Characterization of Bone Material Properties and Microstructure in Osteogenesis Imperfecta/Brittle Bone Disease

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CHARACTERIZATION OF BONE MATERIAL PROPERTIES AND
MICROSTRUCTURE IN OSTEOGENESIS IMPERFECTA/
BRITTLE BONE DISEASE

by

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Osteogenesis imperfecta (OI) is a genetic disorder primarily associated with mutations to type I collagen and resulting in mild to severe bone fragility. To date, there is very little data quantifying OI cortical bone mechanics. The purpose of this dissertation was to investigate bone microstructure, mineralization, and mechanical properties in adolescents with OI. Characterization studies were performed on small osteotomy specimens obtained from the extremities during routine corrective surgeries.

Nanoindentation was used to examine the longitudinal elastic modulus and hardness at the material level for mild OI type I vs. severe OI type III. Both modulus and hardness were significantly higher (by 7% and 8%, respectively) in mild OI cortical bone compared to the more severe phenotype. Lamellar microstructure also affected these properties, as the younger bone material immediately surrounding osteons showed decreased modulus (13%) and hardness (11%) compared to the older interstitial material.

A high resolution micro-computed tomography system utilizing synchrotron radiation (SRµCT) was described and used to analyze the microscale vascular porosity, osteocyte lacunar morphometry, and bone mineral density in OI vs. healthy individuals. Vascular porosity, canal diameter, and osteocyte lacunar density were all two to six times higher in OI cortical bone. Osteocytes were also more spherical in shape.

Finally, three-point bending techniques were used to evaluate the microscale mechanical properties of OI cortical bone in two different orientations. Elastic modulus, flexural yield strength, ultimate strength, and crack-growth toughness were three to six times higher in specimens whose pore structure was primarily oriented parallel vs. perpendicular to the long bone axis. There was also a strong negative correlation between the elevated vascular porosity of OI cortical bone and its elastic modulus, flexural yield strength, and ultimate strength. This relationship was independent of osteocyte lacunar density and tissue mineral density.

In summary, these findings highlight new material and microstructural changes within OI cortical bone that help contribute to its fragility. They also underscore a deep connection between bone structure and mechanical integrity at multiple length scales.
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John R. Jameson, B.S.

Trying to recognize all of the people who made this dissertation possible is like trying to summarize an epic poem in a haiku. I would start by thanking all of the OI patients who donated to this study. The data herein is anonymous, yet I feel an irrevocable bond with each of you that I will carry with me always. You have inspired me with your bravery, outward positivity, and strength. My sincerest hope is that I have contributed to the basic science behind OI in a small, yet meaningful way.

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I. INTRODUCTION

A. Objectives and Significance

A-1. Summary of the Problem

Osteogenesis imperfecta (OI), or brittle bone disease, is a genetic disorder of connective tissue that causes mild to severe bone fragility. Classical OI is commonly characterized by mutations in the genes that code for type I collagen\(^1,2\). Bone tissue is formed by mineral deposition onto this collagen scaffolding, so it is not surprising that deficiencies in the template cause altered mineral crystal formation\(^3-8\) and have negative implications on bone mechanical integrity\(^9-15\). Although symptoms vary widely among patients, the hallmarks of OI include fragile bones and skeletal deformities. The reduced quantity and poor quality of bone in these individuals poses unique and major orthopaedic challenges. However, little is known about the underlying mechanical properties of young OI bone.

A-2. Bone Fragility

Bone fragility is typically defined in terms of mechanical parameters including stiffness, strength, brittleness, and toughness\(^16,17\). Few studies have attempted to characterize these properties in individuals with OI. The lack of biomechanical information can be attributed to several factors including the misplaced focus on bone mineral density (BMD) as the primary determinant of bone strength, the scarcity of human OI bone samples, and the absence of appropriate mechanical testing methods\(^18,19\). Using nanoindentation, elastic modulus (i.e., stiffness) has been shown to be higher in
patients with OI compared to healthy peers\textsuperscript{20}. However, the significance of this finding is not fully understood because none of the other aforementioned mechanical properties describing bone fragility have been studied in humans.

Moreover, bone fragility is influenced by the structural organization of the tissue, which is normally characterized by properties such as bone size, shape, and internal architecture\textsuperscript{16,17}. Most research on the structure of human OI bone has focused on histological analysis of bone biopsies taken from standard anatomical locations\textsuperscript{21–23}. Recently, X-ray micro-computed tomography (\(\mu\)CT) has been used as an alternative method for performing a more quantitative, 3D analysis of OI bone microstructure and mineral properties\textsuperscript{24–26}. First pioneered by Feldkamp and colleagues in the 1980s\textsuperscript{27}, \(\mu\)CT is now the gold standard for \textit{ex vivo} characterization of bone morphology and microarchitecture in small bone specimens\textsuperscript{28}.

\textbf{A-3. OI Statistics and Early Development}

According to the National Institutes of Health, there are between 20,000 and 50,000 people affected with OI in the United States alone, making it the most common genetic bone disorder in humans\textsuperscript{29}. Through a partnership with Shriners Hospitals for Children – Chicago, our group has access to one of the largest pediatric OI populations in the Midwest region. As a part of their normal clinical care, some of these patients undergo surgeries for fracture repair, as well as elective procedures for correction of limb deformities via insertion of metallic rods\textsuperscript{30}. In more severe cases, bowing of the femur and/or tibia is present at birth and progressively worsens during development due to recurrent fractures at the apex of deformity\textsuperscript{31}. Many of these patients have limited
mobility and therefore require assistive devices ranging from foot and ankle orthotics to walkers, crutches, and wheelchairs\textsuperscript{32–34}.

Despite these challenges, physical activity is encouraged to the extent possible to prevent muscle contractures and immobility-induced bone loss\textsuperscript{35–37}. Adolescents with moderate to severe forms of the disorder also have a high risk of losing motor skills during the second decade of life, and this has been attributed to progressive spinal deformity, decreased motivation in physical therapy (PT) activities, and increased wheelchair use\textsuperscript{38}. Since participation in regular sports activities is often not an option, the development of effective PT and rehabilitation programs remains critical in the treatment of OI\textsuperscript{35,39}. Physical training programs have recently been developed that can improve aerobic capacity in children with OI by as much as 18\% or more\textsuperscript{40}. Other alternative therapeutic devices have been developed to apply low-magnitude musculoskeletal loading using whole body vibration. Preliminary studies using these devices on children and adolescents with OI have shown improvements in mobility and lower extremity muscle strength\textsuperscript{41,42}.

A-4. Computational Modeling in OI

As stamina, mobility, and confidence increase in these patients, there is growing interest in the development of computational models that can be folded into clinical treatment programs as a way of assessing fracture risk during different activities. Similar models have been used extensively in adults with varying degrees of osteopenia to identify patients at a higher risk for hip fracture during falls\textsuperscript{43–48}. Our lab has developed a preliminary computational model for a patient with mild OI based on kinematics,
kinetics, and muscle strength data\textsuperscript{49–52}. To date, the geometry of the model has been based on manipulation of an open-source “standardized femur” using 2D clinical radiographs from the OI patient\textsuperscript{53}. Material property inputs (i.e., elastic modulus, strength, and Poisson’s ratio) are also required, and these have been estimated from nanoindentation studies of children with moderate to severe OI\textsuperscript{54–56}, as well as tensile and bending studies on healthy young bone\textsuperscript{57–62}.

The accuracy of these computational bone models can be greatly improved through incorporation of subject-specific data\textsuperscript{63}. In the past, efforts to obtain such information have been hampered by limited access to tissue samples and appropriate testing methods. As a result, the mechanical properties and microstructure of OI cortical bone have not been thoroughly investigated in humans. This dissertation seeks a more definitive understanding of the stiffness, strength, toughness, and pore structure of OI cortical bone in pediatric and adolescent patients. Such an understanding will help to address many important questions about the underlying fragility of OI bone, the differential effects of various mutations or phenotypes, and the fracture risks associated with physical activities. This work also examines possible relationships among mechanical bone fragility descriptors (i.e., stiffness, strength, and toughness), microstructural properties (i.e., cellular and vascular porosity), and mineralization. Knowledge about these relationships may improve our understanding of the etiology of OI bone fragility, which is useful in developing and evaluating treatment methods.
B. Background

B-1. OI Types

In reality, OI is a group of bone fragility disorders characterized by many different connective tissue mutations (Table I-1). The clinical range of phenotypes seen in OI is exceptionally broad, ranging from cases that are lethal in the perinatal period to cases that may be almost indiscernible from healthy individuals. Biochemical and molecular studies continue to uncover new OI types, such that diagnosis can be challenging. This is especially true in children, since a positive family history is not usually present, and the many OI types can vary in the timing of their onset. Indeed, although OI types I-V have autosomal dominant heritability, most mutations occur spontaneously. Because OI mutations affect connective tissue, patients may present varying degrees of skeletal growth deficiency, defective tooth formation (i.e., dentinogenesis imperfecta), hearing loss, macrocephaly (i.e., enlargement of the head), eye discoloration (blue sclerae), scoliosis, barrel chest, and ligament laxity.

OI types I-IV are considered the classical types of the disorder and were first identified in the late 1970s. Patients are usually diagnosed into one of the classical types on the basis of clinical and radiographic features. Accounting for nearly 90% of all cases, classical OI is generally characterized by mutations to genes that code for the two chains (COL1A1 or COL1A2) that are folded into the type I collagen molecule triple helix. To date, over 1000 distinct mutations in COL1A1 and COL1A2 have been identified in OI patients, and these are maintained in an online database. Mild OI type I is the most common form of the disorder and is associated with a null COL1A1
allele that leads to a reduction in the amount of type I collagen produced in the tissue\textsuperscript{69}.

On the other hand, OI types III and IV are related with structural mutations in the above genes that cause deteriorated quality but a normal quantity of type I collagen\textsuperscript{70}. The remaining OI types are mainly caused by mutations to chaperone proteins involved in collagen folding and cellular transport\textsuperscript{71–74}. The genetic etiology of approximately 5\% of OI cases remains unknown\textsuperscript{2}.

<table>
<thead>
<tr>
<th>Clinical OI type</th>
<th>Severity</th>
<th>Affected gene (and associated protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mild (non-deforming)</td>
<td>Null COL1A1 (type I collagen)</td>
</tr>
<tr>
<td>II</td>
<td>Lethal (perinatal)</td>
<td>COL1A1/2 (type I collagen)</td>
</tr>
<tr>
<td>III</td>
<td>Severe (progressive deforming)</td>
<td>COL1A1/2 (type I collagen)</td>
</tr>
<tr>
<td>IV</td>
<td>Moderate</td>
<td>COL1A1/2 (type I collagen)</td>
</tr>
<tr>
<td>V</td>
<td>Moderate</td>
<td>IFITM (bone-restricted interferon-induced transmembrane protein-like protein, BRIL)</td>
</tr>
<tr>
<td>VI</td>
<td>Moderate to severe</td>
<td>SERPINF1 (pigment epithelium-derived factor, PEDF)</td>
</tr>
<tr>
<td>VII</td>
<td>Severe to lethal</td>
<td>CRTAP (cartilage associated protein)</td>
</tr>
<tr>
<td>VIII</td>
<td>Severe to lethal</td>
<td>LEPRE1 (leucine proline-enriched proteoglycan 1, leprecan 1)</td>
</tr>
<tr>
<td>IX</td>
<td>Moderate to lethal</td>
<td>PPIB (cyclophilin B protein, CyPB)</td>
</tr>
<tr>
<td>X</td>
<td>Severe to lethal</td>
<td>SERPINH1 (heat shock protein 47, HSP47)</td>
</tr>
<tr>
<td>XI</td>
<td>Severe (progressive deforming)</td>
<td>FKBP10 (FK506 binding protein 65, FKBP65)</td>
</tr>
</tbody>
</table>

This dissertation focuses on specimens obtained from the main shaft, or diaphysis, of long bones during routine surgical procedures at Shriners Hospitals for Children in Chicago, IL. Of the more than 130 specimens collected to date by our group, most come from patients diagnosed with moderate OI type IV or severe OI type III. This is because surgical intervention is usually only required in the more progressively deforming forms.
of the disorder. The remaining specimens typically come from mild OI type I patients undergoing fracture repair (e.g., from a trauma).

B-2. Hierarchical Bone Structure

Bone fracture is a complex phenomenon that requires insight on the hierarchical nature of the composite bone material (Figure I-1). Healthy bone tissue is composed of organic constituents like cells (e.g., osteocytes, osteoblasts, and osteoclasts) and extracellular matrix proteins, as well as inorganic compounds such as hydroxyapatite (HA). The mechanical properties of bone are intermediate between type I collagen and HA\textsuperscript{75}. HA is arranged in crystals along the length of collagen fibrils, which are themselves complex arrangements of triple helices (Figure I-1). This structure combines the mechanical advantages of both materials. Namely, HA provides structural rigidity and compression strength, while the more flexible collagen fibers prevent brittle cracking and provide improved tensile properties.

![Figure I-1. Hierarchical structure of bone (with associated length scales).](image)

OI has been shown to have an effect on the mineralized collagen fibril at multiple levels. For example, abnormal fibrillar diameters have been reported\textsuperscript{76–79}, and changes in mineral composition\textsuperscript{80}, crystal size\textsuperscript{3,4}, and packing density have also been noted\textsuperscript{6}. It has been suggested that these abnormalities may negatively impact fibrillar mechanics\textsuperscript{81,82}. 
Such changes have been implicated in the increased bone fragility associated with advanced aging\textsuperscript{83}.

In long bones like the femur, cortical bone makes up the hard outer shell surrounding the bone marrow cavity, and the epiphyseal or end regions are filled with the more sponge-like trabecular bone (Figure I-2)\textsuperscript{84}. Typical values for porosity range from 5-10\% for cortical bone to 50-95\% for trabecular bone\textsuperscript{85,86}. The prevailing cortical bone structural unit (BSU) in humans is the osteon, which generally has a diameter ranging from 50-300 $\mu$m and a length of several millimeters. The anatomy of an osteon consists of a central Haversian canal, which contains capillaries and nerves, surrounded by concentric lamellae or layers of mineralized fiber arrays at alternating orientations. Adjacent osteons are connected via transverse Volkmann’s canals (Figure I-2).

