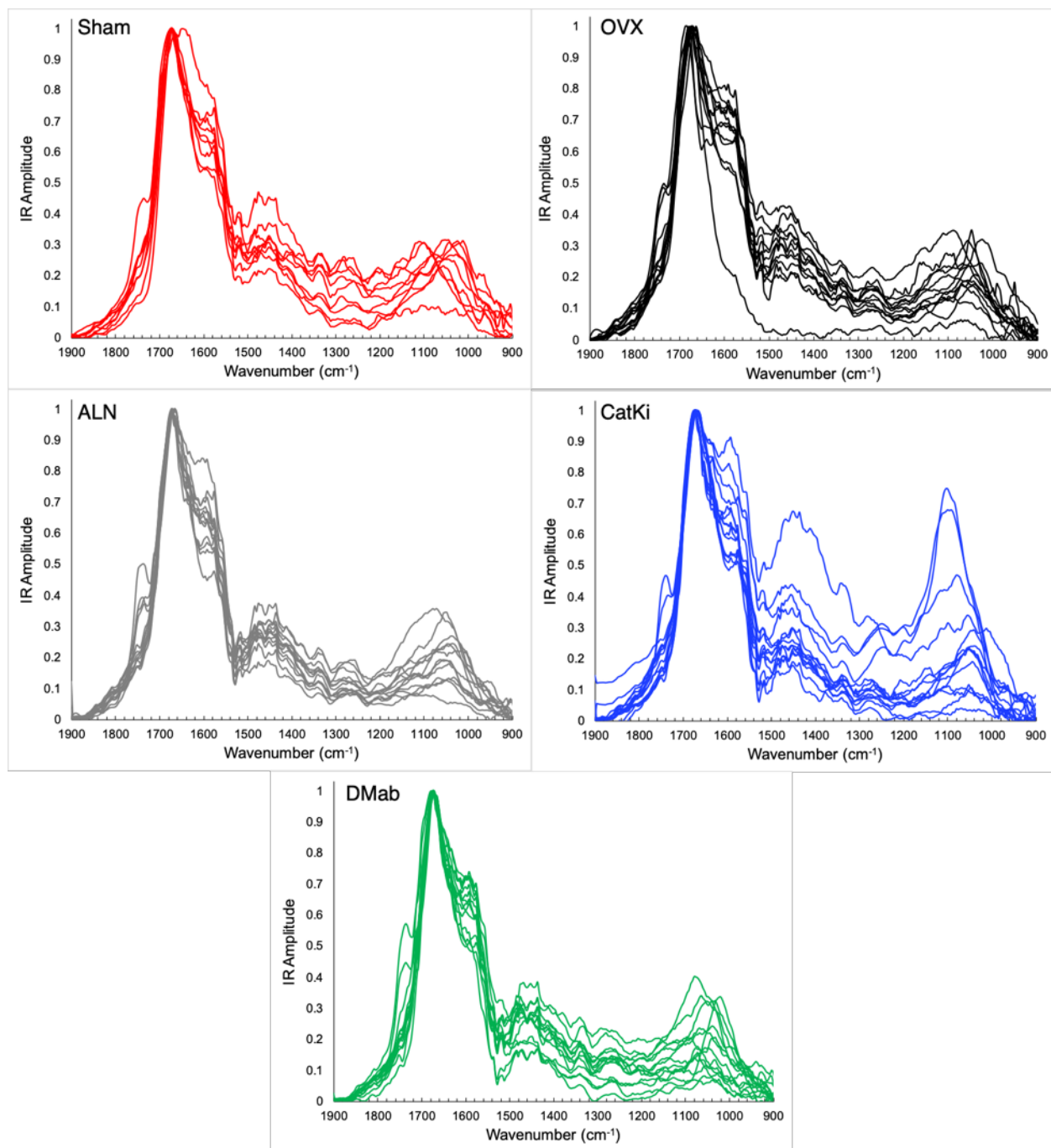


Figure 3.4 Average Localized IR Spectra for all Samples in each Treatment Group

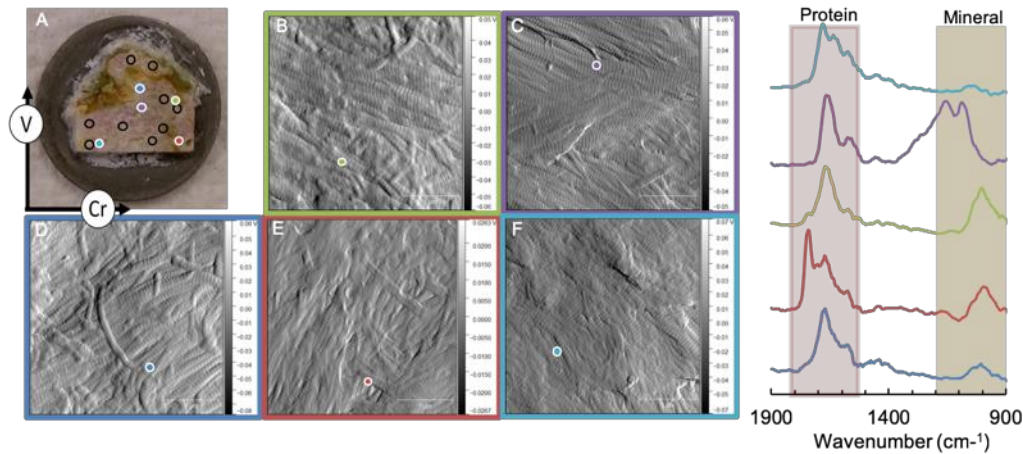


Figure 3.5 AFM-IR Spectra from ~100 nm regions of trabecular surface taken from 5 different locations.

Table 3.4. Summary of Data Collected with AFM-IR per Treatment Group

Treatment Group	Number of Samples	Number of 10x10 μm Images	Number of 3.5x3.5 μm Images	Number of Averaged Localized IR Spectra	Number of Raw Localized IR Spectra
Sham	9	144	288	288	1,152
OVX	13	208	416	416	1,664
ALN	13	208	416	416	1,664
CatKi	14	224	448	448	1,792
DMab	14	224	448	448	1,792

3.5 Discussion

With this data set, we are able to better understand the chemical compositional, structural and spatial heterogeneity differences of protein and mineral content as a function of OVX and treatment. In addition, we have been able to understand their effects on mechanical properties of trabecular lumbar vertebrae as discussed in **Chapter 2**. In upcoming chapters unraveled collagen as a biomarker for osteoporosis, mineralization heterogeneity and chemical heterogeneity's effect on mechanical properties will be discussed.

As discussed in **Chapter 2**, mechanical properties of lumbar vertebrae were preserved with CatKi treatment despite no detectable changes in bone microstructure by

μ CT analysis were observed. These results suggest that important changes in bone material composition contribute to the observed mechanical differences.

In **Chapter 4**, we will discuss how collagen disorder can be used as a biomarker for osteoporosis. We observe a prominent peak that has been reported in the bone IR literature before simply as a carbonyl stretch (C=O). This peak results from an unwinding of the triple helix that exposes collagen's backbone's carbonyls generating a prominent 1740 cm^{-1} peak.²¹ We hypothesize this peak is a key marker of the increased bone turnover associated with osteoporosis as stated in **H1a**.

In **Chapter 5**, we observe differences in the mineral-to-matrix ratio (900 cm^{-1} - 1200 cm^{-1} / 1550 cm^{-1} - 1800 cm^{-1}) and in acid phosphate substitution (1128 cm^{-1} / 1096 cm^{-1} intensity ratio).^{18,22} In bone, the mineral peak is a manifold of about a dozen vibrational groups that appear in similar IR ranges. Acid phosphate substitution indicates younger mineral, and mineral to matrix ratios indicate chemical heterogeneity in the bone.^{18,22} **Chapter 5** will further discuss **H1b** and **H2a**.

For the remainder of this thesis, the reader can expect in depth discussions on the chemical compositional heterogeneity, spatial distributions and variations observed as a function of disease and treatment, possible mechanisms that lead to these variations, and the biomechanical implications of these variations.

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Chapter 4

Unraveled Collagen as a New Marker for Osteoporosis in Vertebral Bone

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4.1 Abstract

Nano- to microscale chemical heterogeneity plays a role in fracture prevention for osteoporotic bone. In this study, we seek to understand changes in unraveled collagen linked to disease and treatment in lumbar vertebral trabecular bone using a treatment-based animal model. A group of cynomolgus monkeys were either ovariectomized (OVX) or submitted to sham surgery. Two years post-surgery, OVX animals were treated with vehicle (VEH), alendronate (ALN; 0.03 mg/kg weekly), the cathepsin K inhibitor MK-0674 (CatKi; 0.6 mg/kg daily), and denosumab (DMab; 2.5 mg/kg weekly subcutaneously) for two years and compared to a control Sham surgery group. To understand changes in structure and composition, atomic force microscopy - infrared spectroscopy (AFM-IR) was used to obtain topographical information coupled to chemical composition/structure for cross-sections of lumbar vertebrae 4 (LV4). A total of 16 locations were imaged per sample, a 10 by 10 μm and two 3.5 by 3.5 μm images were obtained as well as a localized IR spectrum per 3.5 by 3.5 μm image. The average unraveled collagen content for each group was evaluated. Average unraveled to intact collagen integrated peak area ratios

(1732 - 1748 cm^{-1} /1656 - 1672 cm^{-1}) obtained for Sham, OVX, ALN, and CatKi groups were 0.26 (0.1), 0.33 (0.1), 0.29 (0.1) and 0.25 (0.1) which all differ from the 0.14 (0.06) value for the D Mab group respectively ($p < 0.05$). These results for each treatment indicate the level of unraveled collagen for CatKi is similar to Sham whilst ALN is similar to OVX. D Mab treatment differs from all other groups with a substantially lower level of unraveled collagen present. AFM-IR unraveled collagen ratio maps were collected in five 20 x 20 μm areas per treatment group for total of 50 ratio maps. Maps showed unraveled collagen domain sizes ranging ~300 nm to 2 μm . We hypothesize that unraveled collagen heterogeneity that appears as a function of OVX is a chemical marker of increased bone turnover.

4.2 Introduction

Osteoporosis is a degenerative bone disease characterized by bone loss due to a net increase in bone turnover. This disease commonly leads to bone fractures at the wrist, vertebrae and hip.¹⁻⁵ Current diagnostic methods, such as bone mineral density (BMD) and Fracture Risk Assessment Tool (FRAX), consider mineralization, yet fail to capture the role of collagen material changes in bone fragility.^{1,6-8} The role of collagen in bone and its effects on biomechanical properties are not fully understood; however, recent studies have demonstrated the importance of considering collagen's nanoscale structure and microscale organization.^{1,7-21}

As discussed in **Chapter 1** and briefly in **Chapter 2**, nano- to microscale bone heterogeneity likely plays a crucial role in understanding bone mechanical properties and their role in fracture-risk.^{16,19,22-29} To further understand the impact of compositional

heterogeneity on elasticity and crack propagation, a computational study using finite element modelling was developed and published to understand fracture resistance in cortical bone.²⁵ This computational study demonstrated that the presence of younger bone, which contains small or imperfect mineral crystals and immature crosslinks, and more mature tissue in bone is important for fracture resistant bone.²⁵ Reduced mineral and protein matrix heterogeneity was observed in patients treated with bisphosphonates when compared to non-treated patients (both human and canine).^{16,19} This reduction in heterogeneity affects tissue-level toughening mechanisms.^{16,17,19,30} In addition, lack of heterogeneity in hardness and modulus, two important mechanical properties, have been observed to worsen bone quality at the nano-scale in cortical and trabecular bone.¹⁹ Taking this into consideration, inelasticity variation plays a dominant role in energy dissipation in bone as explored experimentally and computationally.^{15,20}

In **Chapter 2**, compressive yield and peak load increases were observed for CatKi treatment when compared to OVX despite no significant differences in trabecular microstructure as measured by μ CT.³¹ This is the first time a preservation in mechanical properties has been observed for an antiresorptive osteoporosis drug administered in *treatment mode*.³¹ These results implied protein material changes were occurring and that μ CT is not sensitive to the nature of protein-level chemical changes as they may not induce a net change in the electron density of the material. We hypothesize these material changes occurring at a nanoscale correct for deficiencies in the bone quantity and/or density.

In this study, we use atomic force microscopy coupled to infrared spectroscopy (AFM-IR) to explore bone composition changes as a function of OVX and drug

treatments. More specifically, we noted the presence of increased *unraveled collagen* as compared to Sham for OVX treatment. Upon OVX followed by drug treatment, the extent of unraveled collagen decreases and even returns to the same level as Sham for the case of CatKi. DMab treatment shows an even lower level of unraveled collagen than Sham. Unraveled collagen has been previously observed in tendon³², ligament³³ and parchment¹⁸ with the presence of a 1740 cm⁻¹ feature in IR spectra. We hypothesize that unraveled collagen, as detected by the 1740 cm⁻¹ peak present in bone, is a key marker for the increased bone turnover associated with osteoporosis.

4.3 Methods

4.3.1 Treatment Animal Model

The *treatment* animal model used in this study has been previously published and was discussed in detail in **Chapter 2 and Chapter 3**.³¹ Briefly, Merck, Inc. collected 63 cynomolgus monkeys, performed ovariectomies, and two years post-surgery treated them with the bisphosphonate Alendronate (ALN), a cathepsin K inhibitor MK-0674 (CatKi), and the antibody therapeutic denosumab (DMab) for two additional years. Longitudinal dual-energy x-ray absorptiometry (DXA), in-vivo periphery quantitative computer tomography (CT), and biomarkers were utilized to track the progression of the disease for each subject as well as post-necropsy μ CT and mechanical testing (3-point bending of femurs and compression of LV) to further understand mechanical properties and microstructure of the bones.³¹ Data collected in-vivo during the *treatment* animal study will not be discussed. For a visual summary, see **Table 3.1** and **Scheme 3.1**.

4.3.2 Sample Preparation, AFM-IR Spectra Collection, AFM Imaging and Processing

Methods used to prepare each lumbar vertebrae (LV) section for AFM imaging and IR spectra collection were discussed in **Chapter 3**. Briefly, each LV was sectioned, mounted on an AFM puck, polished and demineralized using EDTA to expose collagen fibrils (**see Scheme 3.2**). Each sample was imaged in a total of 16 locations using AFM-IR (nanoIR2, Anasys Instruments, Goleta, CA) in contact mode. Each location consisted of a 10 x 10 μm and two 3.5 x 3.5 μm images as shown in **Figure 3.1**. For each 3.5 x 3.5 μm image, a total of 4 IR spectra were collected in the same spot and averaged using Analysis Studio (Anasys Instruments, Goleta, CA). For a summary of all images and spectra collected, see **Table 3.2**.

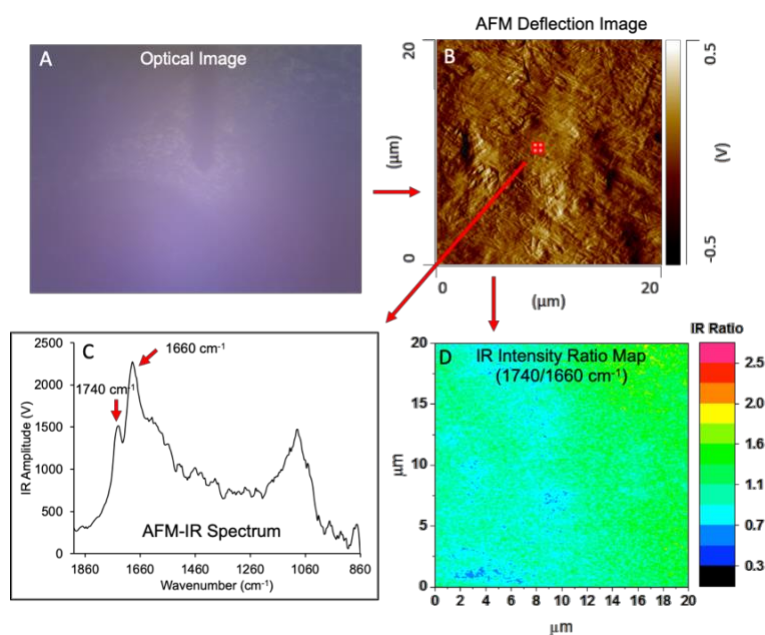
Each averaged spectra was baseline corrected using Origin. After baseline correction, each spectra was normalized to the Amide I peak using Excel (Microsoft Office). To obtain integrated peak area ratios of each baseline-corrected AFM-IR spectra, an Excel (Microsoft Office) macro was used to integrate all the areas under the curve. A one-way analysis of variance (ANOVA) was used to determine treatment group differences for unraveled collagen integrated peak area ratios. Tukey's Test was used to detect differences in ratio means between specific treatment groups. Skewness was evaluated to understand the types of distributions found in our data. All statistical analysis was performed using R. All data are presented as mean $\pm$ (standard deviation).

4.3.3 AFM-IR Mapping and Image Analysis

AFM-IR maps were collected using a nanoIR2 System (Anasys Instruments, Goleta, CA, USA) in contact mode using nIR2 probes (Anasys Instruments, Parameters:

gold coated silicon cantilever, nominal radius 25 nm, force constant 0.07 - 0.4 N/m, resonance frequency 13 ± 4 kHz). Before each map, four IR spectra were taken at the minimum required power and averaged for the region's spectrum. The IR spectrum detection range was set between 860-1900 cm^{-1} . One IR map was collected for Amide I with unraveled collagen triple helix (1740 cm^{-1}), Amide I with intact triple helix (1660 cm^{-1}), and mineralization at 1128 cm^{-1} and 1096 cm^{-1} . Unraveled-to-intact collagen ratio map ($1740/1660 \text{ cm}^{-1}$) was calculated using Analysis Studio (Anasys Instruments, Goleta, CA, USA). For a visual summary, see **Scheme 4.1**.

Scheme 4.1: AFM-IR Spectrum and Intensity Ratio Map Collection



To quantify the domain sizes, each AFM-IR ratio map was analyzed using Fiji (National Institute of Health, United States).³⁴ Each map was imported into Fiji, the scale was set to 0.01 pixel/nm, the threshold set to 0.93 – 1.60 (range of values that corresponds to green in all AFM-IR maps), and the minimum particle size was set to 224 nm. The image was converted to a mask and then converted to a watershed image to

obtain particle areas using the Analyze Particle feature. Assuming the domains were circular, particle areas were converted to domain sizes using Excel (Microsoft Office).

4.4 Results

In **Figure 4.1**, we observe variations in protein content as a function of disease. The black arrow indicates the Amide I peak at 1740 cm^{-1} indicative of collagen triple helix unraveling, or denaturation.^{18,35}

For Sham control group, one out of 9 samples has an unraveled collagen peak at 1740 cm^{-1} . The mineralization band for that sample was similar to the other samples in the Sham group. In addition, one out of 9 samples had an Amide I peak maximum at 1648 cm^{-1} as well as a remarkably low level of mineralization, when the rest exhibited the Amide I maximum at the same wavenumber (1672 cm^{-1}). The mean unraveled collagen ratio for Sham was 0.26 (0.1). For OVX control group, 4 out of 13 samples have an unraveled collagen peak at 1740 cm^{-1} . One of the 4 samples with an unraveled collagen peak had remarkably low mineralization. The mean unraveled collagen ratio for OVX was 0.33 (0.1). For ALN treatment group, 4 out of 13 samples have an unraveled collagen peak at 1740 cm^{-1} . One of the 4 samples with an unraveled collagen peak had remarkably low mineralization. The mean unraveled collagen ratio for ALN was 0.29 (0.1). For CatKi treatment group, 2 out of 14 samples have a unraveled collagen peak at 1740 cm^{-1} . The mean unraveled collagen ratio for CatKi was 0.25 (0.1). Two out of 14 CatKi spectra also exhibited large increases in mineralization at 1100 cm^{-1} , and in general, CatKi showed the greatest dispersion of mineralization ratios.

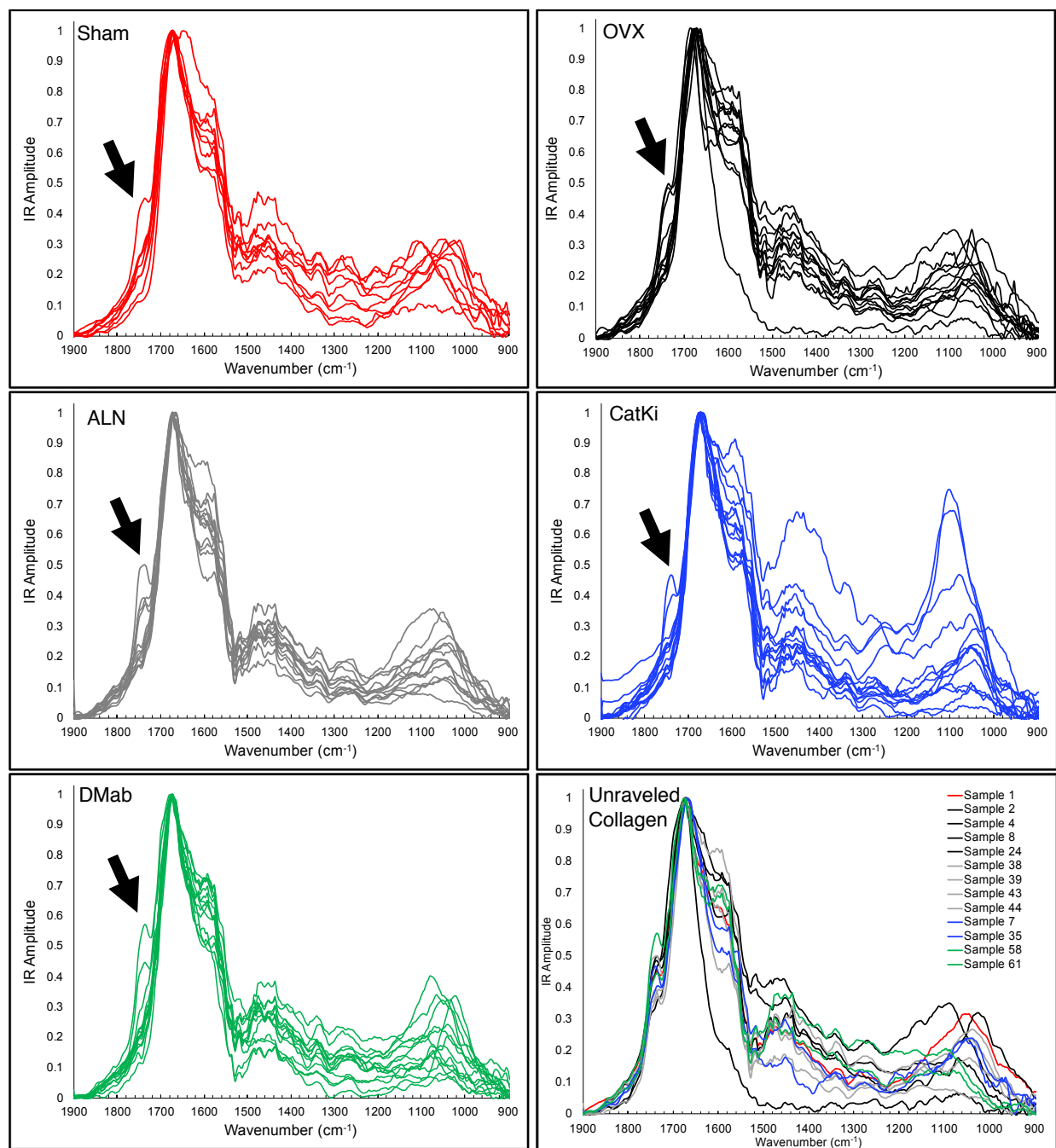


Figure 4.1 IR spectra averaged across all locations for each vertebra sample *shown grouped by treatment*. For each individual average spectrum, at least 32 spectra taken from representative regions across the vertebra and normalized to Amide I peak ($\sim 1672\text{ cm}^{-1}$) intensity. The black arrow indicates the presence of an Amide I peak at 1740 cm^{-1} indicative of collagen triple helix unraveling (denaturation). The bottom right panel is a compilation of all spectra with a prominent collagen triplex helix unravelling peak.

For DMap treatment group, 2 out of 14 samples show an unraveled collagen peak at 1740 cm^{-1} . The mean unraveled collagen ratio for DMap was 0.14 (0.06). The presence of an unraveled collagen peak did not show a connection with mineralization level.

In **Figure 4.2**, the degree of protein heterogeneity observed in an ALN sample is illustrated. These data are representative of the level of heterogeneity observed throughout the entire data set. Chemical differences may also contribute to differences in triple helix/water stoichiometry, variation in cross-linking, and variation in collagen types. Panel A is a photo of the mounted sample with black circles showing each imaged location with dots showing the imaged locations that are presented in 2B, 2C, 2D, 2F, 2E, and the spectra shown. Spectra colors and image borders correspond with the color of the dot in 2A. Panels B, C, D, E, and F are $3.5 \times 3.5\text{ }\mu\text{m}$ deflection images, and the colored dots represents where the IR spectra were taken on each image. The size of the circles on each deflection image are representative of the spatial resolution from which the IR data was obtained.

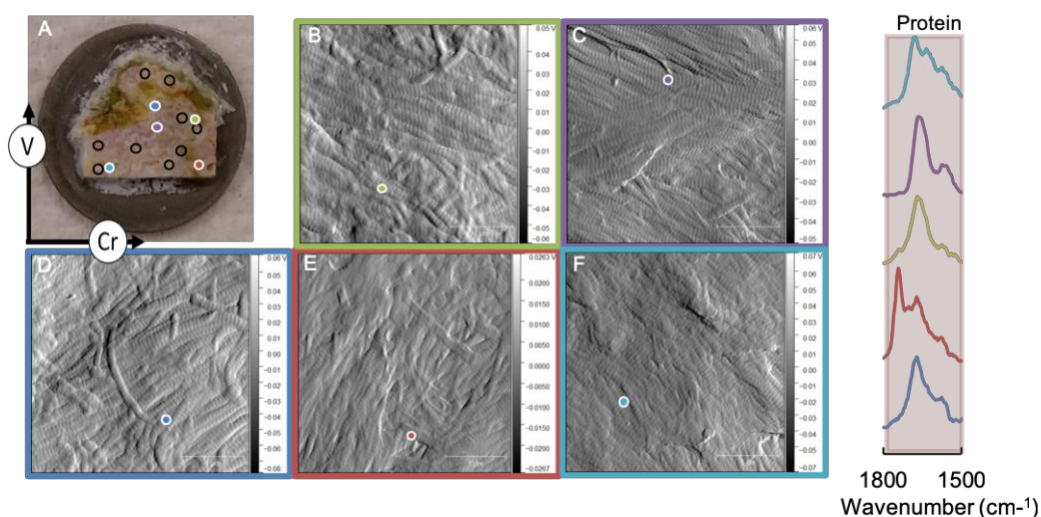


Figure 4.2 Heterogeneity in collagen protein structure in an individual vertebrae ALN sample. Spectral changes as a function of location include the presence of collagen triple helix denaturation leading to the presence of a feature at 1740 cm^{-1} . Each spectrum's color corresponds to the AFM image and locations on Panels A-F.

In **Figure 4.3**, unraveled collagen integrated peak area ratio box and whisker plots for each treatment group show the range of ratios present in all treatment groups with ALN having the largest range of values and D Mab with the smallest range of values. D Mab was significantly different from all other groups ($p < 0.05$). No significant difference was observed between ALN, CatKi, Sham and OVX. Unraveled collagen ratios (1732 - 1748/1656 - 1672 cm^{-1}) obtained for Sham, OVX, ALN, and CatKi groups were 0.26 (0.1), 0.33 (0.1), 0.29 (0.1) and 0.25 (0.1) respectively, which all differ from the 0.14 (0.06) value for the D Mab group ($p < 0.05$). These values correspond the filled dots in **Figure 4.3**.

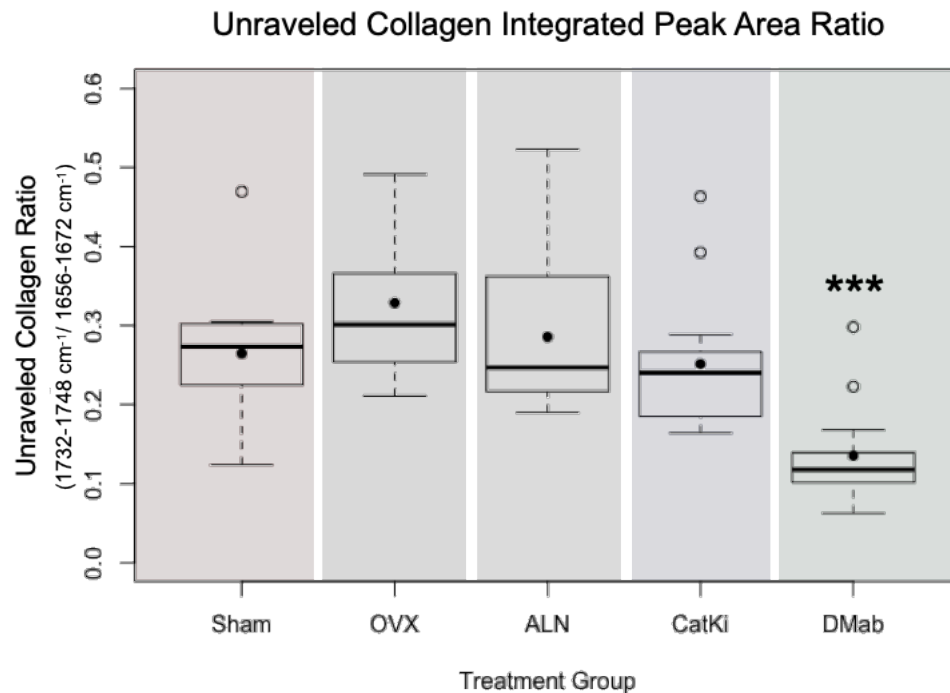


Figure 4.3 Unraveled Collagen Integrated Peak Area Ratio Box and Whisker Plot

Unraveled collagen integrated peak area ratio box and whisker plot for each treatment group show the range of ratios present in all treatment groups with D Mab being significantly different from all other groups. No significant difference was observed between ALN, CatKi, Sham and OVX. All Sham, OVX, ALN, CatKi and D Mab samples are highlighted with boxes in red, black, grey, blue and green, respectively.