*Figure I-2. Bone anatomy.* (Right) Diagram of a human femur, indicating the relevant terms for each bone region. (Left) Magnification of boxed region, showing osteons and their anatomical features. This figure is a derivative of “Bone structure” by Servier Medical Art, used under CC BY 3.0\textsuperscript{84}. For licensing details, visit http://creativecommons.org/licenses/by/3.0/us/.
The formation and adaptation of osteons is controlled by various bone cells through the processes of bone modeling and remodeling, whereby osteoclasts resorb old or damaged bone, while osteoblasts deposit new organic material (i.e., type I collagen) called osteoid that acts as a matrix for subsequent mineralization. Interstitial lamellae are the remnants of older osteons that have been replaced during remodeling. Some osteoblasts become trapped within the matrix during mineralization, where they are transformed into osteocytes (Figure I-2). These osteocytes are encased within spaces called lacunae, but they maintain communication with the bone surface and each other through small dendritic connections called canaliculi that are believed to be important for mechanotransduction. Mechanotransduction is the process by which external loads felt by the skeleton are converted into biochemical signals that are used to drive cellular responses such as microdamage repair and bone remodeling. Although difficult to measure experimentally, it is thought that external loads lead to microscopic pressure gradients within bone tissue that drive fluid flow through canaliculi and across osteocytes. This fluid flow generates shear stresses and tiny electrical potentials that are sensed by the cell membrane.

B-3. Bisphosphonates

Bisphosphonates are a class of bone augmenting drugs initially developed for adults with osteoporosis and Paget’s disease. Their chemical structure is similar to inorganic pyrophosphate, a naturally occurring substance in the body that is responsible for preventing calcium deposits in soft tissues such as skin, kidneys, and blood vessels. Bisphosphonates attach to the surface of HA crystals and tend to
accumulate in regions that have undergone high bone resorption (i.e., where the mineral is most exposed). It is thought that they are selectively internalized (via endocytosis) by osteoclasts, which create a highly acidic microenvironment during bone resorption that normally dissolves HA bone mineral\(^{94,95}\). Beyond simply preventing HA dissolution, unbound bisphosphonates cause morphological changes within the cell, and they have been implicated in the disruption of normal osteoclast functions such as recruitment, differentiation, and resorptive activity. Moreover, there is evidence that bisphosphonates may actually induce osteoclast apoptosis (i.e., programmed cell death)\(^{92}\).

One of the main purported benefits of bisphosphonates therapy is improved bone mass, which is often correlated with bone mechanical properties\(^{96}\). Clinical justification has been provided via osteoporosis studies, which have noted increases in whole body BMD in excess of 2% after bisphosphonates treatment, with corresponding reductions in the incidence of fragility fractures\(^{90,91}\). Despite limited empirical data in children, these drugs have therefore become widespread in the treatment of OI\(^{37,97–100}\). The two most common bisphosphonates used to treat OI are pamidronate (i.e., Aredia\(^{®}\)), which is typically administered via periodic intravenous injection, and alendronate (i.e., Fosamax\(^{®}\)), which can be taken orally in tablet-form.

Unfortunately, the long-term safety, efficacy, and duration of action of these bisphosphonates is poorly understood\(^{101}\), especially in children\(^{36,102}\). For example, the rate of bone turnover (i.e., formation and resorption) is known to be elevated in OI\(^{21,103–105}\). However, recent work on children with OI who had been treated with pamidronate reported suppressed bone turnover below the rate of healthy children\(^{106}\), prompting
B-4. Assessment of Bone Structure

Characterization of bone structure and remodeling is typically performed via histomorphometry, a strategy that is based on histological sectioning and analysis of biopsies collected from the iliac crest (Figure I-3)\(^84,116\). This skeletal site, which is located at the superolateral or top outer portion of the pelvis, is preferred because of its accessibility and reduced complication rate\(^117\). Human iliac crest biopsies are generally

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**Figure I-3. Histomorphometric analysis of bone structure.** (Left) Pelvis anatomy, showing typical location for iliac crest biopsy. (Right) Histological section containing both cortical and trabecular bone. This figure is a derivative of “Skeletons and bones” and “Bone structure” by Servier Medical Art, used under CC BY 3.0\(^84,116\). For licensing details, visit http://creativecommons.org/licenses/by/3.0/us/. 
cylindrical in shape, with a diameter of 5-8 mm\textsuperscript{118}. Histological sections typically contain both cortical and trabecular bone tissue (Figure I-3). Nevertheless, traditional histomorphometry focuses on trabecular bone because the internal geometry of iliac cortical bone is not well understood\textsuperscript{118}.

Static characteristics of the bone structure are determined from histological sections using measurements such as distance (e.g., perimeter or width of an object), area, or the number of objects. Staining and/or fluorescent labeling techniques are also commonly used to enhance visualization of specific structures, or for viewing bone formation during a predetermined period of time\textsuperscript{119}. Accurate 3D structural information is necessary for proper comparisons (e.g., between bones, species, pathologies, etc.), or when results are used in computational models. To address this issue, the branch of mathematics called stereology is used to convert 2D measurements into 3D\textsuperscript{120}.

Stereological theorems require many assumptions about the sampling, geometry, and distribution of objects within a planar section\textsuperscript{121–123}. These conditions are not always met in bone histomorphometry, and they can be difficult to justify on the basis of 2D images\textsuperscript{27}. For example, all stereological formulas require random and unbiased sampling, and most also assume that bone structures are isotropic (i.e., randomly oriented)\textsuperscript{118}. Inherent in these assumptions is the idea that bone structures are being viewed and measured along some oblique “cut” through the material. To correct for this effect, an “obliquity factor” of $4/\pi$ has been derived for estimating 2D trabecular width calculations as 3D thicknesses\textsuperscript{118,122}. Moreover, measurements based on counting the number of objects in a planar image are susceptible to biasing via overestimation. This error can
occur when oblique cuts are made through an irregular structure, producing multiple instances of the same object on the section \(^{124}\).

In addition to these challenges, traditional histomorphometry has other practical limitations. Histology involves embedding the biopsy in plastic resin, which limits the ability for further testing \(^{125}\). Analyses are also time-consuming, expensive, and often require specialized lab equipment and training, especially when staining and/or fluorescent labeling are needed. Because of these constraints, researchers typically sample only two to four sections per biopsy \(^{119}\). Thus, sampling bias is a concern, since any regional variations within the biopsy may not be adequately captured \(^{27}\).

Because of the scarcity of human OI bone specimens, to date there is surprisingly limited data examining how OI affects bone microstructure and development. A few groups have used histomorphometry to examine iliac crest biopsies \(^{22,23}\). The most extensive of these studies reported significant decreases in trabecular bone volume compared to age-matched controls, which was explained primarily by a 40-60% reduction in the number of trabeculae (i.e., trabecular bone units), as well as a 15-30% thinning of trabecular structures \(^{21}\). The same study also found increases in several bone remodeling parameters for OI vs. healthy subjects.

Despite the fact that cortical bone accounts for 80% of the total mass of the skeleton \(^{126}\), very little is known about its structure in OI. Reductions in cortical wall thickness have been noted via histomorphometry \(^{21-23}\). Another study using high-resolution peripheral quantitative CT (HR-pQCT), a form of clinical CT that enables in vivo analysis of distal skeletal sites, found reduced cortical bone area at the tibia in adults.
with mild OI\textsuperscript{127}. Nevertheless, HR-pQCT is mainly used to analyze trabecular bone, since the resolution ($\approx 80 \, \mu\text{m}$) is not fine enough to visualize individual osteons.

Alternatively, the spatial resolution and efficiency of commercial and lab-based µCT systems has improved greatly over the last decade, such that it is now the gold standard for \textit{ex vivo} characterization of bone structure\textsuperscript{28}. Rather than relying on stereology to extrapolate histomorphometric measurements into a third dimension, µCT enables direct 3D quantitative analysis of bone structure and connectivity\textsuperscript{24–27}. As part of a preliminary analysis performed during the initial stages of this dissertation, a lab-based µCT system was used to study osteotomy specimens from the mid-diaphysis of young individuals with OI at a resolution of approximately 35 \, \mu m. The bone contained extensive porosity, with a hybrid-like structure that appeared to be intermediate between cortical and trabecular bone (Figure I-4)\textsuperscript{128}.

\textbf{Figure I-4. Preliminary µCT analysis of OI bone structure.} (Left) Typical wedge osteotomy collected from the mid-diaphysis during deformity correction. (Right) µCT volume rendering of osteotomy specimen. The left part of this figure is a derivative of “Skeletons and bones” by Servier Medical Art, used under CC BY 3.0\textsuperscript{116}. For licensing details, visit http://creativecommons.org/licenses/by/3.0/us/.
Additional improvements in resolution (e.g., \( \approx 1\mu m \)) and compositional sensitivity can be achieved through the use of synchrotron light sources, which are capable of producing highly brilliant, monochromatic (i.e., uniform) X-rays\textsuperscript{129–131}. Synchrotron radiation \( \mu \)CT, or SR\( \mu \)CT, allows for analysis of small microstructural features within cortical bone including Haversian canals, Volkmann’s canals, resorption spaces, cement lines (i.e., the outer boundaries of osteons), and osteocyte lacunae\textsuperscript{131–139}. Access to synchrotron facilities is limited, so experimental beamtime is highly competitive. However, some government facilities such as the Advanced Light Source (ALS) in Berkeley, CA allow graduate students to submit proposals during the later stages of their dissertation.

B-5. Assessment of Bone Mechanical Properties

Physiological loads are typically characterized by some combination of external forces including tension, compression, torsion, and/or bending. Bones respond to these forces by deforming through processes such as elongation (i.e., stretching), compression, twisting, and flexion (i.e., bending), respectively. As long as the external loads remain sufficiently low, there is a linear relationship between the load and deformation. Under these “elastic” conditions, the bone acts like a spring, and it returns to its original shape upon unloading. The structural stiffness (i.e., rigidity) of the bone can be measured from the ratio between the applied load and the resulting deformation\textsuperscript{140}. Once the loading reaches a certain threshold called the structural strength, permanent damage occurs.

However, the structural stiffness and structural strength are not usually considered as true mechanical properties, since they vary based on a bone’s size and geometry, as
well as the loading configuration. As an example, consider the simple case of a bone pulled in tension. It is perhaps intuitive that a bone from a very large person (e.g., an offensive lineman in football) would support higher tensile loads than the same bone from a smaller person (e.g., an Olympic marathon runner). This behavior can be understood by considering the cross-sectional area and chemistry of the two bones. If one visualizes each chemical bond within the bone as a spring with a certain characteristic stiffness and strength, it follows that the bone with the larger cross-sectional area will have more springs (i.e., bonds) and will therefore have a higher structural strength.

It is therefore useful to define stiffness and strength in a relative sense, so that one can determine whether the difference between two bones is caused by some change in the intrinsic properties of the bone material, or if it can be explained simply by variations in bone size, shape, and organization. The engineering concepts of stress ($\sigma$) and strain ($\varepsilon$) have been developed to address such questions. Stress may be thought of as the internal force distribution experienced throughout a material as a result of some external force, and it is defined in terms of a force per unit cross-sectional area. On the other hand, strain characterizes the local deformations (i.e., changes in size and/or shape) that occur within a material as a result of the local stresses. The physical meaning of strain can be grasped by imagining a short “fiber” or line segment that passes through an arbitrary point within the bone. When an external force is applied, internal stresses cause a change in the length and/or orientation of this fiber. In this scenario, the strain is defined as the change in length of the fiber divided by its original length.
Similar to many other engineering and biological materials, bone exhibits a linear relationship between stress and strain up to a certain point called the yield point (Figure I-5). Bone is thus considered a “linear elastic” material, and the ratio between stress and strain (i.e., the slope of the stress-strain curve) in this linear region is a constant that is independent of the bone’s size. This constant is a material property known as the elastic modulus ($E$), and it describes a bone’s intrinsic stiffness. As shown in Table I-2, normal values for elastic modulus in the longitudinal direction range from 10-18 GPa in weight-bearing bones (e.g., femur, tibia, etc.) to 3-5 GPa in non-weight-bearing bones (e.g., ribs). Drawing on the previous example, a bone from a large person may have a much higher structural stiffness than the same bone from a smaller person, yet the two should have the same intrinsic stiffness (i.e., elastic modulus).

**Figure I-5. Stress-strain curve of healthy bone.** (Left) Mechanical properties typically reported for bone include elastic modulus ($E$), yield strength ($\sigma_y$), and ultimate strength ($\sigma_{max}$). (Right) Influence of microstructure on stress-strain behavior. In this example, the curves indicate the stress-strain profile on the tensile surface of the beam during bending.
Beyond the yield point, irreversible damage occurs and the relationship between stress and strain becomes non-linear (Figure I-5). The stress threshold associated with this point is known as the yield strength ($\sigma_y$). The physical meaning of the yield strength can be understood as the point at which permanent or “plastic” deformations (e.g., microcracking) initiate within the tissue\textsuperscript{140}. Even as this damage accumulates, the bone continues to support additional loading up to its ultimate strength ($\sigma_{max}$), after which catastrophic failure (i.e., a bone fracture) occurs (Figure I-5). Healthy values for the yield strength and ultimate strength of bone are highly dependent on variables such as the skeletal testing site, donor age, and testing method, among others (Table I-2).