** $p < 0.05$ vs. Sham, OVX, ALN, and CatKi

Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

From **Figure 4.3** we can observe that the means (filled dot) and medians (line) differ for all treatment groups prompting the evaluation of their distribution skewness. Skewness measures the symmetry of data distributions. The skewness obtained for Sham, OVX, ALN, CatKi and DMab were 1.3, -0.01, -0.6, -0.3, and 0.8, respectively. Sham has a highly skewed data distribution. ALN and DMab have a moderately skewed data distribution. OVX and CatKi have fairly symmetrical data distributions. When skewness is negative, means of the data are less than the medians, and the distributions are skewed toward higher values of the unraveled/intact ratio.. When skewness is positive, means of the data are larger than the medians, and data distributions are skewed toward lower values of the unraveled/intact ratio. A discussion of biological significance of these results can be found in the **Discussion** section.

Given the unraveled collagen heterogeneity observed, the spatial distribution of unraveled collagen across a sample in each treatment group was explored. The nanoIR2 AFM-IR has the capability of mapping one specific wavenumber over the surface of a sample.³⁶ Because of this, when running the experiment wavenumbers representative of the maximum peak intensity for unraveled (1740 cm^{-1}) and ordered (1660 cm^{-1}) collagen were chosen. After each wavenumber map is collected, IR intensity ratio maps were calculated using Analysis Studio. IR intensity ratio maps are used, instead of individual wavenumber maps, to eliminate any tip effects or bias introduced by the probe while imaging.

In **Figure 4.4**, exemplar AFM-IR unraveled collagen ratio maps show collagen unraveled-to-intact ratio heterogeneity and spatial distribution in a $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m}$ area. The left column has 4 IR intensity ratio maps that summarize the domain sizes and areas

observed across all IR intensity ratio maps collected. Each IR intensity map has their corresponding IR spectrum to the right. All IR spectra have 1740 cm^{-1} and 1660 cm^{-1} highlighted with a black dotted line. For maps with higher unraveled-to-intact collagen ratios, the spectra show a similar trend. Each IR spectrum was taken at, or as close as possible to, the middle of the image.

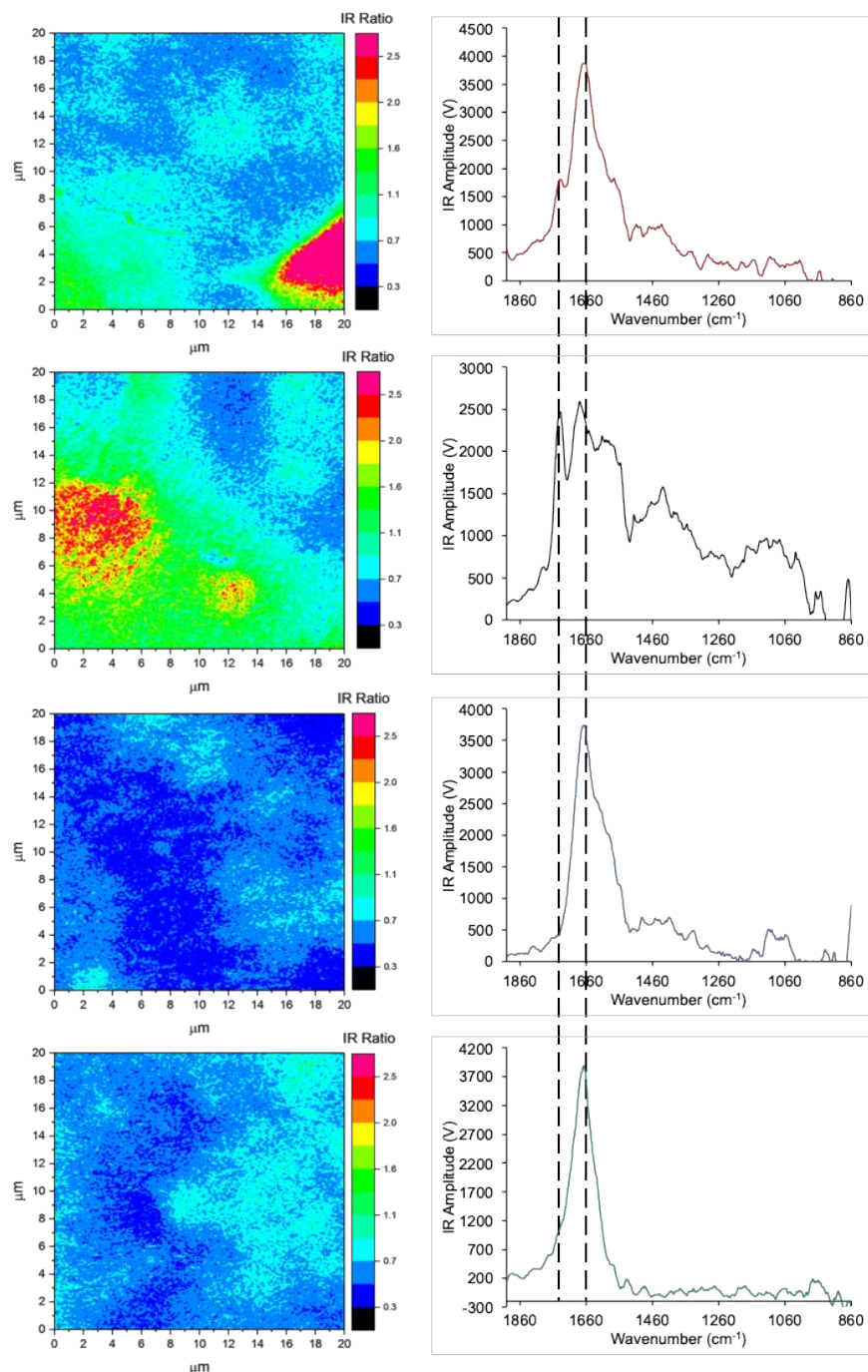


Figure 4.4 IR Maps of Unraveled/Intact Collagen 1740/1660 Intensity Ratio.

Each map represents a region of 20x20 μm and shows measured IR ratios of unraveled collagen (as measured by 1740 cm^{-1} intensity; shown with a black dotted line) over intact Amide I (as measured by 1660 cm^{-1} intensity; shown with a black dotted line). Each map has the corresponding AFM-IR spectrum to the right. All regions imaged can be found in **Appendix A**.

The ratio range presented in all maps is 0.25 (blue) to 2.5 (red). All maps from high unraveled collagen samples show overall higher ratios than low unraveled collagen samples. Most maps show circular shaped domains with higher and lower ratio values that range from ~300 nm to 2 μ m in size, as shown in **Figure 4.5**. This trend is observed across all maps obtained shown in **Appendix A**.

In order to determine the domain sizes in each map, image analysis of all ratio maps was performed using Fiji³⁴ assuming the domains were circular. The domain size analysis had the minimum set to 224 nm due to the program determining a single pixel was a particle. **Figure 4.5** summarizes the domain sizes obtained for all AFM-IR ratio maps collected. Domain sizes were as large as 2 μ m for one Sham and one CatKi ratio map collected. Most domain sizes were 500 nm to 1.6 μ m in diameter. No discernable trend is observed between level of unraveled collagen and domain sizes in each treatment group.

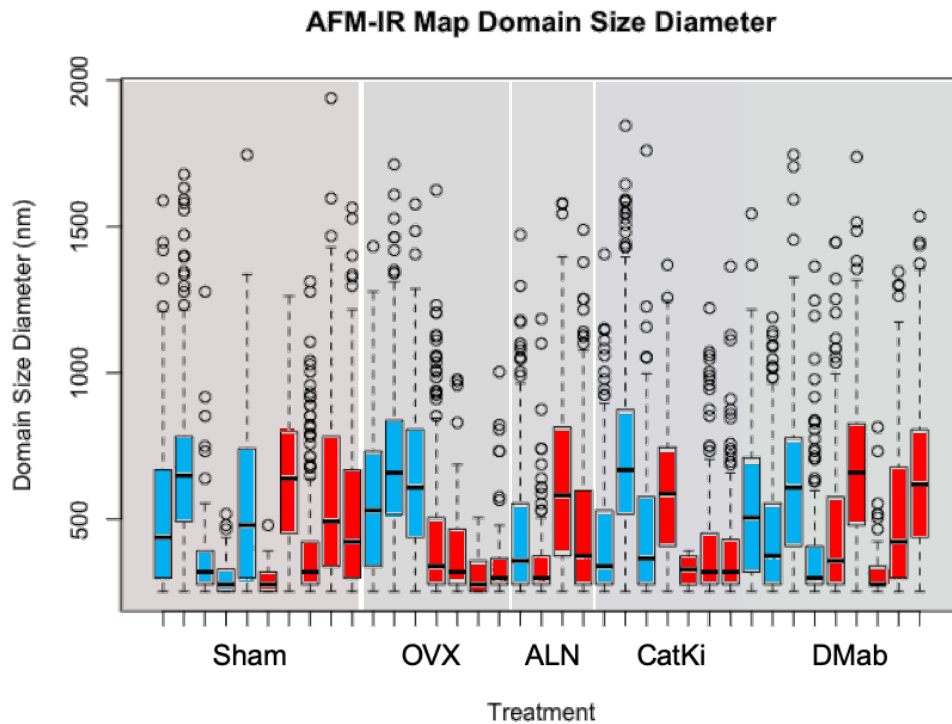


Figure 4.5 AFM-IR Unraveled Collagen Ratio Map Domain Sizes

All Sham, OVX, ALN, CatKi and D Mab samples are highlighted with boxes in red, black, grey, blue and green, respectively. Domain sizes go out to $\sim 2 \mu\text{m}$. For this analysis, domain size minimum was set to 224 nm. All Sham, OVX, ALN, CatKi and D Mab samples are highlighted with boxes in red, black, grey, blue and green, respectively. Blue boxes represent higher unraveled collagen samples, and red boxes represent lower unraveling samples. Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

4.5 Discussion

Osteoporosis is a disease characterized by higher bone turnover rates that lead to a net bone loss.^{1–5} We hypothesize higher unraveled-to-intact collagen ratios present as a function of OVX is associated to the increased bone turnover characteristic of osteoporosis. In this study, we observe higher unraveled-to-intact collagen ratios as a function of OVX, lower ratios in Sham group, and ratios that fall in between Sham and OVX control for ALN and CatKi groups. OVX has the highest unraveled collagen ratio at 0.33 (0.1), followed by ALN with a ratio of 0.29 (0.1), Sham with 0.26 (0.1) and CatKi with 0.25 (0.1). However, D Mab was significantly different from all other groups ($p < 0.05$) with

an unraveling ratio of 0.14 (0.06). Notably, 10 of the 14 DMab subjects developed a neutralizing anti-drug anti-body during the study.

The difference observed between the means (filled dot) and medians (line) for all treatment groups prompted an evaluation the data skewness. Skewness measures the asymmetry of data distributions. Sham has a highly skewed data distribution, ALN and DMab have a moderately skewed data distribution, and OVX and CatKi have fairly symmetrical data distributions. The high levels of skewness result from a subset of each treatment group having higher collagen unraveled-to-intact ratios. This led us to separate each treatment group into samples with and without unraveled collagen present, as shown in **Figure 4.6**. In **Figure 4.6**, we see that all groups of the samples with unraveled collagen have higher ratio values with ranges that do not overlap with each other, with the exception of OVX. Also, DMab has overall the smallest values, with the highest ratio group having values similar to the normal range of unraveled/intact collagen ratio as defined by the bulk of the Sham samples.

Assuming that unraveled collagen can be used as a chemical marker of bone turnover, we can identify 3 distinct groups represented in **Figure 4.6**. Based on this assumption, these groups are: normal turnover (ratios ranging 0.15 – 0.35), high turnover (0.35 and up) and low turnover (0.15 and lower). The normal range is defined by Sham (N=8) set. One Sham sample exhibited a high turnover rate similar to that of OVX (N=5), ALN (N=4) and CatKi (N=2) and we speculate that the one Sham animal may have had *osteoporosis*, or a related bone turnover disease. The high turnover range population increase with OVX treatment (N=5). Interestingly, ALN treatment has only one less number of animals in the normal (N=9) and high turnover (N=4) ranges. The overall level

of turnover, as indicated by the unraveled/intact ratio, decreased when compared to OVX. CatKi has a lower number of samples in high turnover range (N=2) than ALN and OVX with most of the animals falling in the normal range (N=12). Curiously, D Mab treatment results in only 2 animals in the normal turnover range, with 12 animals in low turnover range. Low turnover is even lower than the nominal normal range defined by Sham. In D Mab's case, given the neutralizing anti-drug antibody development, we hypothesize that the drug, or the developed antibody, induced a substantial decrease in bone turnover for these animals, potentially completely shutting down turnover.

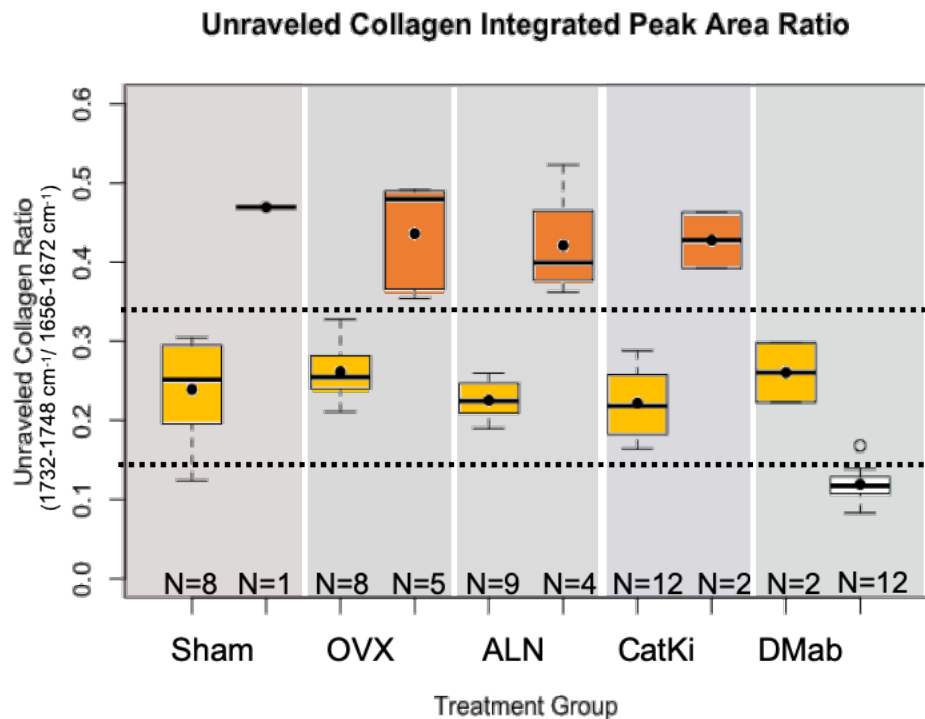


Figure 4.6 Unraveled Collagen Integrated Peak Area Ratio Box and Whisker Plots Showcasing 3 Distinct Groups All Sham, OVX, ALN, CatKi and D Mab samples are highlighted with boxes in red, black, grey, blue and green, respectively. Yellow boxes represent higher turnover samples, Orange boxes represent normal turnover samples, and white boxes represent lower turnover samples.

*** $p < 0.05$ vs. Sham, OVX, ALN, and CatKi

Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile,

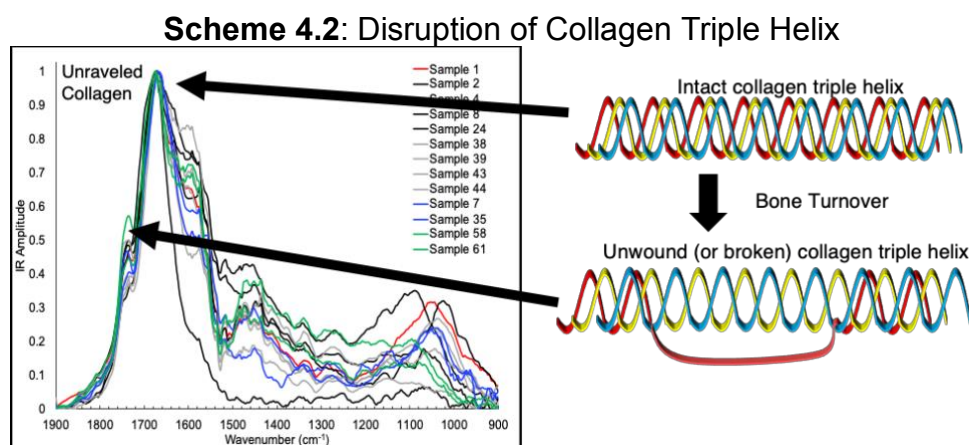
Given the unraveled-to-intact collagen heterogeneity observed, we explored the spatial distribution using IR intensity ratio maps. Maps show the extent of the unraveled-to-intact collagen heterogeneity with maps showing areas of low ratios next to areas of high ratios. Low ratios indicate low intensities for unraveled collagen, and vice versa for high ratios. Most areas in these maps are circular with domain sizes ranging from 500 nm to 1.6 μ m. We hypothesize these domains could correspond to bone turnover activity. This will be further discussed in **Chapter 6**.

No noticeable (with plain eyesight) morphology changes were observed as a function of unraveled collagen. This makes areas with unraveled collagen indistinguishable from intact collagen areas based on morphology (that can be observed with plain eyesight). This calls for micrometer-level morphology analysis to be conducted on AFM images collected for this data set. This will be further discussed in **Chapter 6**.

ALN, CatKi and DMab are antiresorptive drugs that inhibit the bone resorption that we hypothesize leads to the production of unraveled collagen during remodeling processes, particularly for CatKi, which directly targets cathepsin K, the enzyme found in osteoclasts that degrades collagen.^{37,38} Given that cathepsin K degrades collagen, inhibiting this enzyme may result in the preservation of intact collagen leading to a smaller unraveled/intact collagen ratio when compared to OVX. Regarding DMab, we know from the biomarker data collected by Merck, Inc. (data not shown) developed neutralizing anti-drug anti-body explaining the lack of BMD recovery in this group (**Figure 3.1**). We hypothesize the neutralizing anti-drug anti-body development could have played a role to fully shut down bone turnover leading to an increase in the unraveled collagen present. Compression testing was done on DMab samples and no significant difference was

detected. We chose not to publish DMap data in **Chapter 2** due to the development of neutralizing anti-drug anti-bodies.

The unraveled collagen we observe was assigned in the literature as an unwinding of the triple helix which exposes collagen's backbone carbonyls generating a prominent 1740 cm^{-1} peak, as illustrated in **Scheme 4.2**.¹⁸ Unraveled collagen has been previously observed in tendon³², ligament³³ and parchment¹⁸ with the presence of a 1740 cm^{-1} feature in IR spectra.



This unraveled collagen assignment to a 1740 cm^{-1} feature was also done on parchment.¹⁸ In this study, they used nanoscale and non-linear optical spectroscopy to finding a non-invasive way to probe collagen denaturation. They tested gelatin (unraveled collagen) and collagen standards using AFM-IR, and showed a new band at 1738 cm^{-1} for gelatin. This feature most like comes from acidification from degradation. They note that this new band may not have been observed before due to the spatial resolution of conventional techniques when compared to the nano-scale resolution of AFM-IR.

In a recent study, nanoscale chemical heterogeneity was evaluated in anterior cruciate ligament (ACL) of the knee.³³ This was a comparative study on cadaver ACLs

where the chemical composition heterogeneity and microstructure of the tissue was explored to help understand what causes ACL failure in non-contact accidents.³³ They found evidence of unraveled collagen, or the unwinding of the collagen molecule, caused by mechanical loading.³³ Their evidence consisted of a 1740 cm^{-1} feature in nanoscale infrared spectroscopy (AFM-IR), previously assigned in the literature as denatured collagen.¹⁸ To confirm this assignment, they employed a collagen hybridizing peptide (CHP) sequence conjugated to TAMRA dye designed to have a strong and specific binding to Type I collagen.^{32,33} CHP binds specifically to unfolded, or denatured, collagen triple helix making it the ideal tool to determine and quantify the presence of unraveled collagen.^{32,39} They used this conjugated CHP-TAMRA dye to stain and identify regions with unraveled collagen.³³ In this study, authors concluded that greater amounts of unraveled collagen meant degraded physical properties.³³

The nano-scale resolution used in this study sheds light on compositional differences that were not previously reported with conventional techniques. In addition, the lack of reporting 1740 cm^{-1} as a peak related to collagen in the literature could be due to the practice of embedding bone in poly(methylmethacrylate) (PMMA), which generates an overlap peak in the 1740 cm^{-1} region. In addition, for traditional IR microscopy, bone is dehydrated and this process itself generates a substantial degree of collagen denaturation and therefore the presence of the 1740 cm^{-1} peak. The estimated size of compositional heterogeneity is estimated to be $\sim 200\text{ nm}$.²⁷ However, most conventional spectroscopic techniques have spatial resolution on the order of micrometers. This combination of effects gives the AFM-IR technique some distinct differences as compared to conventional spectroscopic techniques employed in the bone field.

In conclusion, we hypothesize higher unraveled-to-intact collagen ratios present as a function of OVX is associated to the increased bone turnover characteristic of osteoporosis. We were able to identify 3 ranges of the unraveled to intact parameter: a normal range defined by a group of Sham animals; a high ratio range induced by OVX and partially remediated by drug treatments that we hypothesize is related to increased bone turnover rates; a low ratio range resulting from DMab treatment associated with a decreased bone turnover rate. With nano-scale resolution, we are capable of detecting compositional changes smaller than those detected with conventional spectroscopic techniques. More work is needed to test the hypothesis that unraveled collagen can be used as a chemical marker of increased bone turnover and/or osteoclastic activity.

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Chapter 5

Changes in Nanoscale Mineral and Organic Matrix of Lumbar Vertebrae Bone induced by Osteoporosis and Drug Treatments

In collaboration with Phillip Yang, Taeyong Ahn, Dr. Le T. Duong, Brenda Pennypacker, Dr. Meagan A. Cauble, Dr. Sriram Vaidyanathan, Dr. James Henderson Prof. Kenneth M. Kozloff, Prof. Bradford Orr, and Prof. Mark M. Banaszak Holl

5.1 Abstract

Nano- to microscale mineral and organic matrix heterogeneity plays a role in fracture prevention for osteoporotic bone. To further understand its role in fracture prevention, we seek to understand compositional changes linked to disease and treatment in lumbar vertebral trabecular bone using a *treatment animal model*. A group of cynomolgus monkeys were either ovariectomized (OVX) or submitted to sham surgery. Two years post-surgery, OVX animals were treated in *treatment mode* with vehicle (VEH), Alendronate (ALN; 0.03 mg/kg weekly), the cathepsin K inhibitor MK-0674 (CatKi; 0.6 mg/kg daily), and Denosumab (DMab; 2.5 mg/kg weekly subcutaneously) for two years and compared to a control Sham surgery group. To understand compositional changes, atomic force microscopy with infrared spectroscopy (AFM-IR) was used to obtain topographical information coupled to chemical composition for cross-sections of lumbar vertebrae 4 (LV4). A total of 16 locations were imaged per sample, a 10 by 10 μm and two 3.5 by 3.5 μm images were obtained as well as a localized IR spectrum per 3.5 by

3.5 μm image. The average mineral-to-matrix ratio and acid phosphate (PO_4^{3-}) substitution for each group was evaluated. Average mineral to matrix (900-1200/1500-1800 cm^{-1}) ratios obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.31 (0.09), 0.24 (0.09), 0.25 (0.09), 0.28 (0.2) and 0.25 (0.1), respectively. However, no significant difference was detected. Average acid phosphate (PO_4^{3-}) substitution (1128/1096 cm^{-1} intensity ratio) obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.86 (0.06), 0.90 (0.07), 0.90 (0.01), 0.79 (0.1) and 0.91 (0.1) respectively. CatKi was significantly different ($p < 0.05$) from OVX, ALN and DMab, yet no significant difference was detected from Sham. CatKi shows a preservation, or recovery, to normal levels of acid phosphate substitution. On average, acid phosphate substitution in CatKi is most similar to Sham.

5.2 Introduction

Bone is primarily composed of hydroxyapatite, or mineral, collagen, non-collagenous protein and water. One of the most common diseases affecting bone is osteoporosis, a degenerative bone disease that causes a net bone loss due to increased bone resorption.¹ It has been well documented that the mineralization level of bone has an impact on bone fragility and fracture risk.^{1,2} Bone Mineral Density (BMD) measurements are commonly used in the clinic as a diagnostic tool for osteoporosis.¹⁻³ However, BMD alone is not sufficient to distinguish when a patient is high or low risk for osteoporotic fractures making it imperative to gain a deeper understanding of how chemical composition changes as a function of osteoporosis and drug treatment.^{1,3}

To better characterize the effects of osteoporosis on mineral-to-matrix ratio and mineral content, Fourier transform infrared spectroscopy (FTIR) and FTIR imaging have been widely used and validated using independent techniques (Table 5.1).^{4–13} Commonly evaluated parameters are mineral-to-matrix ratio, carbonate to phosphate ratio, collagen maturity, mineral crystallinity, and acid phosphate substitution.³ In this study we will mainly focus on mineral-to-matrix ratio and acid phosphate substitution as these changes were most pronounced in this sample set.

Mineral-to-matrix ratio is proportional to mechanical properties such as bending stiffness and failure moment, and it is known to decrease with osteoporosis.^{9,12} Acid phosphate substitution is inversely proportional to mineral maturity and indicative of new bone formation.^{3,14} In addition, studies have shown that the heterogeneity and spatial distribution of these parameters changes as a function of disease.^{3,14–17}

As discussed in **Chapter 2**, compressive yield and peak load increases were observed for CatKi treatment when compared to OVX despite no significant differences in trabecular microstructure as measured by μ CT.¹⁸ These results implied protein material changes were occurring and that μ CT is not sensitive to the nature of protein-level chemical changes as they may not induce a net change in the electron density of the material. Hence, the employment of a nano-scale spectroscopic technique in this study.

We use atomic force microscopy coupled to infrared spectroscopy (AFM-IR) to address the question of what mineral-matrix compositional changes and mineralization changes are driving material changes as a function of osteoporosis and treatment while preserving microstructure of the vertebrae. The key advantages of using AFM-IR are the spatial resolution of ~ 50 nm¹⁹ (approximately the size of hydroxyapatite crystals and

collagen fibrils), capability of imaging fully hydrated tissue (unlike traditional IR of tissue), and the capability of capturing topography and morphology data alongside chemical characterization.

5.3 Methods

5.3.1 Treatment Animal Model

The *treatment animal model* used in this study has been previously published and was discussed in detail in **Chapter 2 and Chapter 3**.¹⁸ Briefly, Merck, Inc. collected 63 cynomolgus monkeys, performed ovariectomies, and two years post-surgery treated them with the bisphosphonate Alendronate (ALN), a cathepsin K inhibitor MK-0674 (CatKi), and the antibody therapeutic Denosumab (DMab) for two additional years. Longitudinal dual-energy x-ray absorptiometry (DXA), in-vivo periphery quantitative computer tomography (CT), and biomarkers were utilized to track the progression of the disease for each subject as well as post-necropsy μ CT and mechanical testing (3-point bending of femurs and compression of LV) to further understand microstructure and mechanical properties of the bones, respectively.¹⁸ Data collected in-vivo during the treatment animal study will not be discussed. For a visual summary, see **Table 3.1** and **Scheme 3.1**.

5.3.2 Sample Preparation, AFM-IR Spectra Collection, AFM Imaging and Processing

Methods used to prepare each lumbar vertebrae (LV) section for AFM imaging and IR spectra collection were discussed in **Chapter 3**. Briefly, each LV was sectioned, mounted on an AFM puck, polished and demineralized using EDTA to expose collagen

fibrils (**see Scheme 3.2**). Each sample was imaged on a total of 16 locations using AFM-IR (nanoIR2, Anasys Instruments, Santa Barbara, CA) in contact mode. Each location consisted of a 10 x 10 μm and two 3.5 x 3.5 μm images as shown in **Figure 3.1**. For each 3.5 x 3.5 μm image, a total of 4 IR spectra were collected in the same spot and averaged using Analysis Studio (Anasys Instruments, Santa Barbara, CA). For a summary of images and spectra collected, see **Table 3.2**.

Each averaged spectra was baseline corrected using Origin. After baseline correction, each spectra was normalized to the Amide I peak using Excel (Microsoft Office). To obtain integrated peak area ratios of each baseline-corrected AFM-IR spectra, an Excel (Microsoft Office) macro was used to integrate all the areas under the curve. A one-way analysis of variance (ANOVA) was used to determine treatment group differences for mineral-to-matrix integrated peak area ratios and acid-phosphate substitution peak intensity ratios. Tukey's Test was used to detect differences in ratio means between specific treatment group. All statistical analysis was performed using R. All data are presented as mean $\pm$ (standard deviation).

5.3.3 AFM-IR Mapping and Image Analysis

Methods used to prepare each lumbar vertebrae (LV) section for AFM imaging and IR spectra collection were discussed in **Chapter 4**. Briefly, AFM-IR maps were collected using a NanoIR2 system (Anasys Instruments, Goleta, CA, USA) in contact mode using nIR2 probes (Anasys Instruments, Parameters: gold coated silicon cantilever, nominal radius 25 nm, force constant 0.07 - 0.4 N/m, resonance frequency 13 ± 4 kHz). The IR spectrum detection range was set between 860-1900 cm^{-1} . One IR map was collected for

Amide I with unraveled collagen (1740 cm^{-1}), Amide I with intact triple helix (1660 cm^{-1}), and mineralization at 1128 cm^{-1} and 1096 cm^{-1} . The mineral-to-matrix and acid phosphate substitution ratios were calculated ($1096/1660\text{ cm}^{-1}$ and $1128\text{ cm}^{-1}/1096\text{ cm}^{-1}$ respectively) using Analysis Studio (Anasys Instruments, Goleta, CA, USA). For a visual summary, see **Scheme 4.1**.