An important caveat to this discussion is the concept of ductility vs. brittleness. These terms relate to the amount of post-yield strain a material can sustain before fracturing. By this definition, ductile and brittle can be taken as large and small post-yield regions, respectively (Figure I-5). Notice that these terms are not related to the strength (i.e., stress). This key distinction means that a brittle material could potentially have a high strength, while a ductile material could have a low strength. Indeed, thus is the case for many ceramics and plastics, respectively. The fact that OI is commonly referred to as “brittle bone disease” is a clinical misnomer, or at least an incomplete description. Perhaps a more appropriate name for OI would be “fragile bone disorder”, since fragility encompasses many properties including elastic modulus, strength, ductility, and toughness\textsuperscript{16,17}.
Toughness is a complex mechanical property that quantifies a bone’s ability to resist fracture. To date, there is no standard method that has been established to assess bone toughness. The total area under the stress-strain curve, or “work to fracture”, represents the amount of energy required to fracture a bone, and it has historically been reported as a measure of toughness.\textsuperscript{126,142} However, quantities such as the work to fracture, yield strength, and ultimate strength are highly dependent on the distribution of flaws within bone.\textsuperscript{147} Examples of “flaws” that act as local stress concentrations in bone include osteocyte lacunae, osteocyte canaliculi, vasculature, and resorption spaces.\textsuperscript{148–154}
Fracture mechanics is a field of materials science that attempts to account for these internal flaws. In its simplest form, fracture mechanics involves machining a sharp pre-crack in a specimen and loading it, using derived relationships between the specimen geometry, pre-crack geometry, and method of loading to calculate a stress intensity factor ($K$). The value of $K$ describes the local stress (and displacement) fields at the edge of the pre-crack. The critical value at which the pre-crack begins to grow is known as the fracture toughness ($K_c$, or “single-value toughness”). Just as the yield strength describes the limiting elastic value of stress (Figure I-5), the fracture toughness can be considered the limiting stable value of stress intensity. The single-value toughness provides a better representation of the bone’s fracture resistance than the work to fracture, because it reduces the role of smaller, pre-existing defects such as microcracks. Nonetheless, the value of $K_c$ is perhaps only a conservative estimate of the true fracture toughness of bone, since by definition it does not incorporate any contributions from plastic deformation.

Often there is a period of stable crack growth in bone such that it can sustain additional loading. During this time, the fracture resistance increases due to several toughening mechanisms including microcracking, crack deflection, and uncracked ligament bridging (Figure I-6). Toughening mechanisms offer relief by lowering the stress intensity in the so-called “plastic zone” near the crack tip. If imaging is performed in concert with a fracture toughness test, it is possible to monitor crack growth as well as the evolution of toughening mechanisms in real time. This information can be used to visualize how the stress intensity changes as a function of crack extension ($\Delta a$). When plotted together, the result is known as a crack-resistance or $R$-curve. The $y$-intercept and slope of this curve represent the crack-initiation
toughness ($K_{J,o}$) and crack-growth toughness ($dK_J/d\Delta a$), respectively. These calculations are performed by using a special branch of fracture mechanics known as elastic-plastic fracture mechanics, or the “$J$-integral method”\textsuperscript{169}. This two-valued approach has been suggested as a truer estimate of the fracture resistance of bone, since it not only describes the toughness associated with crack initiation, but also accounts for plastic deformation that occurs prior to fracture\textsuperscript{160,170,171}. Typical values for $K_c$, $K_{J,o}$, and $dK_J/d\Delta a$ are presented in Table I-3.

![Common toughening mechanisms in bone](image)

**Figure I-6. Common toughening mechanisms in bone.** (a) Microcracking; (b) Uncracked ligament bridging; (c) Crack deflection. The shaded area indicates the “plastic zone”, the size of which depends on the stress conditions within the body.

**Table I-3. Fracture toughness of wet cortical bone in bending.**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Bone (location)\textsuperscript{a}</th>
<th>Ages (years)</th>
<th>Method</th>
<th>Single-value toughness, $K_c$ (MPa·m$^{1/2}$)</th>
<th>Crack-initiation toughness, $K_{J,o}$ (MPa·m$^{1/2}$)</th>
<th>Crack-growth Toughness, $dK_J/d\Delta a$ (MPa/m$^{1/2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>Femur (M.D.)</td>
<td>35-92</td>
<td>Bending</td>
<td>5.6 ± 0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>143</td>
<td>Femur (M.D.)</td>
<td>19-49</td>
<td>Bending</td>
<td>5.1 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>143</td>
<td>Femur (M.D.)</td>
<td>50-69</td>
<td>Bending</td>
<td>5.4 ± 0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>143</td>
<td>Femur (M.D.)</td>
<td>70-89</td>
<td>Bending</td>
<td>4.3 ± 0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>160</td>
<td>Humerus (M.D.)</td>
<td>37-41</td>
<td>Bending</td>
<td>-</td>
<td>$L$: 1.0</td>
<td>$L$: 48.0</td>
</tr>
<tr>
<td>83</td>
<td>Humerus (M.D.)</td>
<td>34-41</td>
<td>Bending</td>
<td>-</td>
<td>$C$: 0.5</td>
<td>$C$: 4.2</td>
</tr>
<tr>
<td>172</td>
<td>Iliac crest</td>
<td>85-99</td>
<td>Bending</td>
<td>-</td>
<td>$C$: 0.4</td>
<td>$C$: 2.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}M.D. = mid-diaphysis, P.D. = proximal diaphysis, D.D. = distal diaphysis, W = whole bone, P.M. = proximal metaphysis (see Figure I-2 for review of bone anatomical regions).

\textsuperscript{b}$L$ = longitudinal direction, $C$ = circumferential direction (see Figure I-5 for schematic of orientations). Note: values in the table that do not include a defined direction can be assumed to be longitudinal properties.
Similar to other biological materials like wood, healthy bone has a “grain” or preferred orientation, along which its mechanical properties are higher\textsuperscript{140}. In materials science, this behavior is known as anisotropy. It should be no surprise that bone’s mechanical anisotropy is strongly tied to its microstructural anisotropy, which is primarily associated with the dominant orientation of Haversian canals in osteons\textsuperscript{145}. As shown in Figure I-5, Table I-2, and Table I-3, the elastic modulus, yield strength, ultimate strength, and fracture toughness are highest in the longitudinal direction, where the osteons are aligned parallel to the long axis of the bone. However, these mechanical properties do not differ significantly when osteons are aligned in the remaining two directions (circumferential and radial with respect to the long bone axis)\textsuperscript{140,146,173–175}. Because of this behavior, healthy bone is known as a “transversely isotropic” material.

Due to the extremely limited availability and irregular size of pediatric bone specimens, there are very few studies describing mechanical properties in OI cortical bone. The majority of this work has focused on nanoindentation, which relies on a sharp indenter tip pressing into the surface of a highly polished, embedded material. This technique provides local measurements of elastic modulus and hardness (i.e., localized plastic deformation) at the level of osteons and interstitial lamellae. One study in a young population reported a 13\% increase in elastic modulus and a 21\% increase in hardness for pediatric OI bone compared to controls\textsuperscript{20}. The authors concluded that these observations were mainly caused by an increase in the degree (but not necessarily the overall amount) of bone matrix mineralization. Another recent study found no difference between the elastic modulus and hardness in the longitudinal direction compared to the circumferential/radial direction in patients with severe OI type III\textsuperscript{54}. In related work, the
same authors showed that these properties did not vary with variables such as disease severity (i.e., severe OI type III vs. moderate OI type IV) or skeletal sampling site (i.e., iliac crest vs. femur/tibia)\textsuperscript{55,56}.

Together, these results were presented as proof that OI cortical bone may have isotropic mechanical properties (compared to the transversely isotropic behavior of healthy bone). However, it is not possible to produce a stress-strain curve using nanoindentation, so other properties governing bone fragility (i.e., yield strength, ultimate strength, and fracture toughness) have not been explored in humans with OI. Moreover, specific indentation locations were not rigorously described in the above studies, so the effect of the local microstructure on elastic modulus and hardness has not been addressed. To date, there have also not been any studies that compared the mechanical properties in mild OI type I (which affects collagen quantity) to the more severe types (which primarily affect collagen quality).

Other mechanical testing methods such as three-point bending are well-suited for small specimen testing, provided that the sample can be machined into a standardized geometry. Indeed, numerous examples in the literature have shown reduced yield strength, ultimate strength, and toughness (i.e., work to fracture) in animal models of OI using three-point bending\textsuperscript{9–14,176}. A few others have also reported an increased susceptibility for microdamage accumulation in mice with similar genetic mutations to OI\textsuperscript{177–179}. However, the microstructure of mouse bone is vastly different from humans and does not contain osteons, so the relevance of these findings is unclear\textsuperscript{180}.
C. Hypotheses and Specific Aims

Accurate material property and microstructural information are needed to better understand bone fragility in OI. This data is also useful in the development and evaluation of treatment strategies for the disorder, as well as subject-specific fracture risk assessment models. The objectives of this dissertation are to characterize bone microstructure (i.e., vascular porosity and osteocyte lacunar porosity), mineralization (i.e., volumetric bone mineral density and tissue mineral density), and mechanical properties (i.e., elastic modulus, yield strength, ultimate strength, and fracture toughness) in cortical bone from children and adolescents with OI. The hypotheses are:

1. Bone material properties vary with microstructure in OI and can help to distinguish clinical severity.
2. OI cortical bone shows altered mineral and microstructural properties at the tissue and cellular levels compared to healthy controls.

To achieve the study objectives, the following specific aims were accomplished:

1. Elastic modulus and hardness were investigated in osteonal and interstitial lamellar bone using nanoindentation, and the results were compared between mild OI type I and severe OI type III.
2. SRμCT was used to assess the microstructural and mineralization properties of OI and healthy cortical bone in 3D.
3. The mechanical isotropy of OI cortical bone was evaluated, and correlations were performed between mechanical, microstructural, and mineralization parameters.
II. BONE PROPERTIES BY NANOINDENTATION IN MILD AND SEVERE OSTEOGENESIS IMPERFECTA

A. Abstract

Previously there have been few studies on the mechanical properties of bone in OI, especially for mild OI type I, which is the most common clinical form of the disorder. In the current study, nanoindentation was used to investigate differences in the elastic modulus ($E_{\text{nano,L}}$) and hardness ($H_{\text{nano,L}}$) in the longitudinal direction between children and adolescents with mild OI type I vs. severe OI type III. A total of 168 indents were performed in bone specimens from 11 pediatric OI donors. Mild OI type I bone showed significant (i.e., $P$-value < 0.05) increases compared to that of severe OI type III in both elastic modulus (17.53 ± 0.47 GPa vs. 16.3 ± 0.55 GPa, respectively) and hardness (0.656 ± 0.026 GPa vs. 0.602 ± 0.018 GPa, respectively). Lamellar bone microstructure was also found to affect these properties, as osteonal bone regions showed decreases in both $E_{\text{nano,L}}$ (13%) and $H_{\text{nano,L}}$ (11%) compared to interstitial bone. Other covariates such as anatomical site, donor gender, and history of bisphosphonates therapy also had a small (i.e., < 10%) yet significant ($P < 0.05$) effect on nanoindentation properties. This study presents the first data describing elastic modulus and hardness in children with mild OI type I, and it underscores the importance of considering microstructure when determining bone mechanical properties. Note: A version of this chapter has been published in a recent journal article\textsuperscript{181}. 
B. Introduction

Currently, the increased bone fragility associated with OI is thought to be related to a combination of bone mass deficiency and compromised bone material properties\textsuperscript{36,182}. However, to date there is little data available describing bone material properties in individuals with OI. To complicate matters, the severity of the disorder varies widely among patients, where most are generally classified into one of at least 11 known clinical types based on their specific genetic mutation and/or symptoms\textsuperscript{1,2,64}. OI type I is the most common and least severe form of the disorder. These patients generally suffer fewer fractures during development and have near normal stature and basic physical function\textsuperscript{49}. On the other hand, OI type III is a severe form of the disorder characterized by progressive spine and limb deformity and extreme bone fragility throughout skeletal development\textsuperscript{1,64}.

Both structural and material bone abnormalities have been noted previously in OI. For example, individuals with OI tend to have lower bone mass or bone mineral density than their peers, as evidenced by lower cortical wall thickness and decreased trabecular bone volume fraction in histomorphometric analyses\textsuperscript{21,183}. At smaller scales (i.e., \( \leq 1 \) \( \mu \)m), differences in type I collagen fibrillar diameter have been reported between OI and healthy bone\textsuperscript{76,79}. Hydroxyapatite mineral abnormalities have also been noted, including changes to mineral content, shape, size, and composition\textsuperscript{3,4,77,80}. Despite a net decrease in overall bone density, several studies have found that OI bone matrix has a higher than normal mineralization density\textsuperscript{20,183,184}. These alterations to the collagen and mineral template suggest corresponding changes to the intrinsic bone material behavior. Indeed,
a few studies have used nanoindentation to characterize elastic modulus and hardness in bone specimens from children and adolescents with OI types III and IV\textsuperscript{20,54–56}.

The nanoindentation technique generally involves pressing a sharp diamond-tip probe into the surface of a finely polished specimen. Indentations are highly localized (i.e., on the order of a few hundred nanometers deep) and thus provide a means for determining elastic modulus and hardness even in small or irregularly shaped specimens. These properties are reportedly higher in OI type III bone compared to age-matched controls\textsuperscript{20}, yet no difference has been found between OI types III and IV\textsuperscript{55,56}. To date, there is no data in the literature describing bone mechanical properties in the more common mild OI type I. In previous nanoindentation studies, wide ranges of values for both elastic modulus and hardness have been reported for severe OI cortical bone. The variability in nanoindentation results may be attributed in part to the selection of indentation sites\textsuperscript{185–188}. Indeed, prior work in healthy bone has indicated that these properties tend to be higher in interstitial compared to osteonal bone regions\textsuperscript{185,187}.

The primary goal of this study was to determine whether the elastic modulus and hardness of bone differ between pediatric patients with OI types I and III. A second aim was to evaluate whether these properties differ in interstitial vs. osteonal bone regions.

C. Methods

C-1. Human OI Cortical Bone Specimens

Bone specimens were collected under informed consent/assent from the lower extremity of young individuals (aged 5-18 years) with OI under an approved IRB
A total of 12 bone samples were gathered at Shriners Hospitals for Children – Chicago during routine orthopaedic procedures (e.g., deformity correction or fracture repair). One of these samples was excluded due to the presence of extensive fracture callous, indicating recent bone remodeling around a fracture site. A total of 11 specimens from 10 donors were thus included in this study: 6 samples from donors classified with mild OI type I (aged 7-16), and 5 samples from donors with severe OI type III (aged 7-14) (Table II-1). There was no significant difference in donor age between the two groups (Student’s $t$-test, $P$-value = 0.5). All specimens were stored unfixed and undecalcified at -85°C until testing. A prior study reported that long term storage at a similar temperature (-80°C) did not significantly alter the mechanical properties of bone\textsuperscript{189}.