To quantify the domain sizes, each AFM-IR ratio map was analyzed using Fiji (National Institute of Health, United States).²⁰ Each map was imported into Fiji, the scale was set to 0.01 pixel/nm, and the threshold set to 0.78 – 1.3 for mineral-to-matrix ratio maps and 0.89 – 1.28 for acid phosphate substitution ratio maps (these ranges correspond to green in all corresponding AFM-IR ratio maps), and the minimum particle size was set to 224 nm. The image was converted to a mask and then converted to a watershed image to obtain particle areas using the Analyze Particle feature. Assuming the domains were circular, particle areas were converted to domain sizes using Excel (Microsoft Office).

5.4 Results

In bone, the mineral peak is a manifold of about a dozen vibrational groups that appear in similar IR ranges as summarized in **Table 5.1**. Total area under the peak indicates different mineralization levels, and acid phosphate (HPO_4) substitution ($1128\text{ cm}^{-1}/1096\text{ cm}^{-1}$ intensity ratio) indicate younger mineral.

In **Figure 5.1**, we observe variations in protein and mineral content as a function of disease. For detailed results observed for protein, see **Chapter 4**. The mineral peak manifold consists of a dozen different vibrational groups reflecting the presence of

nanocrystals.^{5,10} In addition, shifts in the maximum mineral peak location is indicative of tissue age.^{5,10} When the maximum mineral peak is closer to 1000 cm⁻¹, it is indicative of younger mineral.³ When the maximum mineral peak is closer to 1100 cm⁻¹, it is indicative of more mature mineral.³

Table 5.1. FT-IR Vibrational Peak Assignments for Protein and Mineral in Bone

Author	Group	Assignments	Peak Position (cm ⁻¹)	Reference
Paschalis	Boskey	ν_1, ν_3 PO ₄ ³⁻ Amide I Poorly Crystalline Apatite	960 - 1143 1625 - 1693 1109	<i>Clin. Orthop. Relat. Res.</i> 2011 , 469 (8), 2170–2178.
Gourion-Arsiaud	Boskey	ν_1, ν_3 PO ₄ ³⁻ Amide I	916 - 1180 1592 - 1712	<i>J. Bone Miner. Res.</i> 2013 , 28 (1), 150–161. <i>Bone</i> 2010 , 46 (3), 666–672.
Imbert	Boskey	Acid Phosphate Substitution ν_1, ν_3 PO ₄ ³⁻ Amide I	1128 / 1096 920 - 1200 1596 - 1712	<i>PLoS One</i> 2018 , 13 (9), 1–15.
Gu	Katti	C=O Stretch Amide I ν_1, ν_3 PO ₄ ³⁻ Poorly Crystalline Apatite	1747 ~1652 927 - 1180 1120	<i>JOM</i> 2016 , 68 (4), 1116–1127.
Boskey	Recker	Acid Phosphate Substitution	1128	<i>J. Bone Miner. Res.</i> 2016 , 31 (5), 1070–1081.
Greenwood	Zioupou	ν_1, ν_3 PO ₄ ³⁻ Amide I	900 - 1200 1600 - 1750	<i>Bone</i> 2016 , 93 (September), 55–63.

In the Sham group, one out of 9 samples had a remarkably low level of mineralization (<0.1 IR intensity), which coincided with an Amide I peak maximum at 1648 cm⁻¹, while the rest are at the same wavenumber (1672 cm⁻¹). Also, 2 out of 9 samples had a maximum mineral peak at 1030 cm⁻¹ or lower, and 2 out of 9 samples had a maximum mineral peak at 1100 cm⁻¹.

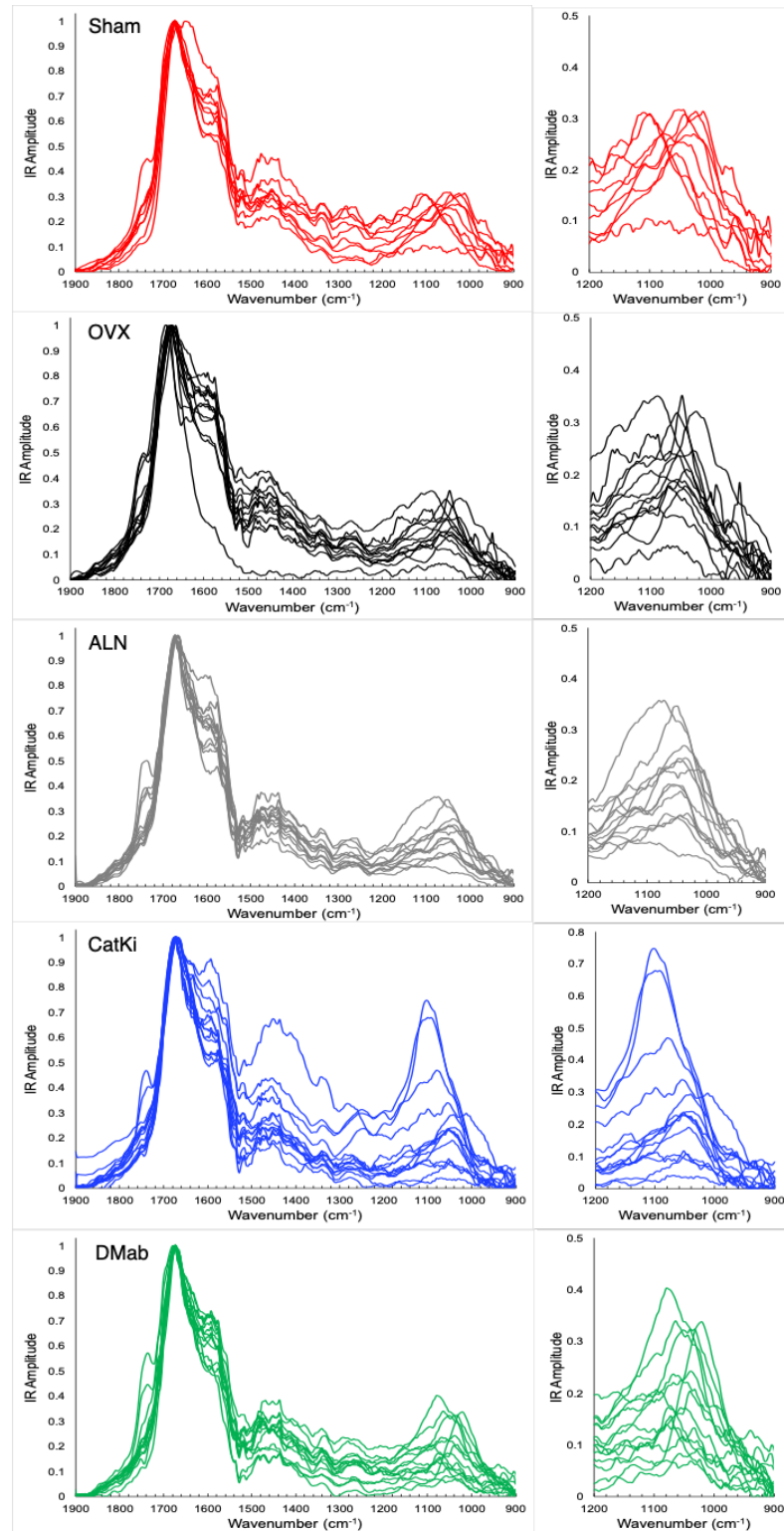


Figure 5.1 Average Localized IR Spectra Show Mineralization Heterogeneity Observed across Treatment Groups. Each individual plot in the left column represents one the averaged spectra of one treatment group. All spectra are normalized to Amide I peak. The right column contains the average spectra of mineralization manifold for each treatment group.

For OVX group, one out of 13 samples had a remarkably low level of mineralization (< 0.1 IR intensity), which coincided with a collagen disorder peak at 1740 cm^{-1} . Also, 2 out of 13 samples had a maximum mineral peak at 1030 cm^{-1} or lower, and 1 out of 13 samples had a maximum mineral peak at 1100 cm^{-1} .

For ALN group, one out of 13 samples had a remarkably low level of mineralization (< 0.1 IR intensity), which coincided with a collagen disorder peak at 1740 cm^{-1} . Also, 2 out of 13 samples had a maximum mineral peak at 1030 cm^{-1} or lower.

For CatKi group, 4 out of 14 samples had remarkably low levels of mineralization (< 0.1 IR intensity). For one of those 4 low mineral samples had an unraveled collagen peak at 1740 cm^{-1} , which is discussed in **Chapter 4**. Also, 2 out of 14 samples had a maximum mineral peak at 1100 cm^{-1} . CatKi shows a greater range of mineralization including much greater mineralization than seen in other groups. A total of 3 samples had a mineral peak with an IR intensity of 0.5 or higher. A similar trend can also be observed for mineral-to-matrix ratios as shown in **Figure 5.3**.

For DMab group, 2 out of 14 samples had a remarkably low level of mineralization (< 0.1 IR intensity). Also, 1 out of 9 samples had a maximum mineral peak at 1030 cm^{-1} or lower.

In **Figure 5.2**, the degree of protein and mineral heterogeneity observed in one ALN sample is illustrated. These data are representative of the level of heterogeneity observed throughout the entire data set. Panel A is a photo of the mounted sample with black circles showing each imaged location with dots showing the imaged locations that are presented in 5.2B, 5.2C, 5.2D, 5.2F, 5.2E, and the spectra shown. Spectra colors and image borders correspond with the color of the dot in 5.2A. Panels B, C, D, E, and F are

3.5 x 3.5 μm deflection images, and the colored dots represents where the IR spectra were taken on each image. To the left of the panels, the spectra are shown highlighting the organic matrix (in pink), and the mineral (in yellow) areas of the spectrum. The size of the circles on each deflection image are representative of the spatial resolution from which the IR data was obtained.¹⁹

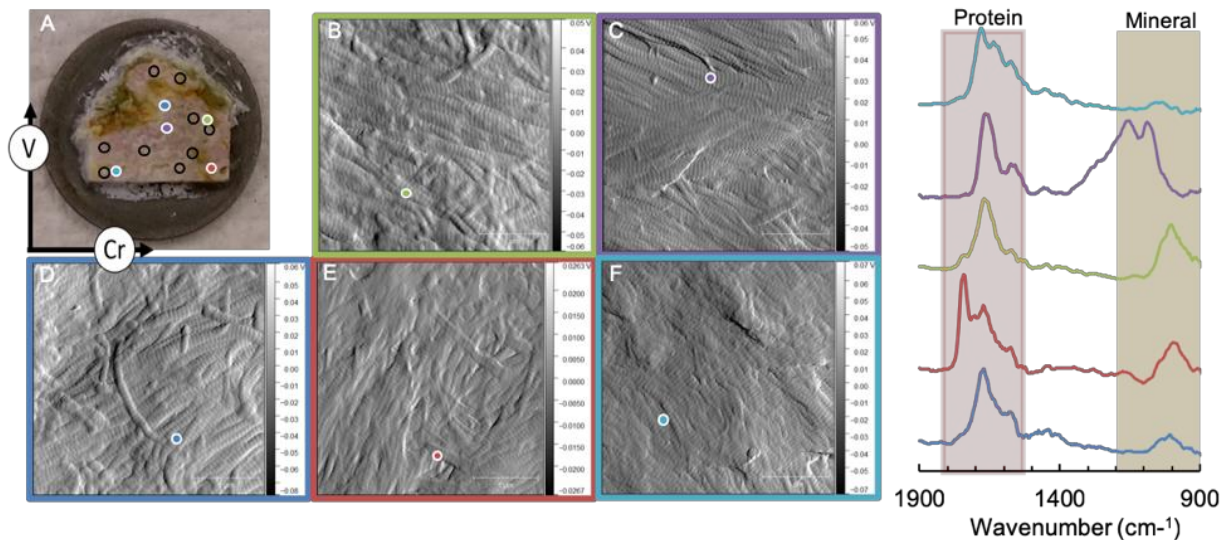


Figure 5.2 Individual AFM-IR Spectra Demonstrate Organic and Mineral Matrix Compositional Heterogeneity in Lumbar Vertebrae Bone

In **Figure 5.3**, mineral-to-matrix integrated peak area ratio boxplots for each treatment group show the range of ratios present in all treatment groups with CatKi having the largest range of values and Sham with the smallest range of values including two outliers. No significant difference was observed between treatment groups. Average mineral to matrix (900-1200/1500-1800 cm^{-1}) integrated peak area ratios obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.31 (0.09), 0.24 (0.09), 0.25 (0.09), 0.28 (0.2) and 0.25 (0.1), respectively. However, no significant difference was detected. Average acid phosphate (PO_4^{3-}) substitution (1128/1096 cm^{-1} intensity ratio) obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.86 (0.06), 0.90 (0.07), 0.90 (0.01), 0.79 (0.1) and 0.91 (0.1) respectively. CatKi was significantly different ($p < 0.05$) from

OVX, ALN and DMab. These ratio means presented here correspond the filled dots in **Figure 5.3**.

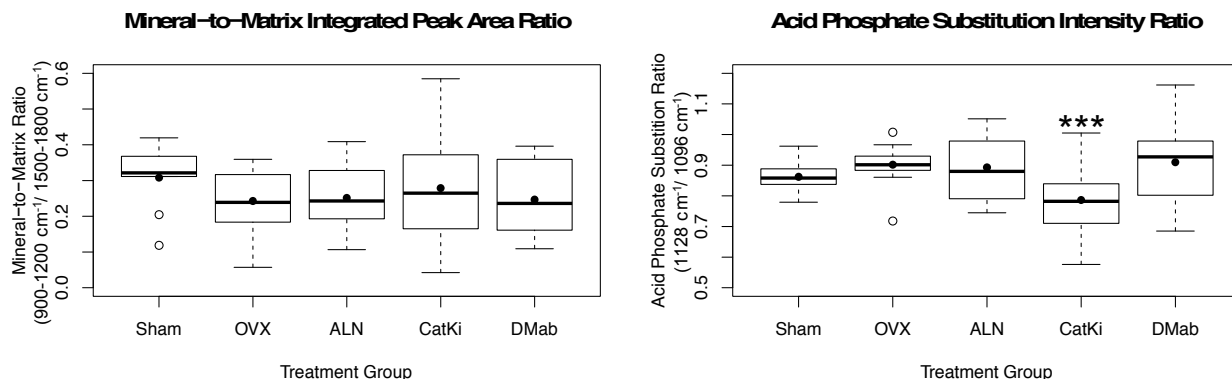


Figure 5.3 Mineral to Matrix Ratio Heterogeneity and Acid Phosphate Substitution Ratios Observed across Treatment Groups

Mineral to Matrix integrated peak area ratio box and whisker plots for each treatment group show the range of ratios observed for all treatment groups. No significant difference was observed between treatment groups. Acid Phosphate Substitution intensity ratio box and whisker plots for each treatment group show the range of ratios observed for all treatment groups with CatKi being significantly different all other groups. All Sham, OVX, ALN, CatKi and DMab samples are highlighted with boxes in red, black, grey, blue and green, respectively.

*** $p < 0.05$ vs. OVX, ALN, and DMab

Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

Given the mineral-to-matrix heterogeneity observed, the spatial distribution of mineral-to-matrix ratio and acid phosphate substitution across a sample in each treatment group was explored. The nanoIR2 AFM-IR has the capability of mapping one specific wavenumber over the surface of a sample.²¹ To evaluate the spatial distribution of mineral-to-matrix ratio, we mapped wavenumbers representative of the maximum peak intensity for mineral (1096 cm⁻¹) and matrix (1660 cm⁻¹). To evaluate acid phosphate substitution, we used the field's established assignments: 1128 cm⁻¹ and 1096 cm⁻¹. After each wavenumber map is collected, IR intensity ratio maps were calculated using Analysis Studio. IR intensity ratio maps are used, instead of individual wavenumber maps, to eliminate any tip effects or bias introduced while imaging.

In **Figure 5.4**, the left column has exemplar AFM-IR mineral-to-matrix ratio maps show mineral-to-matrix ratio heterogeneity and spatial distribution in a 20 μm x 20 μm area. In addition, the middle column has exemplar AFM-IR acid phosphate substitution ratio maps show acid phosphate substitution ratio heterogeneity and spatial distribution in a 20 μm x 20 μm area. The left and middle columns each have 4 IR intensity ratio maps that summarize the domain sizes and areas observed across all IR intensity ratio maps collected. Each IR intensity map has their corresponding IR spectrum on the right column. All IR spectra have 1660 cm^{-1} , 1096 cm^{-1} and 1128 cm^{-1} highlighted with a black dotted line. Each IR spectrum was taken at, or as close as possible to, the middle of the image. The ratio range presented in all mineral-to-matrix maps is 0.25 (blue) to 2.0 (red), and the range for all acid phosphate substitution maps is 0.5 (blue) to 1.8 (red). Most maps show circular shaped domains with higher and lower ratio values that range from ~ 300 nm to 2 μm in size, as shown in **Figure 5.5**. This trend is observed across all maps obtained shown in **Appendix B**.

In order to determine the domain sizes in each map, image analysis of all ratio maps was done using Fiji²⁰ assuming the domains were circular. The domain size analysis had the minimum set to 224 nm. **Figure 5.5** summarizes the domain sizes obtained for all AFM-IR ratio maps collected. Domain sizes were as large as 2 μm for one Sham and one CatKi ratio map collected. Most domain sizes were 500 nm to 1.6 μm in diameter.

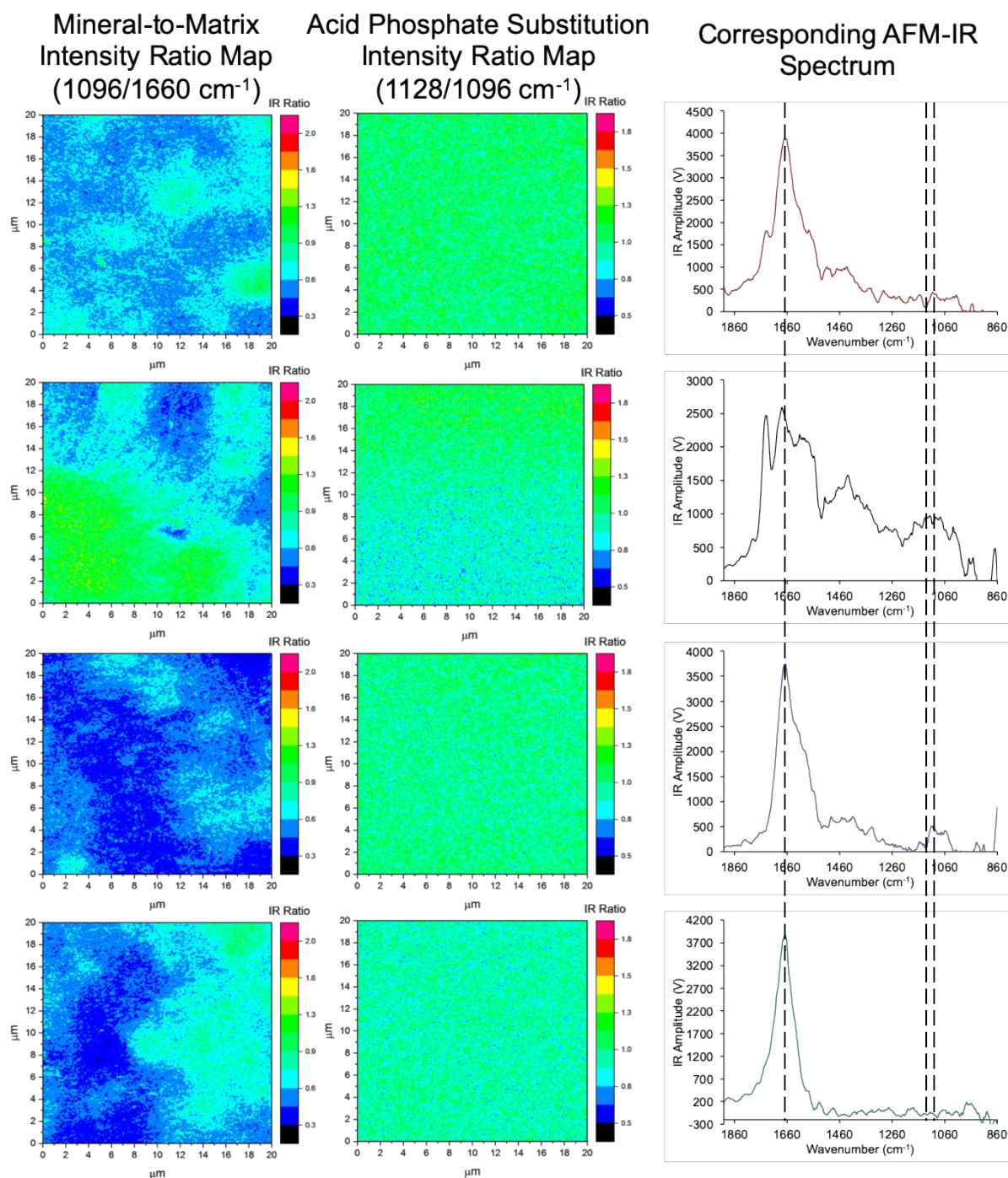


Figure 5.3 IR Intensity Ratio Maps Show Chemical Heterogeneity in Mineral-to-Matrix Ratio Across Treatment Groups and Compositional Consistency in Acid Phosphate Substitution Throughout Treatment Groups

Each map represents a region of 20x20 μm . Maps on the left column show calculated IR intensity ratios of mineral (as measured by 1096 cm^{-1} intensity; shown with a black dotted line on the corresponding spectrum) over matrix (as measured by 1660 cm^{-1} intensity; shown with a black dotted line on the corresponding spectrum). Maps in the middle column show calculated IR intensity ratios of acid phosphate substitution measured by 1128 cm^{-1} /1096 cm^{-1} (1128 cm^{-1} and 1096 cm^{-1} are shown with a black dotted line on the corresponding spectrum). Column to the right shows each corresponding AFM-IR spectrum collected for each sample with all wavenumbers of interest highlighted with black dotted lines. All regions imaged can be found in **Appendix B**.

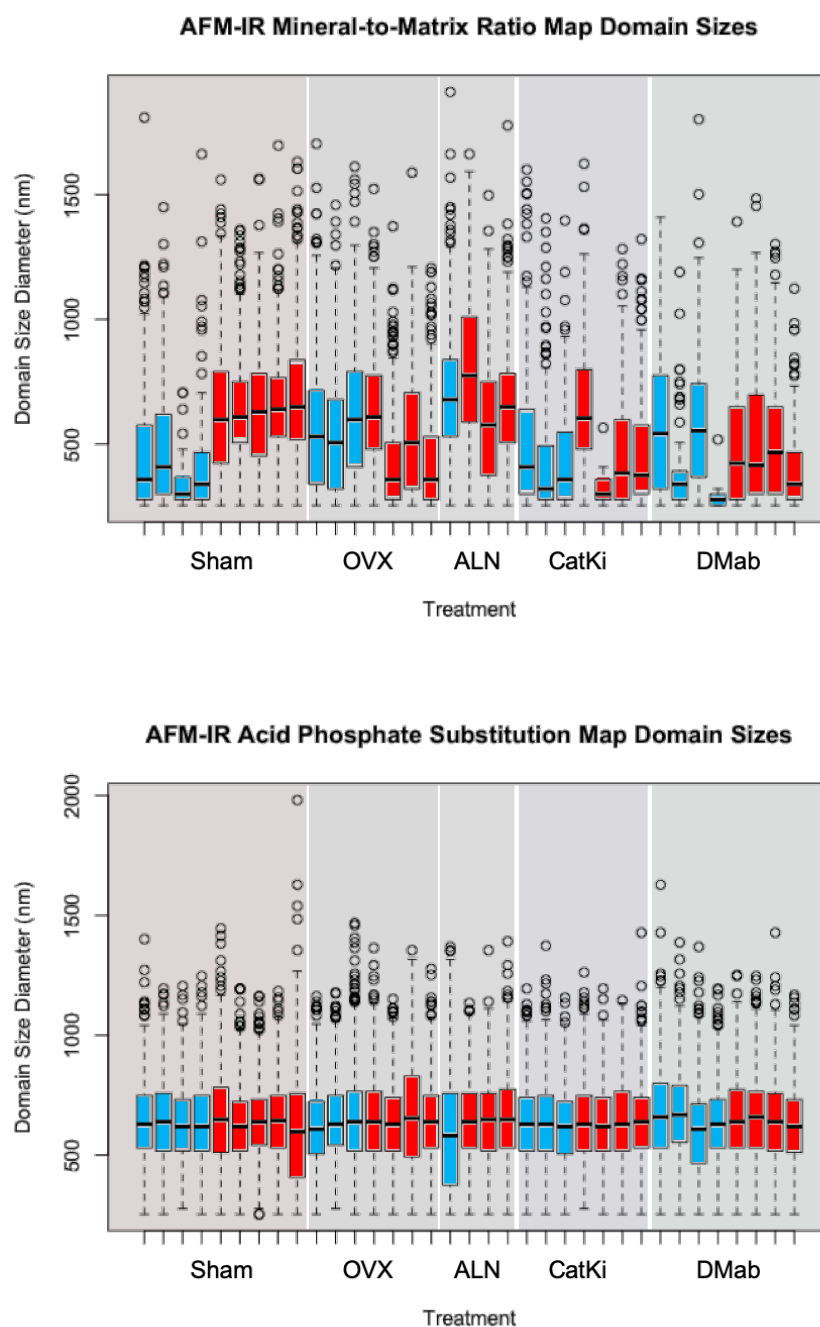


Figure 5.5 AFM-IR Mineral-to-Matrix Ratio Map and Acid Phosphate Substitution Ratio Map Domain Sizes
Domain sizes go up to ~2 μm , and all acid phosphate substitution ratio map domain sizes have medians close to 600 nm. For this analysis, domain size minimum was set to 224 nm. All Sham, OVX, ALN, CatKi and DMab samples are highlighted with boxes in red, black, grey, blue and green, respectively. Blue boxes represent samples with higher levels of unraveled collagen (presence of Amide I peak at 1740 cm^{-1} indicative of collagen triple helix unraveling), and red boxes represent samples with lower levels of unraveled collagen (as assigned in **Chapter 4**). Box and Whisker plots showing mean (filled dot), median (line), 25% and 75% percentile (lower and upper quartile, respectively), maximum and minimum values (lower and upper whiskers, respectively), and outliers (circle).

5.5 Discussion

The current gold standard in the clinic for diagnosing osteoporosis is BMD.¹⁻³ However, BMD only measures the mineral component of bone and is not able to evaluate the role of protein, or organic matrix.^{1,3} In this study, we seek a deeper understanding of how mineral and matrix heterogeneity changes as a function of osteoporosis and treatment. Average mineral to matrix (900-1200/1500-1800 cm^{-1}) integrated peak area ratios obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.31 (0.09), 0.24 (0.09), 0.25 (0.09), 0.28 (0.2) and 0.25 (0.1), respectively. However, no significant difference was detected. Average acid phosphate (PO_4^{3-}) substitution (1128/ 1096 cm^{-1} intensity ratio) obtained for Sham, OVX, ALN, CatKi and DMab groups were 0.86 (0.06), 0.90 (0.07), 0.90 (0.01), 0.79 (0.1) and 0.91 (0.1) respectively. CatKi was significantly different ($p < 0.05$) from OVX, ALN and DMab, yet no significant difference from Sham was detected. CatKi and Sham not being significantly difference indicates a preservation or a recovery to normal levels for mineral maturity in animals treated with CatKi. It should be noted that 10 of the 14 DMab subjects developed a neutralizing anti-drug anti-body during this study.

Given the mineral-to-matrix heterogeneity observed, we explored the spatial distribution using IR intensity ratio maps. Maps show the extent of the mineral-to-matrix heterogeneity with maps showing areas of low ratios next to areas of high ratios. Curiously, these maps mimic the patterns for the corresponding unraveled-to-intact collagen maps shown in **Chapter 4** and **Appendix B**. Low ratios indicate low intensities for mineral, and vice versa for high ratios. Most areas in these maps have domain sizes

ranging from 500 nm to 1.6 μ m. We hypothesize these domains correspond with osteoclastic activity, which will be further discussed in **Chapter 6**.

Given the acid phosphate substitution consistency observed, we explored the spatial distribution using IR intensity ratio maps. Maps show consistency in ratio values throughout the data set for acid phosphate substitution with speckles of high or low ratio values. Most samples have average domain sizes of ~600 nm. For all maps collected, see **Appendix B**.

The nano-scale resolution used in this study sheds light on compositional differences that cannot be detected with conventional techniques. The estimated size of compositional heterogeneity is estimated to be ~200 nm.¹⁵ However, most conventional spectroscopic techniques have a spatial resolution on the order of micrometers. This gives the AFM-IR technique an inherent advantage over conventional spectroscopic techniques used in the bone field. In addition, we have the capability of imaging fully hydrated bone without the need to embed the sample in a polymer, such as Poly(methyl methacrylate) (PMMA), a common practice in the bone community.

Curiously, with this smaller spatial resolution, no significant difference in mineral-to-matrix ratio results was obtained. This is inconsistent with a number of FTIR spectroscopic studies of bone chemical composition, yet it is consistent with human iliac crest biopsy studies. It should be noted that changes in mineral-to-matrix ratio are both age- and disease-dependent.²² It should be noted that in this study we are not able to control for age due to lack of age data.