### Table II-1. Specimen descriptions for nanoindentation study.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>OI Type</th>
<th>Age</th>
<th>Gender</th>
<th>Anatomic Site</th>
<th>Bisphosphonate Treatment</th>
<th># Indents Osteonal</th>
<th># Indents Interstitial</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>16</td>
<td>M</td>
<td>Tibia\textsuperscript{a}</td>
<td>Yes</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>7</td>
<td>F</td>
<td>Tibia\textsuperscript{b}</td>
<td>Yes</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>3\textsuperscript{*}</td>
<td>I</td>
<td>14</td>
<td>F</td>
<td>Tibia\textsuperscript{b}</td>
<td>Yes</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>13</td>
<td>M</td>
<td>Femur\textsuperscript{a}</td>
<td>Yes</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>5\textsuperscript{*}</td>
<td>I</td>
<td>11</td>
<td>F</td>
<td>Femur\textsuperscript{a}</td>
<td>Yes</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>15</td>
<td>M</td>
<td>Femur\textsuperscript{c}</td>
<td>Yes</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>III</td>
<td>12</td>
<td>M</td>
<td>Tibia\textsuperscript{b}</td>
<td>No</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>8</td>
<td>III</td>
<td>12</td>
<td>M</td>
<td>Tibia\textsuperscript{b}</td>
<td>No</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>III</td>
<td>14</td>
<td>M</td>
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<td>14</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>III</td>
<td>7</td>
<td>F</td>
<td>Tibia\textsuperscript{b}</td>
<td>No</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>III</td>
<td>12</td>
<td>F</td>
<td>Femur\textsuperscript{a}</td>
<td>Yes</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Indicates specimens from the same donor collected during surgical procedures performed three years apart.

\textsuperscript{a}Proximal diaphysis; \textsuperscript{b}Mid-diaphysis; \textsuperscript{c}Distal diaphysis.

### C-2. Specimen Preparation

Prior to nanoindentation testing, all specimens were thawed, sectioned, dehydrated, and embedded in general agreement with guidelines reported for hard
tissue\textsuperscript{190}. Namely, bone samples were sectioned under constant water irrigation using a low-speed diamond saw (Isomet\textsuperscript{TM} Low Speed Saw; Buehler, Lake Bluff, IL, USA) such that each cut was approximately parallel to the long axis of the bone. Cut specimens were fixed and dehydrated in the following graded solutions of ethanol: 70% (24 hours), 80% (2 hours), 95% (2 hours), 95% (3 hours), 100% (2 hours), 100% (2 hours), and 100% (3 hours). Specimens were then air-dried and embedded under vacuum (to allow full infiltration of the embedding media) in a low viscosity epoxy resin (Epo-Thin\textsuperscript{®}; Buehler, Lake Bluff, IL, USA). To generate the smooth surface required for nanoindentation, embedded samples were sanded using a grinder-polisher (Metaserv\textsuperscript{®} 3000; Buehler, Lake Bluff, IL, USA) with silicon carbide (SiC) paper having progressively finer grit sizes (400, 600, 800, and 1200), and then polished to a final surface roughness of 0.05µm using a polishing cloth and alumina suspension (Micropolish\textsuperscript{®} B; Beuhler, Lake Bluff, IL, USA).

C-3. Nanoindentation Testing

Indentation was performed using a tetrahedral Berkovich diamond tip affixed to a nanoindenter machine (Nano Indenter XP; MTS, Eden Prairie, MN, USA) at the Characterization Facility, a user center operated by the University of Minnesota (Minneapolis, MN). Elastic modulus ($E_{nano,L}$) and hardness ($H_{nano,L}$) were determined in the longitudinal direction using the nanoindenter’s Continuous Stiffness Measurement (CSM) algorithm (Figure II-1), which applies a small harmonic force ($P_h$) superimposed on top of a nominally increasing compressive load ($P$) that drives the indenter into the material according to the following equations:
\[ P = P_h e^{i\omega t} \quad \text{Equation II-1} \]

\[ h(\omega) = h_0 e^{(i\omega t + \phi)} \quad \text{Equation II-2} \]

where \( \omega \) is the excitation frequency, \( t \) is time, \( h(\omega) \) is the magnitude of the oscillating displacement response of the indenter, and \( \phi \) is the phase angle between the force and displacement signals\(^{191} \). Note that the amplitude of the oscillating force \( P_h \) is typically several orders of magnitude smaller than the nominal load.

The nanoindentation process can be generalized as a mechanical model consisting of springs and dashpots according to Figure II-1, where \( m \) is the mass of the indenter, \( K_s \) is the spring constant of the leaf springs supporting the tip, \( K_f \) is the stiffness of the indenter frame (with compliance \( C_f \)), \( C \) is a damping coefficient related to the air in the gaps between capacitor plates in the displacement sensor, and \( S \) is the contact stiffness.

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\[ h(\omega) = h_0 e^{(i\omega t + \phi)} \]

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Note that $K_s$ and $K_f$ are not related to the previous discussion of fracture toughness in Chapter I. Rather, these letters follow the convention typically used to describe spring constants in physical models. In this way, $S$, $E_{nano,L}$, and $H_{nano,L}$ can be calculated in the longitudinal direction at any point along the loading curve from the known frequency and the measured displacement, phase angles, and force according to:

$$S = \left[ \frac{1}{\left( \frac{P_h}{h(\omega)} \right) \cos(\phi) - (K_s - m\omega^2)} - K_f^{-1} \right]^{-1}$$  \hspace{1cm} \text{Equation II-3}

$$E_{nano,L} = (1 - \nu_{bone,L}^2) \left( \frac{\sqrt{\pi}}{2} \frac{1}{\sqrt{A_c}} S \right)$$  \hspace{1cm} \text{Equation II-4}

$$H_{nano,L} = \frac{P_{max}}{A_c}$$  \hspace{1cm} \text{Equation II-5}

$$A_c = 24.56 h_c^2 + C_1 h_c^1 + C_2 h_c^{1/2} + C_3 h_c^{1/4} + \cdots + C_8 h_c^{1/128}$$  \hspace{1cm} \text{Equation II-6}

where $\nu_{bone,L}$ is the Poisson’s ratio of bone in the longitudinal direction, $P_{max}$ is the peak compressive force of the indenter, $A_c$ is the contact area, $h_c$ is the contact depth, and $C_1$ through $C_8$ are all calibration constants that account for deviations from the ideal Berkovich tip geometry due to blunting. Poisson’s ratio is a material property describing a compressed (or stretched) body’s tendency to expand (or contract) in perpendicular directions. For uniaxial compressive loading, a Poisson’s ratio of 0.3 (i.e., 30% expansion in the lateral direction) is common in bone studies. The
calibration constants in Equation II-6 were automatically calculated prior to bone testing through repeated indentation measurements in a fused silica standard sample.

The CSM method is common in bone material property studies, whereby the sharp indenter tip penetrates the specimen surface with a user-defined frequency, amplitude, strain rate, and maximum depth. Similar to other studies\textsuperscript{193,194}, the following input parameters were used: 45 Hz frequency, 2 nm magnitude oscillation, 0.05 s\textsuperscript{-1} strain rate, and 2000 nm maximum penetration depth. For each indent, $E_{nano,L}$ and $H_{nano,L}$ were determined over an approximately constant indentation depth range of 800-1600 nm.

For each specimen, four clusters of five indents were performed in different regions of lamellar bone. Bone microstructure was also described at each indent site using an optical microscope. Indent sites were classified into two microstructural groups: osteonal bone or interstitial lamellar bone. Typical indentations are shown in Figure II-2.

\textbf{Figure II-2. Locations of typical indentation sites.} (Left, A) Cluster of five indents in osteonal bone region. (Left, B) Cluster of five indents in interstitial lamellar bone region. (Right) High magnification image of boxed region, showing sharp residual indent area. The cross-section shown was obtained from the femoral diaphysis of a 16-year-old male with mild OI type I.
Microstructural classification was verified by two blinded observers, and data points for which these observers did not agree were excluded from the dataset. Indents that were either indistinguishable as osteonal/interstitial bone or located in non-lamellar bone regions were also excluded. A total of 168 indents were included in the study, where the number of indents was distributed approximately evenly between specimens from patients with mild OI type I \( (n_{OI-I} = 85) \) and severe OI type III \( (n_{OI-III} = 83) \). Moreover, half of the indents were located in interstitial lamellar bone regions, while the other half were in osteonal regions \( (n_{int} = n_{ost} = 84) \).

C-4. Statistical Analysis

Linear mixed models were used to statistically test the effects of disease severity (OI types I vs. III) and lamellar microstructure (osteonal vs. interstitial) on elastic modulus and hardness. All statistical analyses were performed using an open-source software package (The R Project for Statistical Computing; www.r-project.org). Additional potential covariates were explored including patient age, gender, anatomic site (femur vs. tibia), and patient history of bisphosphonate treatment (yes vs. no). Significant covariates were included in the final statistical models, where significance was defined by a \( P \)-value less than 0.05 (i.e., \( P < 0.05 \)).

D. Results

D-1. Results for Statistical Model of Elastic Modulus (\( E_{nano,L} \))

Elastic modulus and hardness results for each OI type and microstructural designation are shown graphically in Figure II-3. The results from the final linear mixed
model for elastic modulus are shown in Table II-2. Based on the model results, OI disease severity had a statistically significant effect on the longitudinal elastic modulus ($P = 0.024$), where bone specimens from individuals with severe OI type III had a 7% lower average elastic modulus than did those with mild OI type I (i.e., $16.3 \pm 0.6$ GPa vs. $17.5 \pm 0.5$ GPa, respectively). Bone microstructure also had a statistically significant effect on elastic modulus ($P < 0.001$), with osteonal bone having a 13% lower average elastic modulus than interstitial lamellar bone. Of the possible covariates investigated, only anatomic site had a significant effect, where specimens from tibiae showed a 8% higher elastic modulus than those from femora ($P = 0.014$). Donor age, gender, and history of bisphosphonates treatment did not have a significant effect on the elastic modulus and were therefore not included in the final statistical model.

![Figure II-3. Summary of mechanical properties from nanoindentation testing.](image)

(Left) Elastic modulus ($E_{\text{nano,L}}$). (Right) Hardness ($H_{\text{nano,L}}$). Results for mild OI type I are delineated with diamonds: interstitial (♦) and osteonal (◊) bone regions. Results for severe OI type III are shown as circles: interstitial (●) and osteonal (○) regions. All results are plotted as mean ± standard deviation. *Denotes significant difference between OI types I and III ($P < 0.05$), based on linear mixed effects models.
Table II-2. Results of the linear mixed model for elastic modulus ($E_{\text{nano,L}}$).

<table>
<thead>
<tr>
<th></th>
<th>Coefficient (GPa)</th>
<th>SE (GPa)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (OI type I, interstitial bone, femur)</td>
<td>17.53</td>
<td>0.47</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Severity = OI type III</td>
<td>-1.23</td>
<td>0.55</td>
<td>0.024</td>
</tr>
<tr>
<td>Microstructure = Osteonal</td>
<td>-2.21</td>
<td>0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Anatomical site = Tibia</td>
<td>1.40</td>
<td>0.57</td>
<td>0.014</td>
</tr>
</tbody>
</table>

D-2. Results for Statistical Model of Hardness ($H_{\text{nano,L}}$)

The results from the final linear mixed model for hardness are presented in Table II-3. Disease severity had a statistically significant effect on tissue-level hardness ($P = 0.003$), where severe OI type III bone showed an 8% decrease in average hardness compared to mild OI type I (i.e., $0.60 \pm 0.02$ GPa vs. $0.66 \pm 0.03$ GPa, respectively). Microstructural location also had a statistically significant effect on hardness ($P < 0.001$), with osteonal bone showing an 11% decrease in average hardness vs. interstitial bone. The donor gender and history of bisphosphonates covariates were found to have a statistically significant effect on hardness. On average, male bone specimens were harder than their female peers by 6%. Individuals with a history of bisphosphonates therapy prior to tissue donation also showed a 6% reduction in average hardness ($P < 0.04$).

Table II-3. Results of the linear mixed model for hardness ($H_{\text{nano,L}}$).

<table>
<thead>
<tr>
<th></th>
<th>Coefficient (GPa)</th>
<th>SE (GPa)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (OI type I, interstitial bone, female)</td>
<td>0.656</td>
<td>0.026</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Severity = OI type III</td>
<td>-0.054</td>
<td>0.018</td>
<td>0.003</td>
</tr>
<tr>
<td>Microstructure = Osteonal</td>
<td>-0.074</td>
<td>0.013</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender = Male</td>
<td>0.041</td>
<td>0.015</td>
<td>0.006</td>
</tr>
<tr>
<td>Bisphosphonates = Yes</td>
<td>-0.044</td>
<td>0.021</td>
<td>0.039</td>
</tr>
</tbody>
</table>

E. Discussion

There is very little data describing the material properties of OI cortical bone, especially for the most common form, mild OI type I. The primary goal of this study was
to determine whether elastic modulus and hardness differ in the longitudinal direction between pediatric patients with mild OI type I and severe OI type III. A secondary research question was to assess the role (if any) of bone microstructure (i.e., osteonal vs. interstitial lamellar regions) in determining bone material properties in OI. These questions were investigated using nanoindentation, a well-established technique for small, irregularly shaped samples.

Children with OI often suffer from bowing of upper and lower extremity long bones requiring removal of wedge-shaped specimens via routine osteotomy surgeries. These specimens are often sent for histopathology or discarded post-operatively. The current study shows how these small samples can provide a valuable resource for studying mechanical properties in OI cortical bone. Because of its small scale, nanoindentation is well-suited for repeated, localized measurements. Nevertheless, the technique does have several inherent limitations, including the assumption of local isotropy when calculating elastic modulus. A previous nanoindentation study of OI cortical bone did not find significant differences in \( E_{nano,L} \) between specimens tested in both the longitudinal and circumferential/radial direction, suggesting that it may be more isotropic than healthy bone\(^5^4\).

As shown in Equation II-4, modulus calculations also require knowledge (or estimation) of the material’s Poisson’s ratio. Similar to previous bone studies\(^5^4,5^5,1^93–1^95\), a \( \nu_{bone,L} \) value of 0.3 was assumed. Varying this property between 0.2 to 0.4 has been reported to affect modulus results by less than 10%\(^1^8^5,1^96,1^97\). Bone tissue is viscoelastic by nature, and as such factors related to experimental setup including specimen
dehydration\textsuperscript{185,187,198,199}, loading rate\textsuperscript{185,200}, specimen embedding media\textsuperscript{199,200}, time of storage before testing\textsuperscript{200}, and indenter tip geometry\textsuperscript{195,199,201} have been shown to affect modulus calculations from nanoindentation.