A review summarized all mineral content studies to date.¹⁷ In this review, mineral-to-matrix ratio is reported to decrease in osteoporotic canines (cortical bone), OVX

monkey (trabecular), OVX and disuse osteoporosis rat, and postmenopausal women (cortical and trabecular).¹⁷ However, in the largest iliac crest case-matched study, no differences in heterogeneity and mineral-to-matrix ratio was detected.³

In this study, iliac crest biopsies of 60 patients with fragility fractures and 60 patients age- and BMD-matched patient biopsies were collected and analyzed with FTIR imaging. Significant compositional differences were detected between fracture and non-fracture patients. More specifically, a carbonate-to-phosphate ratio decrease was observed for patients with fractures. They emphasize the importance of studying bone heterogeneity at all levels.

In addition, a difference in acid-phosphate substitution was detected for CatKi. CatKi's preservation, or recovery, of acid-phosphate substitution to Sham levels prior to 2-years of bone depletion and 2 years of treatment could shed light on a bone fracture prevention mechanism. Curiously, CatKi was the only treatment group to show significant difference in mechanical properties as discussed in **Chapter 2**, suggesting a link between acid-phosphate substitution and mechanical properties.¹⁸ The process of collagen mineralization in bone happens via self-assembly over a few weeks aided by osteoblasts. Also, collagen maturity is measured by their mineral content and what mineral species are present.

In conclusion, no detectable difference was observed for mineral-to-matrix ratio between treatment groups. The lack of detectable mineral-to-matrix ratio differences was more consistent with results observed in case-matched human biopsies. CatKi showed a different acid-phosphate substitution ratio from other treatment groups. These results

together with the past chapters highlight the importance of studying bone compositional heterogeneity at the nanoscale.

5.7 References

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Chapter 6

Conclusions and Future Directions

6.1 Summary of Key Findings

This is the first study, to the best of our knowledge, done in *treatment mode* to understand chemical composition heterogeneities in osteoporotic monkeys (a non-human primate model). It is especially important that this study was conducted in *treatment mode* because the current state of treatment in the clinic requires a diagnosis for osteoporosis and most animal studies published to date were conducted in prevention mode.

Infrared spectroscopy has been widely employed in the material characterization of bone. However, this technique has some drawbacks that we have been able to overcome. The first one being spatial resolution. Using AFM-IR, we are capable of reaching areal resolutions of ~50 nm, which is the approximate diameter of collagen fibrils and hydroxyapatite nanocrystals. With this technique we are also capable of imaging fully hydrated bone, as opposed to traditional FTIR where samples need to be dehydrated. Third, there is no need to embed samples in a polymer as it is often done. Therefore we do not need to worry about having contamination or spreading of a polymer in our samples.

With the combination of this data set and the AFM-IR technique, we are able to better understand the chemical compositional, structural and spatial heterogeneity differences of protein and mineral content as a function of OVX and treatment. In addition,

we have been able to understand their effects on mechanical properties of trabecular lumbar vertebrae.

As discussed in **Chapter 2**, mechanical properties of lumbar vertebrae were preserved with CatKi treatment despite no detectable changes in bone microstructure by μ CT analysis were observed. These results suggest that important changes in bone material composition contribute to the observed mechanical differences. Therefore, we wanted to explore what chemical variations could be contributing to the differences reported in **Chapter 2**.¹

Chapter 3 is an in depth discussion of the animal model used, and data collection with a concise summary of the main results.

In **Chapter 4**, we hypothesize unraveled collagen is a key marker of the increased bone turnover associated with osteoporosis as stated in **H1a**. We hypothesize unraveled collagen (1740 cm^{-1} peak) results from an unwinding of the triple helix that exposes collagen's backbone's carbonyls generating a prominent 1740 cm^{-1} peak.² We were able to identify 3 groups: normal turnover, high turnover, and low turnover (based on the assumption that unraveled collagen is a chemical marker of turnover). However, more work is needed to confirm that unraveled collagen can be used as a chemical marker of increased bone turn over, or more osteoclastic activity.

In **Chapter 5**, **H1b** and **H2** were explored, and **H1b** was not proven. The hypotheses explored in this thesis were:

H1) Healthy lumbar vertebrae exhibit heterogeneity in protein structure including less unraveled collagen, mineral-to-matrix ratio, and mineral composition at the level of individual trabeculae and within a single trabecula than OVX disease state.

H1a) Unraveled collagen (1740 cm^{-1} peak)^{16,17} present in osteoporotic bone is a key marker of the increased bone turnover associated with osteoporosis.

H1b) Mineral-to-matrix ratio and mineral composition decreases as a function of OVX disease state and drug treatment partially recovers bone towards healthy state as observed for sham animals.

H2) Structure, compositional and spatial heterogeneity decreases in the OVX disease state and drug treatment at least partially recovers the bone towards the original, healthy state observed for sham animals.

H2a) These compositional changes occur independent of mineral age.

In bone, the mineral peak is a manifold of about a dozen vibrational groups that appear in similar IR ranges. Acid phosphate substitution indicates younger mineral, and mineral to matrix ratios indicate chemical heterogeneity in the bone.^{3,4} Curiously, with this smaller spatial resolution, no detectable significant difference in mineral-to-matrix ratio results was obtained. This result is inconsistent with a number of studies reported by the bone FTIR community, although it is consistent with case-matched bone biopsy studies of women with and without fragility fractures. A difference in mineral maturity was detected for CatKi. CatKi's preservation, or recovery, of mineral maturity to Sham levels prior to the experiment suggest that material-level changes happen with treatment.

6.2 Future Directions

As showcased in **Chapter 3**, this project has generated data that can be further analyzed. The analyzes and experiments I think can greatly strengthen the outcome of this project are: 1. Morphology analysis of AFM image, 2. Determine osteoclastic activity

as a function of disease and treatment, 3. Imaging whole samples using new mIRage technique, and 4. In-vivo detection of unraveled collagen.

As noted in **Chapter 4**, no visible differences (to the naked eyes) in morphology were detected in the AFM images for samples with high turnover. Past research in our group suggest differences in nano-scale morphology arise as a function of OVX and treatment.⁵⁻⁸ These analyzes will get to the question of whether chemical changes also drive changes in microstructure and morphology. In addition, it will provide more ways to identify unraveled collagen when chemical characterization is not available.

In **Chapter 4**, we hypothesize that the observed domain sizes could be due to osteoclastic activity. I would propose to explore methods on how to measure osteoclastic activity, or evidence for osteoclastic activity using histology on our bone samples.

In regards to IR intensity ratio maps, one of the limitations is the size of the image, the time it takes to acquire each image, and how much information we can obtain at that size. I would like to image the whole sample using mIRage instrument (Photothermal Spectroscopy Corporation, Santa Barbara, CA, USA). This technique used optical photothermal IR, and provides sub-micron IR imaging in seconds. With the speed of this technique, we could be capable of imaging one whole sample in a reasonable amount of time. Refined domain size analysis can be done on the data obtained with the mIRage to see if specific areas of the sample have higher or lower levels of unraveled collagen, and if these locations of higher or lower levels of unraveled collagen correspond to locations identified as high in osteoclastic activity. In addition, given the nature of the technique, which does not utilize probes scanning the surface, there is no need to calculate ratio images.

Lastly, I would propose an in-vivo study to detect unraveled collagen. A way we have thought about doing this is injecting CHP-TAMRA dye into mice, and use second harmonic generation (SGH) spectroscopy to detect the dye intercalated with unraveled collagen.⁹ This would be a way to confirm our hypothesis about unraveled collagen being a chemical marker of osteoporosis. In order to confirm our hypothesis, we need to quantify unraveled collagen levels, detect unraveled collagen *in vivo*, and relate unraveled collagen with *in life* established biomarkers of high and low turnover.

In conclusion, there is still a lot that can be done with the data set presented in this thesis, and more light can be shed on the knowledge gaps highlighted here. This project spans further than basic science and understanding of bone chemical composition. Some of the broader impacts of the work showcased in this thesis consists of helping close important knowledge gaps on bone's chemical composition and variations as a function of osteoporosis on treatment. The work here also helps understand better the relationship between mechanical properties and chemical composition spanning from macro- to nanoscopic scales in bone.

6.3 References

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Appendix A

Unraveled Collagen as a New Marker for Osteoporosis in Vertebral Bone

In collaboration with Phillip Yang, Taeyong Ahn, Dr. Le T. Duong, Brenda Pennypacker, Dr. Meagan A. Cauble, Dr. Sriram Vaidyanathan, Dr. James Henderson Prof. Bradford Orr, Prof. Kenneth M. Kozloff, and Prof. Mark M. Banaszak Holl

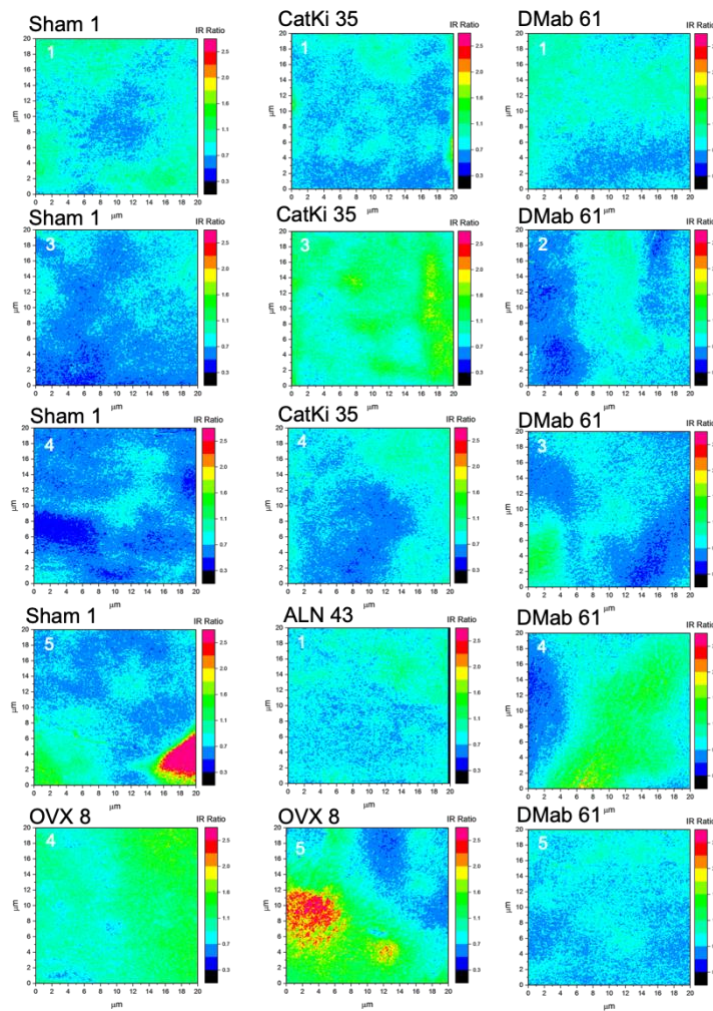


Figure A.1 IR Maps of Unraveled/Intact Collagen Ratio of High Turnover Samples
Each map represents a region of 20x20 μm and shows measured IR ratios of collagen disorder (1740 cm^{-1}) over Amide I (1660 cm^{-1}).

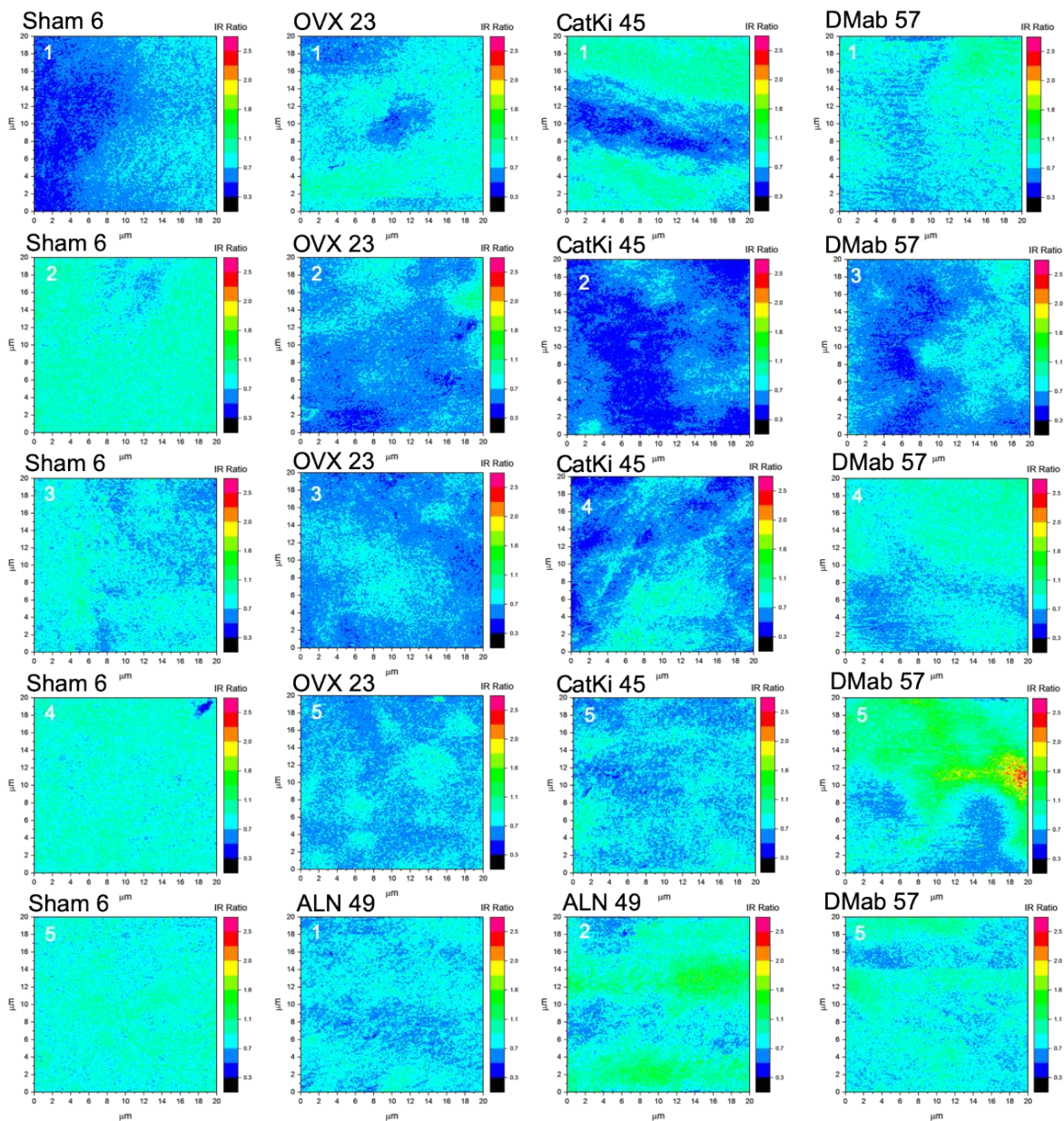


Figure A.2 IR Maps of Unraveled/Intact Collagen Ratio of Normal Turnover Samples
Each map represents a region of 20x20 μm and shows measured IR ratios of collagen disorder (1740 cm^{-1}) over Amide I (1660 cm^{-1}).