To minimize any undue experimental variability, these variables were kept constant for all specimens. Specifically, all specimens were embedded in the same low viscosity epoxy resin and tested on the same day (within three months of embedding) in a fixed, dehydrated state using a Berkovich indenter tip and the CSM method (45 Hz frequency, 2 nm magnitude oscillation, 0.05 s\textsuperscript{-1} strain rate, and 2000 nm maximum penetration depth). Because of the abovementioned limitations associated with nanoindentation testing in general, the relative effects of the current findings are emphasized rather than the absolute values of elastic modulus and hardness. As with most pediatric studies on bone material properties, the current sample size was limited, so any conclusions relied heavily on the chosen statistical model.

The elastic modulus and hardness results of the current study were within the range of values reported in previous nanoindentation studies of human OI cortical bone\textsuperscript{20,54–56}. Current results for osteonal bone regions were similar to another study in which the indentation sites were also located within osteons\textsuperscript{54}. Previously reported values for elastic modulus (19-22 GPa) and hardness (0.7-0.8 GPa) of interstitial bone regions in OI type III are somewhat higher than those from the current study\textsuperscript{20,55}. However, both of these other studies used the Oliver-Pharr method (rather than the CSM approach)\textsuperscript{202}, which requires multiple loading/unloading cycles for each indent and has been shown to underestimate the contact area ($A_c$)\textsuperscript{190,191}. Underestimation of $A_c$ would
lead to elevated modulus and hardness values (e.g., see Equations II-4 and II-5).
Moreover, differences between these studies and the current work could also be
explained by differences in anatomical site (i.e., iliac crest vs. long bones)\textsuperscript{197,203} and/or
differences in indentation sites within interstitial bone. Due to the dynamic behavior of
bone remodeling, the range of relative tissue ages in bone has been suggested as a source
of experimental variability in nanoindentation testing\textsuperscript{203}.

The main finding of this study was that OI severity had a small but statistically
significant effect on the elastic modulus and hardness of pediatric long bone specimens.
Specifically, both properties were higher in donors with OI type I than in those with type
III. The explanation for this observation is not fully understood. Previous studies have
suggested that increased elastic modulus and hardness in OI type III compared to controls
may be attributable to a more highly mineralized matrix\textsuperscript{20,183}. Bone mineralization
density distribution ($\textit{BMDD}$) is a related parameter that measures the spread of mineral
content in the matrix. $\textit{BMDD}$ has been reported to be higher in iliac crest biopsies from
individuals with OI type I than in healthy bone\textsuperscript{182,204}. Similar observations have been
recorded for other bone pathologies including osteomalacia, idiopathic osteoporosis, and
postmenopausal osteoporosis\textsuperscript{182}. Indeed, the relationship between local elastic modulus
and local mineralization (e.g., mineral-to-matrix ratio, bone mineral content, etc.) has
been observed in previous studies of animal\textsuperscript{205,206} and human\textsuperscript{207,208} bones. A similar
comprehensive study using quantitative backscattered electron microscopy (qBSEM)
found increased mineralization density in iliac crest biopsies from patients with OI types
I, III, IV, and V compared to controls\textsuperscript{184}. The same study also noted that the
mineralization density tended to be lower in OI type I than in the latter types.
Thus the current finding of higher elastic modulus and hardness in OI type I vs. III is surprising in that it does not appear to fit the assumption that a higher elastic modulus is associated with higher mineralization. A recent nanoindentation study on the mouse model of severe OI (oim) revealed that modulus was not correlated with local measurements of bone matrix mineralization\textsuperscript{15}. The effect of OI severity on elastic modulus and hardness may therefore be related to other factors such as the size, shape, and packing density of mineral crystals, collagen structure, and/or mechanical interaction between the collagen fibrils and the mineral crystals\textsuperscript{4,6,38,76--82,176,209,210}.

OI type I is typically associated with a null COL1A1 mutation that causes a reduced quantity but a normal quality of type I collagen\textsuperscript{36,64}. On the other hand, OI type III is generally characterized by amino acid substitution defects within type I collagen molecules that lead to reduced quality of collagen. These mutations can be expected to affect downstream micromechanical behavior of collagen fibrils. For example, computational studies have demonstrated that structural mutations within type I collagen molecules could negatively impact fibrillar mechanical properties by altering the stress distribution\textsuperscript{81,82}.

Abnormal type I collagen fibril diameters have also been reported in OI, although there is disagreement as to whether their diameters are higher\textsuperscript{76,77} or lower\textsuperscript{78,79} than normal. Likewise, these studies also report conflicting results regarding changes in fibrillar diameter between phenotypes. One group noted that fibrillar diameter tended to be larger in OI type I and smaller in OI type IV\textsuperscript{77}. Other studies reported that OI types I
and II, the mildest and most severe types respectively, displayed the smallest fibrillar
diameters\textsuperscript{78,79} while OI types III and IV were not statistically different from normals\textsuperscript{78}.

On the mineral side, compositional and structural abnormalities have been reported in OI bone. Calcium-to-phosphorus ratio (\textit{Ca/P}) has been found to be lower than normal in OI\textsuperscript{80}. Hydroxyapatite mineral crystals were also found to be smaller than normal in children with OI types III and IV\textsuperscript{3,4}. A recent study using synchrotron radiation X-ray scattering found that mineral crystals were of similar dimensions but were more tightly packed (i.e., higher density of mineral plates) in OI type I compared to controls\textsuperscript{6}. Similar observations have been made in bovine\textsuperscript{5} and murine\textsuperscript{7,15,211} models of OI. Additional work is warranted to investigate the role of abnormal mineralization kinetics on the mechanical properties of human OI bone.

The current study presents the first mechanical characterization of bone tissue in humans with OI type I. Although the study did not include a healthy control group, the results can be compared against those of other previously published studies to get an idea of how elastic modulus and hardness compare between OI phenotypes and normal bone. A few studies have investigated tensile and flexural mechanical properties of pediatric bone\textsuperscript{59–62}. Elastic modulus results from the current study fall within the range previously reported for pediatric lower extremity long bones (e.g., 10-20 GPa)\textsuperscript{60,62}. Rho et al\textsuperscript{212} conducted nanoindentation and flexural testing on pediatric specimens and found a high correlation ($R^2 > 0.9$) between both moduli. Nevertheless, direct comparison between the two approaches could be misleading, since nanoindentation does not take vascular porosity into account.
Although no difference has been found with nanoindentation between individuals with OI types III and IV\textsuperscript{55,56}, Weber et al\textsuperscript{20} noted a 13\% increase in the elastic modulus of iliac crest biopsies from patients with OI type III compared to age-matched controls. Based on the aforementioned data from fibrillar and mineralization studies, one might expect the elastic modulus and hardness of OI type I bone to fall between that of severe OI type III and normal tissue. Yet results from the current study indicate that this assumption may not be true (Figure II-4). It is possible that the increased matrix mineralization of OI cortical bone causes a general increase in elastic modulus and hardness vs. controls, while the reduced quality of collagen in OI type III reduces its elastic modulus and hardness compared to OI type I. Further work on the fibrillar and mineral properties of OI cortical bone are warranted to help explain this observation.

As mentioned previously, mouse models offer a common surrogate for human OI. Several previous bending studies on murine whole bone properties have reported a higher elastic modulus in \textit{oim/oim} (i.e., homozygous severe OI) and \textit{Btrl} (i.e., knock-in moderate
OI) mice compared to wild-type littermates, however another oim study reported no significant difference. Grabner et al performed microindentation (which measures hardness only) and found that oim/oim bone had a higher hardness than oim/+ (i.e., heterozygous mild OI), and that both genotypes were harder than controls. Recent nanoindentation studies are conflicting. Whereas Vanleene et al noted a decreased elastic modulus in oim bone, Sinder et al observed an increase in Brtl bone modulus compared to healthy littermates. In the Mov13 mouse, which has been used as a model for mild OI type I, elastic modulus was reported to be higher in the anterior femoral region but lower in the posterior region compared to healthy littermates.

The second main finding of the current study was that bone microstructure had a statistically significant effect on elastic modulus and hardness in mild and severe OI cortical bone. Both properties were 11-13% higher in interstitial lamellar regions than in osteonal regions. Similar results have been observed in other nanoindentation studies of bovine and adult human bone tissue. Microstructural differences in elastic modulus and hardness are likely attributable to local differences in the degree of mineralization and tissue age between these regions. Osteonal bone, which has been more recently formed during remodeling, tends to be less mineralized than interstitial bone, and relationships have been reported between elastic modulus and the local degree of mineralization. These observations suggest that any abnormalities in the mineralization kinetics of OI cortical bone affect both osteonal and interstitial bone similarly.
Elastic modulus and hardness measure resistance to elastic (i.e., recoverable) and localized plastic (i.e., irrecoverable) deformation, respectively. Although they are distinct properties, a relationship exists between them in bone. In the present study, there was a statistically significant positive correlation between these properties ($R^2 = 0.8$, $P < 0.001$). This relationship is consistent with previous studies, which attribute it to a dependence of both properties on the local bone mineral content\textsuperscript{185,216,217}.

Bisphosphonates have emerged as the standard treatment for children with OI, with the aim of decreasing bone fragility through increased bone mass\textsuperscript{36}. As a result, 7 of the 10 individuals who donated a specimen for the current study had a documented history of bisphosphonate treatment. This factor was not controlled in the current experimental design. A previous study on human OI bone found no significant changes in histomorphometric, mineralization, or nanoindentation properties after two to three years of pamidronate treatment\textsuperscript{20}. In the current study, history of bisphosphonates treatment had no significant effect on elastic modulus but was associated with a 6% decrease in hardness (Table II-3). To date, mechanical properties such as yield strength, ultimate strength, and fracture toughness have not been characterized in human OI cortical bone. Since they inhibit osteoclasts (which are responsible for resorbing/repairing microdamage), bisphosphonates may have adverse effects on tissue quality, strength, and toughness. While decreased fracture risk associated with the increase in bone mass has been reported in individuals with OI\textsuperscript{20} and osteoporosis\textsuperscript{90,91}, the long term effects of bone augmenting drugs on pediatric bone material properties is not well understood\textsuperscript{36,101,102}. 
Finally, the other significant covariates affecting either modulus or hardness in the current study were anatomical site and gender. In the current OI sample population, a small increase in nanoindentation modulus was observed at the tibia compared to the femur. A similar observation has been reported by Burstein et al\textsuperscript{173} in large-scale mechanical tests performed on human bone samples. Specimens from male OI donors also had a slightly higher hardness than those from females. The meaning behind this observation is unclear. However, a lack of any difference in elastic modulus results between genders is in agreement with a previous study\textsuperscript{218}.

The structural behavior of whole bones is dependent not only on material properties but also on geometry, mineralization, and microstructure. Histomorphometric studies have shown that children with severe OI tend to have less bone tissue than those with mild OI, as evidenced by thinner cortices and reduced trabecular bone volume fraction\textsuperscript{21}. Coupled with the decrease in elastic modulus found in the current study, these findings suggest that the structural stiffness of whole bones in severe OI type III could be appreciably lower than in mild OI type I.

F. Conclusions

Results from the present study of OI cortical bone indicate that the elastic modulus and hardness are higher in interstitial versus osteonal lamellar tissue, and these properties can help to differentiate between mild and severe forms of the disorder. Namely, elastic modulus and hardness are increased in young individuals with OI type I compared to OI type III. For both groups, bone specimens from the tibia displayed a
higher modulus than those from the femur. Other factors such as gender and history of bisphosphonates treatment had a slight effect on hardness but not on modulus.
III. SYNCHROTRON RADIATION X-RAY MICROTOMOGRAPHY (SRµCT) AT THE ADVANCE LIGHT SOURCE

A. Abstract

This chapter describes the synchrotron radiation X-ray microtomography (SRµCT) beamline at the Advanced Light Source (ALS) in Berkeley, CA. Various elements of the ALS, beamline setup (e.g., X-ray microscope setup/lens/camera combinations), and imaging pipeline (e.g., image acquisition, data pre-processing, and reconstruction) are discussed in detail. Relevant features of the beamline’s image pre-processing and reconstruction software are presented, and recommendations are provided for bone imaging. As part of this work, an open-source algorithm that is built into the software has been used to evaluate three levels of phase contrast correction for bone datasets. The phase contrast parameters $\delta$ and $\beta$ are dimensionless quantities related to a material’s index of refraction. At a photon energy of 17 keV, setting $\beta = 5 \times 10^{-10}$ and $\delta = 2.5 \times 10^{-5}$ has been shown to provide improved absorption contrast, with reproducible segmentation of small bone cell compartments. High resolution imaging studies require accurate reporting of resolution beyond simply listing the nominal pixel size of the camera. A Fourier-based method has been used to evaluate the effect of common scan setup parameters on image noise and resolution. Notable improvements have been achieved by collecting a higher number of radiographs over a longer camera exposure time. These observations are relevant for new and experienced users, including the bone studies performed in Chapters IV and V. Note: Portions of this chapter appear in a recently published proceedings paper$^{219}$. 
B. Introduction

First developed for medical applications in the 1970s\textsuperscript{220,221}, X-ray computed tomography (CT) has been used to digitally visualize the 3D structure of a diverse range of materials. The underlying physics governing the technique is based on the differential absorption of X-rays by various compounds or mixtures within a sample. Despite advances in source, gantry, and detector technology, the resolution limit of clinical CT systems, such as multi-detector CT (MDCT) or high resolution peripheral quantitative CT (HR-pQCT), is still on the order of 50-500 µm\textsuperscript{24,127,222–225}. The feasibility of more compact microtomography (µCT) systems was first proposed in the 1980s, using scintillators to convert X-rays into visible light, which could then be imaged by a light microscope and a charge-coupled area detector (CCD)\textsuperscript{226}. These systems have a much smaller field of view (FOV) than clinical scanners, but they offer greatly improved resolution on the order of a few microns\textsuperscript{24}. Thus, µCT has evolved as a preferred method for non-destructive, high-resolution imaging of small animal tissues as well \textit{ex vivo} analysis of biopsy samples.

One of the main limitations of clinical and lab-based CT systems is that they rely on polychromatic (i.e., broad energy spectrum) X-ray sources. The relatively wide spectral bandwidth of the incident beam results in preferential absorption of low-energy or soft X-ray photons causing beam hardening artifacts, ultimately leading to geometric non-uniformities in the reconstructed image data and a non-linear relationship between attenuation and material density. Various beam hardening corrections can be applied during the acquisition or reconstruction process\textsuperscript{227–229}. Assuming a calibrated tissue phantom is scanned along with the sample, the resulting compositional measurements
have been shown to correlate well with those from monochromatic (i.e., uniform energy) synchrotron light sources\textsuperscript{230}.

Nevertheless, synchrotron radiation microtomography (SRµCT) offers many advantages over conventional µCT. The ability to select a monochromatic energy reduces beam hardening artifacts and allows for accurate compositional analysis\textsuperscript{129–131}. Synchrotron radiation is also characterized by a high flux over a wide range of available energies, which improves signal-to-noise ratio and spatial resolution. The parallel beam geometry also enables exact CT reconstruction. Several dedicated SRµCT beamlines have been developed at light sources around the world.

The purpose of this study is to provide an overview of the SRµCT beamline at the Advanced Light Source (ALS) in Berkeley, California. A secondary goal is to present a set of acquisition, pre-processing, and reconstruction parameters that could assist other users and establish reliable default values for future bone experiments.

C. Instrument Description

C-1. Synchrotron Radiation and the ALS

At the heart of the ALS is a linear accelerator, which emits and accelerates bunches of electrons to a velocity of 299,792,447 m/sec, or 99.999996\% the speed of light (Figure III-1). A small circular synchrotron, or booster ring, increases the energy of the electrons to 1.9 GeV, at which point they are injected into a 200 meter long storage ring. A series of powerful bending magnets continuously steers the electron beam in a circular path around the heavily shielded storage ring at a rate of nearly 1.5 million
revolutions/sec. After each bending magnet is a straight section containing additional bending magnets of alternating polarity called undulators and/or wigglers, which cause the electron beam to rapidly oscillate and change course.

![Figure III-1. Schematic of ALS and SRµCT beamline.](image)

The top panel shows an enlargement of the highlighted box, where the synchrotron radiation produced from a 6 T superbend magnet is used for hard X-ray microtomography.

Each time a bending magnet influences the electron beam, synchrotron radiation energy is produced in the form of photons (i.e., tiny packets of light). These photons travel down straight offshoots called beamlines to a monochromator, which is used to select the desired photon energy for a given modality and experiment. The energy of a photon \(E\) is related to its wavelength \(\lambda\) according to the Planck equation:

\[
E = \frac{hc}{\lambda}
\]

(Equation III-1)

where \(h\) is Planck's constant \((h \approx 6.626 \times 10^{-34} \text{ J} \cdot \text{s})\) and \(c\) is the speed of light \((c \approx 3 \times 10^8 \text{ m/s})\). The range of energies produced via synchrotron radiation can be modified by varying the magnetic field, which is usually achieved by changing the spacing between
magnet arrays. In this way, a broad spectrum of electromagnetic radiation is possible at
the ALS (from $\approx 0.00005$-45 keV), allowing for a variety of imaging modalities based on
infrared, visible, ultraviolet, or X-ray radiation.

At the end of each beamline is an experimental end-station or hutch, where an
image is created by focusing the light onto a given sample. In general, the ALS
specializes in lower energy or soft X-ray imaging. However, there are three
superconducting magnets called superbends spaced equally around the storage ring that
are capable of producing high energy or hard X-rays. As shown in Figure III-1, the
SRµCT beamline is located on one of these superbends.

C-2. SRµCT Beamline

The X-ray source for the microtomography beamline is the synchrotron radiation
generated from a 6 T superconducting magnet$^{231}$. A monochromator allows for adequate
flux up to a photon energy of approximately 45 keV for penetration of thick, high-Z
materials (i.e., high atomic number). The sample is illuminated in a lead-lined hutch
located 20 meters from the storage ring. A graphical user interface (GUI) is used for scan
setup and acquisition. Multiple camera and lens combinations are possible, based on the
desired resolution and $FOV$. Raw data is automatically copied to a local storage server,
where it is available for processing. Alternatively, a copy of all data is also automatically
streamed to a supercomputer that is available for data processing, retrieval, and archiving.
The following outlines the relevant data acquisition, image pre-processing,
reconstruction, and visualization pipeline for beamline data.
C-3. Scan Setup

The optimal photon energy depends on the sample thickness and composition. For a given homogenous sample with a diameter \( D \) and an average linear attenuation coefficient \( \mu(E) \), a material property, the theoretical beam energy that minimizes the number of X-rays required in the exposure can be estimated from:

\[
\mu(E) \times D \approx 2.2 \quad \text{(Equation III-2)}
\]

where \( \mu \) depends on the energy\(^{232} \). However, there is an inverse relationship between the photon energy and the flux, as well as a decrease in the overall beam height at high energies (Figure III-2). The flux can also be increased by increasing the exposure time of the camera, where exposures up to 1.5 seconds are possible. In practice, this means that there are tradeoffs between the beam energy, exposure time, beam height, and overall scan time. Most of the bone samples analyzed at the ALS have a thickness on the order of 1 mm, and as such a photon energy of 17 keV has been reported to be a good compromise\(^{172} \). Other bone studies have reported monochromatic X-ray energies ranging from 10-60 keV, depending on the sample geometry and application\(^{134,233} \).

![Figure III-2. Relationship between photon energy, flux, and beam height. At energies above 15-20 keV, both the: flux at the sample (left), and the beam height (right) decrease.](image-url)
The energy resolution of the monochromator is approximately 1%, and it can be calibrated using thin metallic foils having a known composition and thickness. Bright field images (used to normalize the background) have a characteristic non-uniform, horizontal striping pattern commonly seen in SRµCT\textsuperscript{234}. The stripes result from minute phase differences across the illuminated field, which can be attributed to monochromator imperfections. To ensure adequate image normalization, the striping pattern is smoothed by dithering (i.e., vibrating) the monochromator at a constant frequency and amplitude.

C-4. Sample Rotational Stage

Approximately 7 meters downstream from the monochromator is a lead-shielded hutch for sample mounting and image acquisition (Figure III-3). The sample can be attached using standard kinematic mounts, which connect to a circular magnetic plate assembly that is coupled to a rotary air bearing. The rotational stage connects to motorized vertical and horizontal stages via additional kinematic mounts. The magnetically coupled circular plate that sits atop the rotational stage can be pushed in the horizontal (XY) plane by two small motors, to ensure proper alignment between the stage and the sample’s rotational axis.

C-5. X-ray Microscope Setups

After passing through the sample, X-rays are converted into visible light via a single crystal scintillator, magnified using an objective lens, and imaged onto a camera using one of the two X-ray microscope setups shown below (Figure III-3). The standard X-ray microscope setup is housed within a light-tight box that sits atop adjustable rails in the hutch. This setup is more versatile, allowing the user to choose between two cameras.
having different total pixel areas and readout speeds (Table III-1). A turning mirror is employed between the scintillator wheel and the objective lens/camera. The purpose of this mirror is to remove the lens and camera from the plane of the X-rays, which tend to darken the lens over time (and would damage the camera). However, the use of a turning mirror between the scintillator and lens necessitates long working distance objective lenses with lower numerical aperture (NA, a measure of a lens’s ability to gather and resolve detail).

A second, more compact X-ray microscope setup is also available for high resolution experiments (Optique Peter, Lentilly, France). This setup requires the user to slide the standard X-ray microscope out of the way along the camera box rails, after which the separate Optique Peter assembly can be bolted into place. Note that both setups share the same sample rotational stage. The main advantage of the high resolution
setup is that it has an internal turning mirror that is downstream of the lens (Figure III-3), allowing for closer scintillator-to-objective distances, which enables the use of high NA lenses. However, because the scintillators for the high resolution setup are thinner and generally have a low conversion efficiency (and thus require longer exposure times), only the fast camera (see Table III-1 for specifications) is used with this setup.

For both setups, the scintillators are mounted on motorized wheels that allow the user to select from numerous scintillator plates of varying thicknesses and materials. In general, high resolution imaging requires the thinnest scintillators. Thick scintillator plates absorb photons more efficiently, but they increase blurring in the resulting camera image\textsuperscript{235}. Conversely, thin scintillators offer less blurring and higher resolution, but they are limited by lower photon absorption, requiring longer exposure times. For bone imaging applications, a 50 µm thick lutetium aluminum garnet (Lu\textsubscript{3}Al\textsubscript{5}O\textsubscript{12}, or “LuAG”) scintillator was selected for its moderate conversion efficiency and high resolution imaging capability\textsuperscript{236}.

For both X-ray microscope setups, the objective lenses are mounted on motorized rotational turrets (Figure III-3), where higher magnification corresponds with a smaller pixel size and horizontal FOV (Table III-1). As mentioned above, the use of a turning mirror reduces lens darkening for the standard setup. Nevertheless, periodic lens clearing is required via overnight exposure with an ultraviolet (UV) light-emitting diode (LED). For the high resolution setup, the objective lens is in the plane of X-rays, and thus requires frequent clearing (approximately once per day).
Table III-1. Pixel sizes and horizontal \textit{FOV} for various lens/camera combinations.

<table>
<thead>
<tr>
<th>Lens magnification(^a)</th>
<th>pco.4000 (&quot;slow&quot;) camera: 14-bit, 4008 × 2672 pixel area, 0.7 Hz readout speed</th>
<th>pco.edge (&quot;fast&quot;) camera(^b): 14-bit, 2560 × 2160 pixel area, 35 Hz readout speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>20×</td>
<td>Pixel size (µm)</td>
<td>FOV (mm)</td>
</tr>
<tr>
<td>10×</td>
<td>0.9</td>
<td>3.6</td>
</tr>
<tr>
<td>5×</td>
<td>1.8</td>
<td>7.2</td>
</tr>
<tr>
<td>4×</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2×</td>
<td>4.5</td>
<td>18</td>
</tr>
<tr>
<td>1×</td>
<td>9</td>
<td>36</td>
</tr>
</tbody>
</table>

\(^a\)For the standard setup, both cameras can be used, and 1×, 2×, 5×, or 10× lenses are available. For the high resolution setup, only the fast camera can be used, and 2×, 4×, 10×, or 20× lenses are available.

\(^b\)Numbers in square brackets indicate pixel sizes and \textit{FOV} for the high resolution Optique Peter setup.

As shown in Table III-1, the slow camera (pco.4000, PCO-TECH Inc., Romulus, MI) has a larger area (4008 × 2672 pixels) in comparison to the fast camera (pco.edge sCMOS camera, PCO-TECH Inc., Romulus, MI; 2560 × 2160 pixels). The pco.4000 is therefore useful for bigger/thicker samples, where a larger horizontal \textit{FOV} is advantageous. On the other hand, the readout time for the pco.edge is approximately 35 Hz, or 50 times faster than the pco.4000. In practice, the sample thickness/diameter determines the minimum required \textit{FOV}, which in turn defines the camera, magnification, and imaged pixel size. One caveat to this general rule is that high resolution imaging of larger samples that do not fit in the \textit{FOV} is possible via either vertical tiling (i.e., collecting multiple scans in the vertical direction and connecting them during reconstruction) or local tomography (i.e., reconstructing only the region of the sample that remains continuously within the \textit{FOV} throughout the scan). The latter is avoided whenever possible as it complicates image normalization.
D. Imaging Pipeline

D-1. Data Acquisition

After selecting the appropriate X-ray microscope setup, imaging is conducted via a GUI. The sample is rotated over a user-specified range (generally 0-180°), while a series of 513-2049 radiographs are recorded. The rapid readout time of the fast camera allows for continuous sample rotation, rather than the traditional “step-and-shoot” method, which is required when using the slow camera. Users select the exposure time, number of images/angles to collect, and the “blur limit” (which corresponds to the maximum acceptable rotation of the sample, in pixels, during the exposure time). The program then automatically determines a rotational velocity.

Images are collected to a memory buffer on the acquisition computer, and then streamed by a data transfer node to a 10 Gb switch, which temporarily stores the data on a 40 TB storage server. A set of four beamline workstations are also connected to the switch, so that data can be quickly and easily transferred locally. Many users collect upwards of 1 TB of data in two days of beamtime. To alleviate the massive storage bottleneck, a copy of all data is automatically converted into hierarchical data format revision 5 (HDF5, a more efficient file format for high-performance computing) and streamed to a supercomputer at the National Energy Research Scientific Computing Center (NERSC, a division of LBNL) over 10 Gb Ethernet. A NERSC web portal allows one to download raw data onto his or her own workstation for analysis and visualization.
D-2. Image Processing – NormalizeStack832newnaming Fiji Plugin

After collecting the radiographs for a scan, the user can transfer data from the storage server (or NERSC) to a workstation for pre-processing, reconstruction, and visualization. For convenience, the image pre-processing and reconstruction steps have been built into a semi-automated plugin in Fiji (a distribution of ImageJ v1.49f, National Institutes of Health, Bethesda, MD)\(^{237}\). As shown in Figure III-4, plugins can incorporate many functions in the same GUI. The beamline plugin (NormalizeStack832newnaming) combines functions native in Fiji, along with those programmed by beamline staff or provided by the reconstruction software (Octopus v8.6, inCT, Ghent, Belgium).

**Figure III-4. SRµCT plugin in Fiji.** The plugin is accessed via the Plugins toolbar in the main Fiji window (top). Once selected, it opens a log window (middle) and a plugin window (bottom). The typical flow of pre-processing and reconstruction steps has been numbered for convenience.
After selecting the NormalizeStack plugin from the main Fiji window, a log window and plugin window appear. Non-uniformities in the X-ray beam, scintillator, and camera cause defects in the radiographs that can be homogenized via a flat field correction. This preliminary step is computed for a given radiograph as the ratio of the differences between the raw uncorrected, average bright field (i.e., unattenuated X-rays only, with no sample), and average dark field (i.e., ambient light only, with no X-rays) images (Figure III-5). Flat field correction does not require any user input, so it is automatically performed by the plugin, and it is not considered as one of the steps in Figure III-4.