Appendix B

Changes in Nanoscale Mineral and Organic Matrix of Lumbar Vertebrae Bone induced by Osteoporosis and Drug Treatments

In collaboration with Phillip Yang, Taeyong Ahn, Dr. Le T. Duong, Brenda Pennypacker,
Dr. Meagan A. Cauble, Dr. Sriram Vaidyanathan, Prof. Kenneth M. Kozloff, Prof.
Bradford Orr, and Prof. Mark M. Banaszak Holl

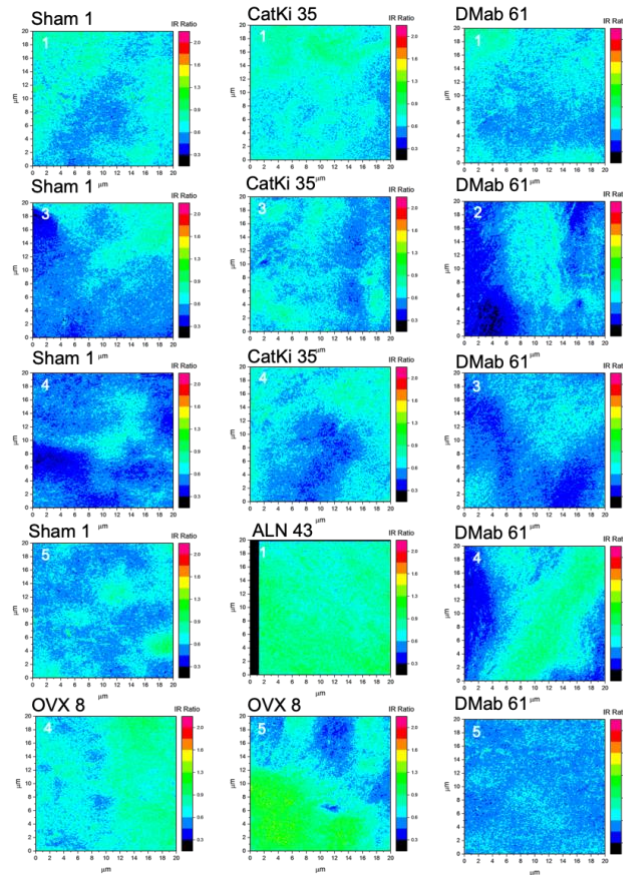


Figure B.1 Mineral to Matrix IR Ratio Maps Show Chemical Heterogeneity Across Treatment Groups in High Turnover Samples.

Each map represents a sample that was previously identified as high or low levels of unraveled collagen per treatment group. Each map is a ratio of 1660 cm^{-1} (traditional Amide I peak) and 1096 cm^{-1} (Mineral peak). Each map is $20 \times 20\text{ }\mu\text{m}$.

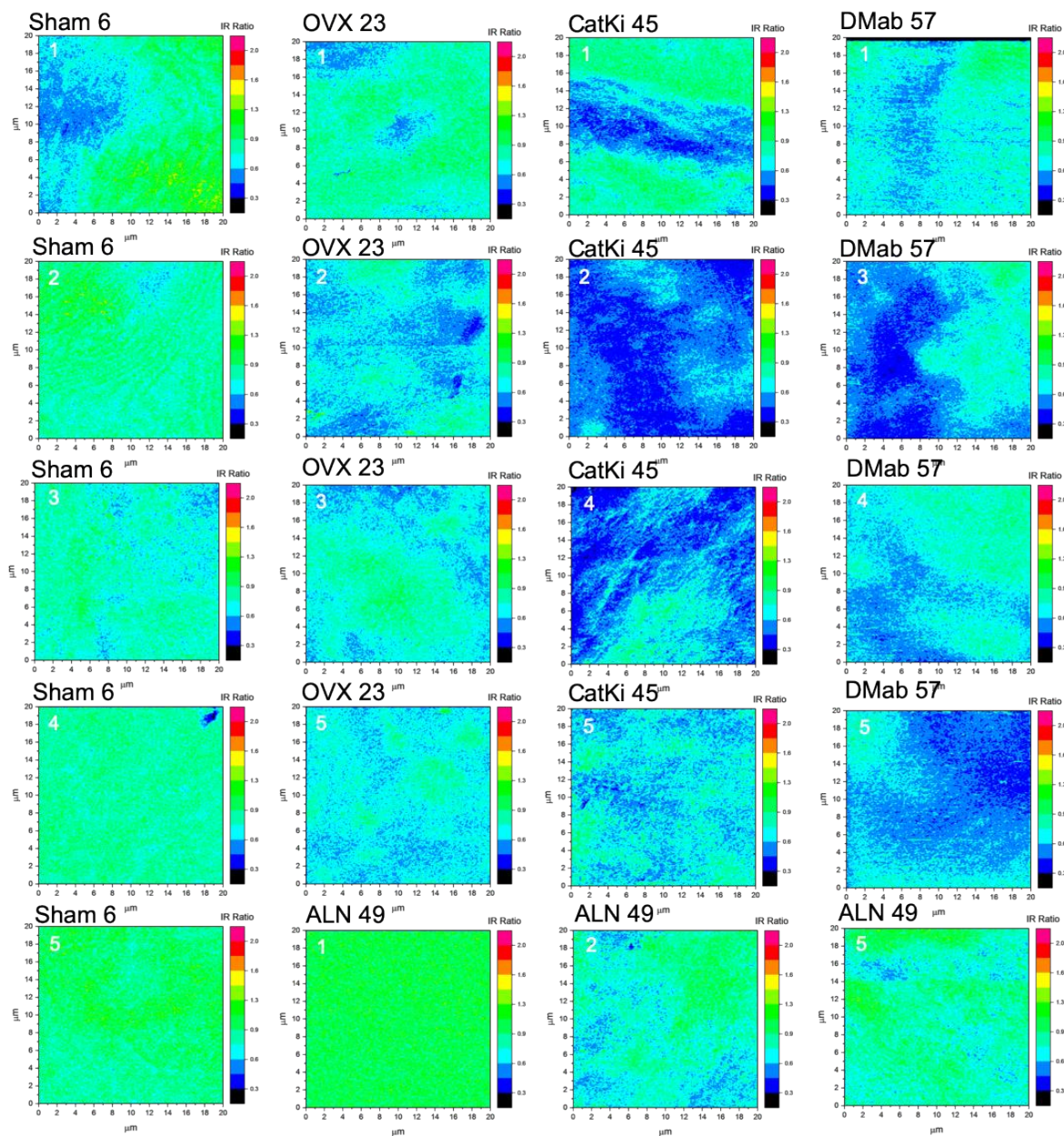


Figure B.2 Mineral to Matrix IR Ratio Maps Show Chemical Heterogeneity Across Treatment Groups in Low Turnover samples.

Each map represents a sample that was previously identified as high or low levels of unraveled collagen per treatment group. Each map is a ratio of 1660 cm^{-1} (traditional Amide I peak) and 1096 cm^{-1} (Mineral peak). Each map is $20 \times 20\text{ }\mu\text{m}$.

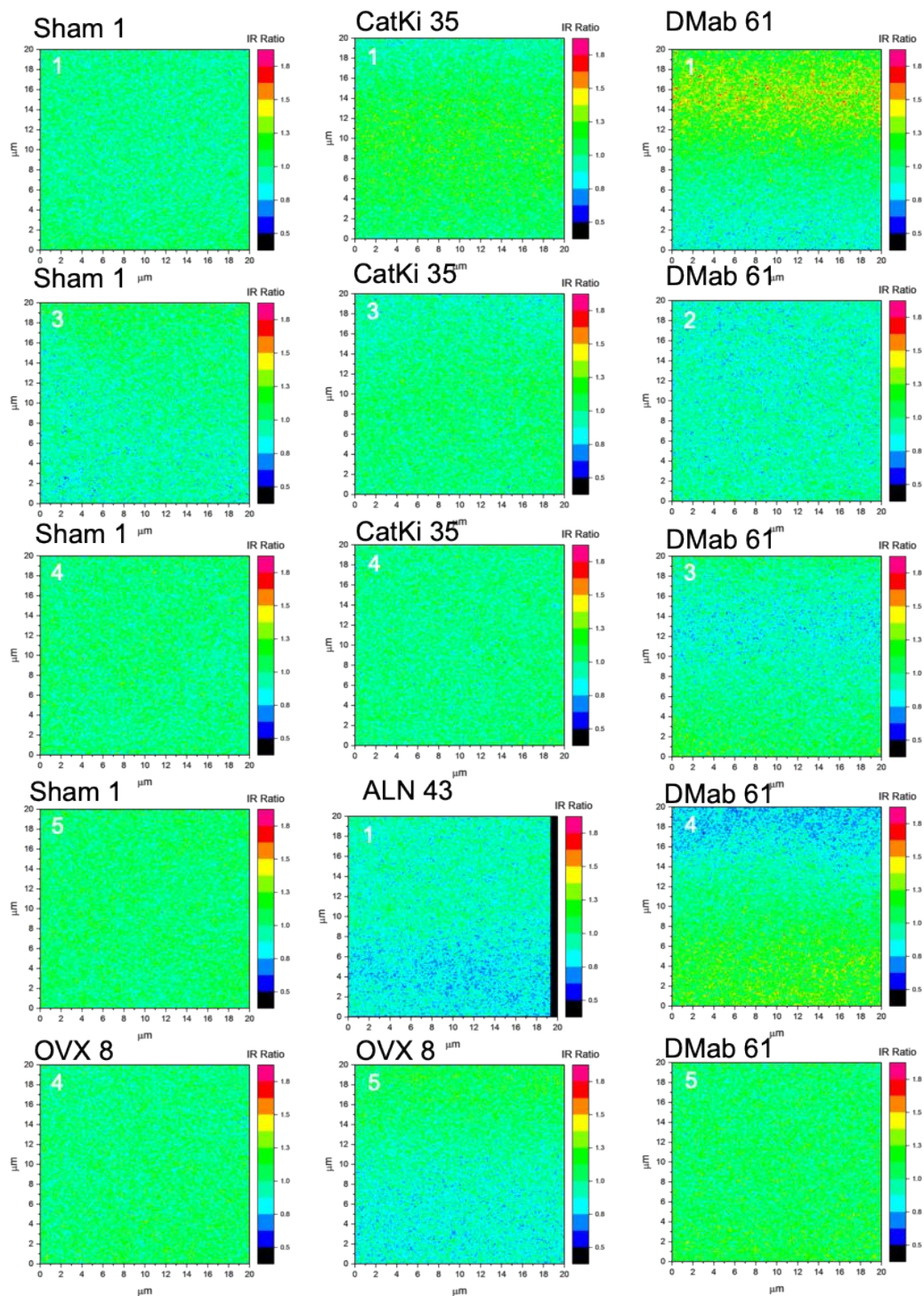


Figure B.3 Acid Phosphate Substitution Ratio Maps Are Consistent Throughout Treatment Groups in high Turnover Samples

Each map represents a sample that was previously identified as high or low levels of unraveled collagen per treatment group. Each map is a ratio of 1128 cm^{-1} (Phosphate peak) and 1096 cm^{-1} (Mineral peak). Each map is $20 \times 20\text{ }\mu\text{m}$.

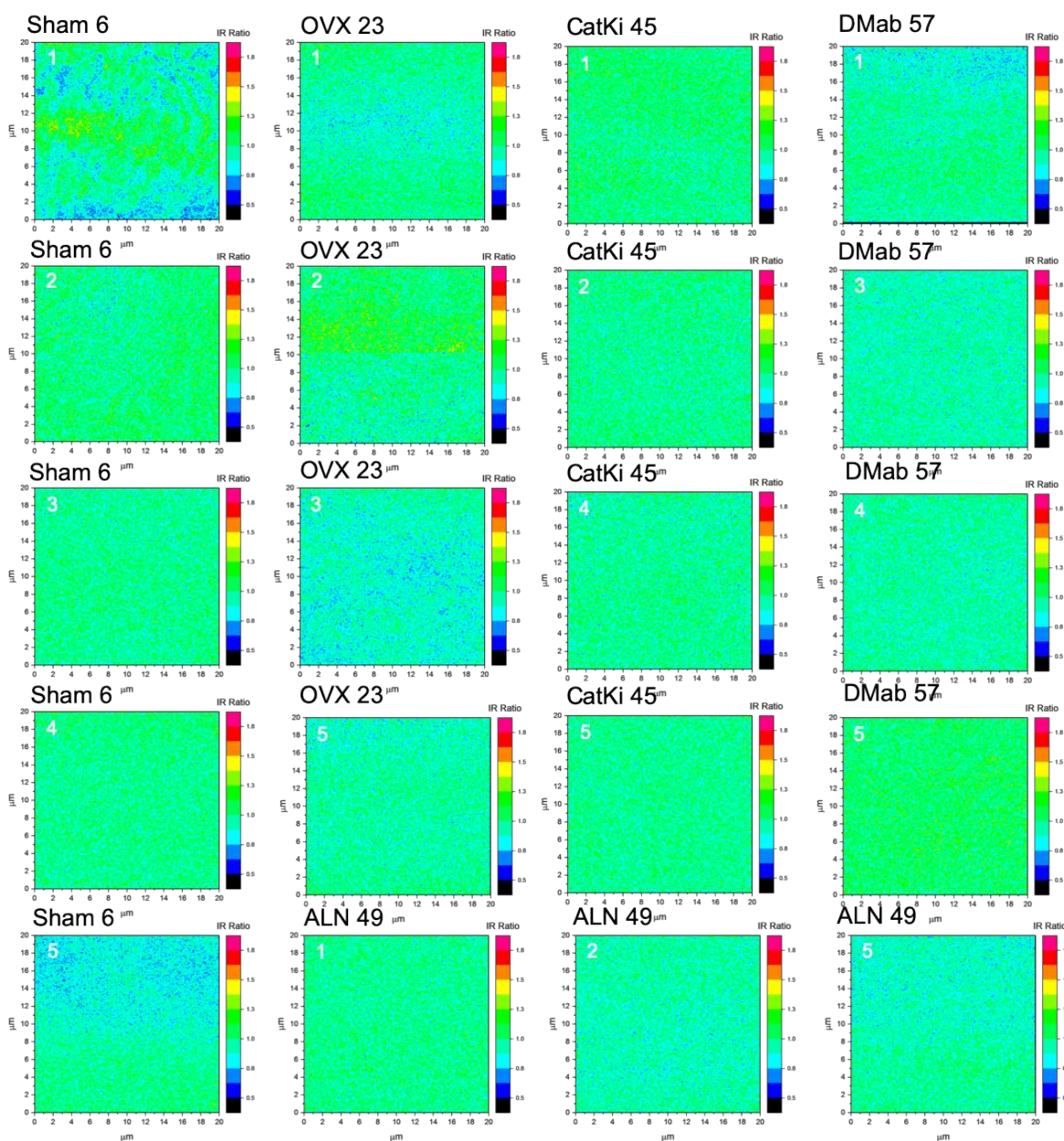


Figure B.4 Acid Phosphate Substitution Ratio Maps Are Consistent Throughout Treatment Groups in Low Turnover Samples.

Each map represents a sample that was previously identified as high or low levels of unraveled collagen per treatment group. Each map is a ratio of 1128 cm^{-1} (Phosphate peak) and 1096 cm^{-1} (Mineral peak). Each map is $20 \times 20\text{ }\mu\text{m}$.