The first two steps in the plugin require the user to select an input directory (i.e., the folder where all radiographs have been stored) and an output directory (i.e., the destination folder for all reconstructed images). The next step is to calculate the center of rotation \( (COR) \), which is automatically determined by clicking the “Detect center of rotation” button in the plugin window. The \( COR \) is calculated based on the first and last radiograph collected at 0° and 180°, respectively. The \( COR \) can be manually adjusted in minimum increments of 0.1 pixels. When adjusting parameters manually, the “Preview reconstruction” button is useful for comparison.

Figure III-5. Flat field correction and normalization. (a) Raw, uncorrected radiograph of a bone sample. The boxed area indicates the region selected by the user for image normalization. (b) Average bright field image, containing only unattenuated X-rays. (c) Average dark field image, containing only ambient light detected by the camera when no X-rays are present.
The fourth step involves normalizing the radiographs by a background region, which the user defines by selecting an area where the sample never enters in any radiograph (Figure III-5 (a)). This step reduces noise and takes advantage of the full dynamic range of pixel values. In the fifth step, a noise filter can optionally be applied in frequency (i.e., Fourier) space by an Octopus function during reconstruction. The noise filter is input as a percentage, where the default value of 0% corresponds to a ramp filter, and larger values correspond to additional attenuation of high frequencies (which usually represent noise). According to the Octopus manual, this filter is considered a “quality parameter”, and it is usually determined visually by previewing multiple slices reconstructed with different noise filter values. Specific details of the filter are not disclosed by Octopus. However, for most materials (including bone), an empirical value of 50-80% is recommended.

In the sixth step, the user defines the output type (i.e., 32-bit real, 16-bit signed, or 8-bit), sets the scaling (for non-32-bit output types), and crops the reconstruction. By default, Octopus reconstructs images in the 32-bit real output type. To conserve disk space, it is common to convert 32-bit images into the 16-bit signed output type by applying a linear scaling equation. After the user inputs the minimum and maximum 32-bit values desired in the reconstructed image, the coefficient and intercept for the scaling equation are automatically determined and stored in the header file for each image by the plugin. Any pixel whose gray value is outside this range is set to the minimum or maximum, respectively, and the remaining pixel values are scaled accordingly.
In the seventh step, the user can optionally filter out any ring artifacts in the reconstruction, which are caused by the accumulation of dust/impurities on the scintillator, individual pixel transfer characteristics, and hardware scaling/offsets (e.g. miscalibrated or defective detector elements). These artifacts appear as concentric rings superimposed onto the reconstructed images. Although many strategies exist, for high resolution imaging using flat detectors, recent work suggests directly filtering the reconstructed images. The beamline plugin uses an algorithm built into Octopus, which transforms each reconstructed image into polar coordinates and applies a filter based on five user-selected parameters. These include the lower value, upper value, ring threshold, maximum ring size, and minimum arc length.

The lower and upper values correspond to the minimum and maximum pixel gray values to be included in the filtering process. The ring threshold is the maximum gray value for detecting rings. The maximum ring size is the thickness (in pixels) of the largest ring to be filtered. Because rings are not always completely closed, the minimum arc length defines the minimum angle (in degrees) for an arc to be considered a ring. In practice, the filter is most sensitive to the maximum ring size and the minimum arc length. For bone imaging, the following values have been found empirically: lower value = -100, upper value = 100, ring threshold = 100, maximum ring size = 30 pixels, and minimum arc length = 30°. The first three parameters are set to extremes to include all pixel values, while the remaining two parameters can be tuned based on the worst rings.

The eighth step in the beamline plugin allows the user to optionally apply a phase contrast filter to the original radiographs prior to reconstruction. X-ray inline phase
contrast is caused by the presence of a gap between the sample and the image detector, and it is a common phenomenon in synchrotron imaging (Figure III-6). As X-rays pass through the sample, tiny diffractions occur along sample edges and internal structures. This interference causes distortion of the X-rays and enhanced contrast of edges\textsuperscript{241,242}. When imaging materials such as bone, which contain thousands of interfaces in a small volume, phase contrast can produce bright halos around sharp changes in composition that can negatively affect segmentation and bone mineral density calculations.

\textbf{Figure III-6. Absorption contrast vs. phase contrast tomography.} The intensity profile at the detector is a combination of the: (Top) Absorption contrast, where lower X-ray intensity is indicated by thinner lines exiting the sample; and (Bottom) Phase contrast, where X-rays are distorted by sample edges and internal structures according to the distance between the sample and the detector (or scintillator in synchrotron imaging), $z$. 
To remove these artifacts, a reconstructed phase map $\varphi(x,y)$ can be determined as:

$$
\varphi(x,y) = \frac{\delta}{2\beta} \ln \left( \mathcal{F}^{-1} \left( \frac{\mathcal{F}[I(x,y)/I_0(x,y)]}{1 + \left[ \frac{\lambda z \delta}{4\pi\beta} (u^2 + v^2) \right]} \right) \right)
$$

(Equation III-3)

where $x$ and $y$ are the Cartesian coordinates in the image (with Fourier conjugates $u$ and $v$, respectively), $\lambda$ is the wavelength of the X-rays at the selected beam energy, $z$ is the distance from the object to the scintillator, $\mathcal{F}$ and $\mathcal{F}^{-1}$ are the forward and backward Fourier transforms respectively, $I(x,y)$ is the intensity distribution in the phase contrast radiograph, $I_0(x,y)$ is the incident intensity just upstream of the object, and $\delta$ and $\beta$ are the real-part decrement and imaginary part of the X-ray index of refraction ($n$) of the material, respectively\textsuperscript{243,244}. The parameters $\delta$ and $\beta$ are related to $n$ according to:

$$
n = 1 - \delta + i\beta
$$

(Equation III-4)

$$
\mu = \frac{4\pi\beta}{\lambda}
$$

(Equation III-5)

where $i$ is the imaginary unit number and $\mu$ is the linear attenuation coefficient of the material being imaged. The parameters $\delta$ and $\beta$ are dimensionless quantities that can be estimated from reference tables based on the X-ray photon energy and the chemical composition of the material being imaged\textsuperscript{245}.

It is clear from Equation III-3 that $\varphi(x,y)$ depends only on the ratio $\delta/\beta$ and not on the absolute values. For the beamline plugin, the variables required for phase retrieval include the X-ray photon energy (in keV), the distance between the sample and the scintillator ($z$, in mm), the imaged pixel size (determined from the X-ray microscope
setup/lens/camera combination selected), and the $\delta$ and $\beta$ parameters. Both the photon energy and imaged pixel size are automatically populated in the plugin. The sample-to-scintillator can be measured using a digital micrometer. The values of $\delta$ and $\beta$ can be estimated using an online calculator from the Center for X-ray Optics (CXRO, Lawrence Berkeley National Lab, Berkeley, CA, USA), which is based on work from Henke et al.\(^{245}\). The values can then be manually tuned by previewing multiple reconstructions based on different combinations of $\delta$ and $\beta$.

For bone, three levels of phase contrast correction have been evaluated: 1) mild correction, $\beta = 5 \times 10^{-10}$ and $\delta = 1 \times 10^{-5}$; 2) moderate correction, $\beta = 5 \times 10^{-10}$ and $\delta = 2.5 \times 10^{-5}$; and 3) aggressive correction, $\beta = 5 \times 10^{-10}$ and $\delta = 5 \times 10^{-5}$. As shown in Figure III-7, there is a strong attenuation of phase contrast effects at all levels of filtering. In the uncorrected reconstructions, edge-enhancement of the sample boundaries, Haversian canals, and osteocyte lacunae leads to errors during segmentation (see top left and bottom left of Figure III-7, respectively). Mild correction (i.e., $\beta = 5 \times 10^{-10}$ and $\delta = 1 \times 10^{-5}$) greatly reduces small edges associated with osteocyte lacunae, but sample boundaries and Haversian canal borders remain largely unattenuated. On the other hand, aggressive correction (i.e., $\beta = 5 \times 10^{-10}$ and $\delta = 5 \times 10^{-5}$) removes all phase contrast effects and improves the absorption contrast between osteons with different mineralization distributions. Unfortunately, it also attenuates many osteocyte lacunar edges enough to prevent their detection during segmentation. The best compromise is achieved with a moderate level of correction (i.e., $\beta = 5 \times 10^{-10}$ and $\delta = 2.5 \times 10^{-5}$), where phase effects are diminished, while still preserving the ability to detect small lacunar particles.
Figure III-7. Manual tuning of phase contrast filter parameters. (Top row) From left to right, the original reconstructed bone slice is shown, along with three different combinations of phase contrast filter parameters. Phase contrast effects become progressively finer with increasing correction, while local changes in bone mineralization become clearer. (Bottom row) From left to right, segmentations of each reconstructed image from the top row show the tradeoff between phase contrast attenuation and detection of osteocyte lacunae (small black particles).

The ninth and final step in the beamline plugin is reconstruction of all slices in the image stack. The general idea behind tomographic reconstruction is that each radiograph represents the sum of a series of line integrals characterizing X-ray attenuation through the sample. The sample can be thought of as a 3D distribution of linear attenuation coefficients ($\mu$), where each line integral represents the attenuation of an X-ray beam as it travels in a straight line through the sample. The Fourier transform of a 1D parallel projection is equal to a radial line of the 2D Fourier transform of the sample, where the
line is oriented perpendicular to the projection direction. Thus, given the Fourier
transform of a projection at enough angles, one could accurately estimate the 2D Fourier
transform, interpolate on a Cartesian grid, and perform a 2D inverse Fourier transform to
get an estimate of the original object. Mathematically this process is known as the
Fourier Slice Theorem\textsuperscript{246}.

In practice, the Fourier Slice Theorem can be implemented computationally in a
variety of ways, the most common of which is a process called filtered backprojection
(FBP). FBP is actually a general class of reconstruction algorithms involving two main
steps: 1) filtering each projection (in either the frequency or spatial domain) and 2)
backprojecting or “smearing” the filtered projection across the image plane. The
beamline plugin relies on a proprietary FBP reconstruction algorithm provided by
Octopus. A series of Radon transforms\textsuperscript{247} (i.e., line integrals) is used to generate a stack
of sinograms, where each sinogram represents all of the attenuation data for a given
horizontal line in the radiographs, combined over all angles. These sinograms are then
used as inputs in the FBP algorithm, which backprojects the results onto a 3D image
stack representing virtual slices through the sample. The resulting data can be visualized,
manipulated, and quantitatively analyzed using either Fiji or Avizo (Avizo 8.1,
Visualization Sciences Group, Burlington, MA, USA).

D-3. Spatial Resolution and Noise Considerations

Reporting the spatial resolution of imaging datasets is a requirement in literature,
as it ensures adequate resolution for the microstructural features being analyzed, and
provides a starting point for other researchers who wish to perform related experiments.
However, image resolution is often difficult to characterize, so most studies list the nominal pixel size of the camera. For tomographic datasets, there is an accumulation of errors such that the reconstructed 2D spatial resolution is often worse than the radiographic 2D resolution. The electron tomography imaging community has attempted to address resolution and noise issues in reconstructed images using various measures such as the signal-to-noise ratio\textsuperscript{248–250} and the Fourier ring correlation (FRC)\textsuperscript{251}. The FRC metric can be used to determine how much the information in a specific radiograph contributes to the overall reconstruction. This approach, called the noise-compensated leave-one-out (NLOO) method, generally involves comparing an original radiograph image to its reprojection, based on a tomogram determined from all other radiographs except the one in question\textsuperscript{252}. By repeating this process for several radiographs, one can estimate the average 2D resolution of a scan (NLOO\textsubscript{2D}).

For a given radiograph \(i\) having Fourier transform components \(m\) and \(n\), the FRC between the 2D discreet Fourier transforms of the original, flat-field corrected image \((\mathcal{F}[C_{m,n}^{(i)}])\) and the corresponding reprojected image \((\mathcal{F}[R_{m,n}^{(i)}])\) is given by:

\[
FRC_{CR}^{(i)}(\omega) = \frac{\sum_{m,n \in A(\omega)} \text{Re}\left\{ \mathcal{F}[C_{m,n}^{(i)}] \mathcal{F}[R_{m,n}^{(i)}]^* \right\}}{\left\{ \left( \sum_{m,n \in A(\omega)} |\mathcal{F}[C_{m,n}^{(i)}]|^2 \right) \left( \sum_{m,n \in A(\omega)} |\mathcal{F}[R_{m,n}^{(i)}]|^2 \right) \right\}^{1/2}} \quad (\text{Equation III-6})
\]

where \(\omega\) is the radial frequency, \(A(\omega)\) is an annular zone (ring) in Fourier space, and the asterisk denotes the complex conjugate\textsuperscript{252}. Note that in Equation III-6 above, \(R^{(i)}\) is the reprojection calculated from a tomogram generated from all input radiographs. Determining the FRC requires the calculation to be repeated and summed over a discreet set of spatial frequencies (i.e., rings in frequency space) defined by the user. If one were
to perform a reconstruction based on all radiographs except $i$, the resulting reprojection $(R^{(i)})$ could be assumed to display different noise statistics than both $C^{(i)}$ and $R^{(i)}$. The ratio between the FRCs of these images provides a measure of a given radiograph’s contribution to the overall resolution. Mathematically, the $NLOO_{2D}$ measure becomes:

$$NLOO_{2D}^{(i)}(\omega) = \frac{FRC_{CR}^{(i)}(\omega)}{FRC_{CR}^{(i)}(\omega)}$$

(Equation III-7)

where the numerator and denominator correspond to the FRCs calculated from the leave-one-out and full reprojections, respectively. Here, the denominator can be viewed as a noise compensation and normalization step. Thus, $NLOO_{2D}$ is bounded between 0-1, and by convention the resolution is estimated at the spatial distance where $NLOO_{2D} = 0.5$.

The resolution depends on many user input parameters including the number of angles (i.e., radiographs) collected during a scan, the number of bright field counts (related to the exposure time of the camera), the total sample rotation, and the degree of monochromator dithering. To assist beamline users in determining the optimal scan settings for their application, the NLOO method has been applied to a test series of bone scans at the ALS. For the image acquisition variables mentioned above, the following values have been evaluated independently: 1) number of radiographs = 513, 1025, or 2049; 2) number of bright field counts $\approx 8 \times 10^3$, $1.6 \times 10^4$, or $3.2 \times 10^4$; 3) total sample rotation = 180° or 360°; and 4) degree of monochromator dithering = 0, 1, or 10 Hz.

All parameters have been evaluated using the most common combination of beamline equipment: standard X-ray microscope setup, fast camera (i.e, pco.edge), 5× objective lens (NA=0.14; Mitutoyo Corporation, Kawasaki, Japan), and 50 µm thick
LuAG scintillator. To date, the NLOO method has not been built into the beamline plugin. Rather, this calculation is performed in Matlab (R2012a, Mathworks, Natick, MA, USA) using a combination of custom functions in combination with the Image Reconstruction Toolbox, a set of open source reconstruction algorithms. Two main outcomes have been reported: noise and $NLOO_{2D}$ resolution. Noise has been estimated by averaging the standard deviation of various homogenous regions in the foreground of the reconstructions. NLOO calculations have been carried out for eight radiographs spaced evenly throughout each scan, and then averaged to obtain a representative resolution curve.

As shown in Figure III-8 (a-b), the number of radiographs has the strongest influence on noise and resolution. For a four-fold increase in the number of radiographs collected, there is a three-fold improvement in resolution (i.e., from $6.7 \pm 1.0$ to $2.1 \pm 0.1 \mu m$). Table III-2 summarizes the noise and NLOO resolution results for the other scan settings. In general, noise is reduced by collecting more radiographs and increasing the bright field counts (i.e., longer cameral exposure time), while NLOO resolution is strongly dependent on projection number. Sample rotation and monochromator dithering are associated with minimal changes in noise and resolution.
Table III-2. Noise and resolution results for various acquisition settings.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Noise (St. dev of foreground)</th>
<th>NLOO(_{2D}) resolution (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiographs (angles):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>513</td>
<td>1203 ± 70</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>1025</td>
<td>1059 ± 91</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>2049</td>
<td>874 ± 63</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Bright field counts:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7345 ± 971</td>
<td>980 ± 64</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>16413 ± 2106</td>
<td>797 ± 38</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>32798 ± 4376</td>
<td>694 ± 47</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>Sample rotation (°):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>1192 ± 84</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>360</td>
<td>1115 ± 116</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Monochromator dithering (Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>980 ± 64</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>998 ± 72</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>1111 ± 66</td>
<td>3.8 ± 0.1</td>
</tr>
</tbody>
</table>

Figure III-8. Effect of acquisition parameters on noise and resolution. (a-b) Increasing the number of radiographs collected from 512 to 2049 produces drastic improvements in both noise and resolution. (c-d) Increasing the number of bright field counts (i.e., the camera exposure time) improves noise and has a subtle effect on resolution. Note: All reconstruction parameters are the same for each inset image. Scalebars represent 100 µm.
E. Discussion

The purpose of the current study was to give a detailed overview of the SRµCT beamline at the ALS. Relevant features of the beamline were described including multiple X-ray microscope/camera/lens setups, as well as the general workflow for image acquisition, pre-processing, reconstruction, and visualization. Insofar as the ALS is a national user facility, the SRµCT beamline was designed to suit a myriad of imaging applications, from materials science to biological studies. One result of this versatility is that users are required to make a series of non-trivial decisions during experimental setup and data processing, which can have a significant impact on the quality of the resulting reconstructions. For both new and experienced users alike, this concept can serve as a potential pitfall, especially considering the scarcity of beamtime.

A related issue is that users are often forced to pool data collected during multiple shifts, which may have been spaced months apart. There is a need for developing protocols designed with consistency in mind. General guidelines in the literature are useful for selecting an appropriate photon energy\textsuperscript{129,232}, however many ALS users require more sophisticated advice. To this end, a second goal of the current study was to provide new and existing users with specific details regarding the impact of various experimental setup variables on noise and resolution. The beamline plugin is also explained, with recommendations for bone imaging that reduce artifacts in the reconstructions.

One of the characteristic features of synchrotron imaging is the production of X-rays having high spatial coherence, a physical property related to wave geometry. As planar wavefronts propagate through a sample, their geometry is altered such that the
boundaries between regions containing microstructural and compositional variations are enhanced in accordance with the distance between the sample and the detector\textsuperscript{241}. This phase contrast phenomenon is relevant when analyzing materials such as bone, which contains multiple levels of hierarchical organization and broad variations in mineralization. Recent dental studies have used phase contrast tomography to examine incremental features of enamel microstructure and mineralization similar in scale to cortical bone lamellae\textsuperscript{233,254,255}. The technique is also practical when imaging biological materials having a low X-ray absorption contrast. Indeed, phase contrast synchrotron X-ray imaging has recently been used to study lung ventilation\textsuperscript{256}, as well as to identify cancer lesions in liver\textsuperscript{257} and breast\textsuperscript{258} tissue.

However, the strong edge-enhancement effects caused by phase contrast interference patterns can produce errors in osteocyte lacunar segmentation and bone mineralization calculations. Numerous phase retrieval methods have been developed, where they are typically differentiated based on assumptions about sample composition and X-ray optical properties, X-ray beam characteristics, and wave propagation\textsuperscript{244,259–264}. At the ALS, an open-source algorithm developed by Wietkamp et al\textsuperscript{243,244} has been integrated into the SR\textmu CT beamline’s Fiji plugin. Three different phase contrast filtering settings have been evaluated at an X-ray photon energy of 17 keV. Significant improvements have been observed in the absorption contrast and the accuracy of small particle segmentation with moderate phase correction (i.e., $\beta = 5 \times 10^{-10}$, $\delta = 2.5 \times 10^{-5}$).

The initial values for $\beta$ and $\delta$ can be estimated from the index of refraction according to a material’s chemical composition, density, and the X-ray photon energy\textsuperscript{245}. 


Thus, it is prudent to use the tabulated values or the CXRO online calculator as a reference point when tuning these parameters. One significant limitation to this technique is that the code in its current form is very computationally intensive, where processing time scales approximately linearly according to the number of radiographs collected. Most beamline users collect 1025 radiographs per scan, which requires nearly three hours for phase retrieval alone, or approximately 10 seconds per projection. Other users seeking the highest resolution collect 2049 radiographs, and they require quasi-real-time feedback on their datasets during beamtime. Areas of future interest include better parallelization of the code, integration of other phase retrieval methods, and full assimilation of phase retrieval as part of the supercomputer pipeline at NERSC.

Many studies require an accurate estimate of resolution beyond simply reporting the pixel size, according to the X-ray microscope/camera/lens setup. Moreover, many beamline users wish to maximize productivity by reducing scan time via sparser sampling (i.e., collecting fewer radiographs) and decreasing exposure time. Common scan settings have been analyzed using the NLOO method, to determine how each parameter contributes to noise and resolution. The number of radiographs has been found to have the most profound effect on noise and 2D spatial resolution, while modest improvements have also been noted with increased bright field counts (i.e., longer camera exposure time). These trends agree with several cone-beam CT studies, which have proposed an inverse square root relationship between image noise, number of projections, and exposure time\textsuperscript{265–267}.
The theoretical number of radiographs needed to completely fill Fourier space up to the highest frequency is defined as approximately 1.5 (or \( \pi/2 \)) times the number of detector pixel rows\(^{268} \). For the pco.edge camera, this would correspond with 3392 radiographs. However, the beamline acquisition code requires the number of angles to be in the form \( N_{a+1} = 2N_a - 1 \), where \( N_{a+1} \) and \( N_a \) are the new and original number of angles, respectively (e.g., 513, 1025, 2049, etc.). Thus, a total of 4097 images would be required to satisfy the theoretical case, corresponding with a data collection rate of almost 23 GB/dataset. This rate is not practical, given the beamline’s current storage and computational constraints. In practice, lab-based CT systems typically collect only about 25% of the theoretical number of angles. Based on the current findings, it is recommended that users collect either 1025 projections (i.e., 30% of the theoretical number; \( \approx 5.5 \) GB/dataset) for most normal scans, or 2049 projections (i.e., 60% of the theoretical; \( \approx 11 \) GB/dataset) for high resolution applications. Additional improvements in image noise and resolution can be achieved through an increased camera exposure time, provided that longer scan times are acceptable.

One limitation of the FRC analysis is that it was restricted to 2D resolution, however this approach can also be generalized to 3D by calculating Fourier shell correlations (FSCs) instead of FRCs\(^{252} \). At the ALS, an estimate of the resolution has been determined by averaging over a representative subset of equally spaced images, for a given set of acquisition parameters. This has been done to speed up the calculation and reduce the computational load, since each NLOO\(_{2D} \) measurement requires user input and repeated ROI selection. Thus, a slight loss in the accuracy of the overall resolution measurement can be expected. Nevertheless, standard deviations of the resolution using
this method have been shown to be on the order of a few hundred nanometers, suggesting good stability of the estimate.

F. Conclusions

This study provides an overview of the hardware, software, image acquisition, and processing pipeline for the SRµCT beamline at the ALS. Setup parameters are critical in high resolution SRµCT studies. Increasing the number of radiographs, as well as the exposure time, can improve image resolution and reduce noise. Based on the application, phase retrieval can also be useful for highlighting or attenuating both external and internal sample edges. This work provides general guidance for new and experienced beamline users, especially those conducting bone research.
IV. ALTERED 3D VASCULAR POROSITY AND OSTEOCYTE LACUNAR MORPHOMETRY IN HUMAN OSTEOGENESIS IMPERFECTA

A. Abstract

In addition to bone density, it has been suggested that the strength of bone is also determined in part by its microstructural organization. This study investigated the 3D microstructure and composition of diaphyseal cortical bone in children with OI as well as healthy controls. A total of 71 specimens from 29 individuals were collected during routine orthopaedic procedures or autopsy and imaged via micro-computed tomography at a synchrotron light source (SRµCT) with 0.65 µm isotropic pixel size. Parameters describing bone composition, vascular porosity, and osteocyte lacunar porosity were measured. At the tissue level, vascular porosity in OI cortical bone was significantly elevated compared to controls, with associated increases in connectivity and canal diameter. OI cortical bone porosity was also more isotropic than in healthy individuals. At the cellular level, osteocyte lacunar porosity was increased in OI cortical bone, and this was explained in part by an increase in lacunar density. Lacunae were also more spherical in shape in OI cortical bone compared to controls. This study presents novel data on osteocyte lacunar characteristics in OI. Osteocytes have been implicated in bone modeling and remodeling processes, and osteocyte morphology is thought to adapt according to mechanical loading. Results from the current study suggest that the abnormal pore network and osteocyte morphology in OI cortical bone may contribute to its increased fragility. Note: a version of this chapter has been accepted for publication as a proceedings paper by the international society for photonics medical imaging conference (SPIE Medical Imaging)\textsuperscript{269}. 
B. Introduction

Bone strength is a function of many factors including composition, mechanical properties, and microstructural organization. The relative importance of these properties in determining effective bone strength is poorly understood in bone pathologies such as OI. Most prior studies on OI bone structure have been limited to histological assessment of biopsies taken from the iliac crest\textsuperscript{21–23}, where it was found that the cortical thickness was significantly decreased compared to age-matched controls. The latter was explained in the context of a deficiency in bone modeling, the process responsible for bone growth during development. X-ray computed-tomography (CT) studies have reported similar trends at the femoral diaphysis\textsuperscript{270} and the proximal radius\textsuperscript{271}. Recent advances in peripheral clinical CT systems have enabled assessment of cortical porosity at a resolution of approximately 80 µm. One such study compared adults with mild OI to age-matched controls, noting decreases in cortical bone area at the ultradistal tibia\textsuperscript{127}. Nevertheless, the resolution of peripheral scanners is still not sensitive enough to capture vascular porosity (i.e., Haversian canals, Volkmann’s canals, and resorption spaces).

Synchrotron radiation micro-computed tomography (SR\textsubscript{μ}CT) is the current gold standard for high-resolution quantitative characterization of bone in 3D. Although access to these facilities is limited, they offer the unique ability to simultaneously assess both osteonal and osteocyte lacunar properties in bone. Osteocytes are the most abundant cells found in bone and are thought to play an important role in mechanical signal sensation and transduction\textsuperscript{272}. These cells are encased within small lacunar spaces in the bone matrix. Since the osteocytes themselves cannot be imaged with X-ray based techniques, the lacunar spaces are used as proxy. The vast number of cells translates to a large
surface area that has been reported to be 400 times larger than the Haversian systems\textsuperscript{273}. Therefore, changes to the osteocyte lacunar network may have significant effects on overall bone microstructure and mechanical behavior. Research on the relative role of osteocyte lacunar properties in adaptation and disease have produced mixed results. In one study, osteoporotic patients showed no difference in lacunar size or shape compared to controls\textsuperscript{274}. However another study reported an increase in mean lacunar volume for vitamin D deficient tissue\textsuperscript{172}.

The purpose of this study was to examine vascular and lacunar porosity, as well as bone mineral parameters, in OI diaphyseal bone from young individuals. A related objective was to compare OI microstructural and mineral properties to healthy pediatric and adult populations.

C. Methods

C-1. Study Population

A total of 24 osteotomy specimens were collected from the long bones of 17 patients diagnosed with a variety of OI types ranging from mild to severe (Table IV-1). All but 4 of the OI patients had been treated with various doses of bisphosphonates as part of a normal clinical regimen to improve bone mass. An additional 14 specimens were harvested from the lower extremity long bones of 12 subjects who had no prior history of bone pathology. The control population was divided into two groups, where 8 specimens originated from pediatric autopsies, and the remaining 4 were from adult cadavers. All tissue samples were collected under written IRB approval (#10101309...
### Table IV-1. Study population and specimen descriptions for SR\(\mu\)CT imaging study.

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\(\textsuperscript{a}\)Specimens 5 and 6 were collected simultaneously from contralateral tibiae during an elective procedure.

\(\textsuperscript{b}\)Specimens 8 and 9 were collected simultaneously from contralateral femora during an elective procedure.

\(\textsuperscript{c}\)Specimens 15-17 were collected within a two-year interval. The donor had not been treated with bisphosphonates prior to donating specimen 15, but received pamidronate treatments prior to donating the other specimens.

\(\textsuperscript{d}\)OI types are based on Sillence clinical classification\(\textsuperscript{1}\). Genotype, if known, is indicated in parentheses.

HP = Healthy Pediatric; HA = Healthy Adult.
C-2. Specimen Preparation

Specimens were fresh-frozen at -80°C prior to sample preparation and imaging. To achieve the required resolution for osteocyte lacunar measurements (i.e., a pixel size $\approx 1 \mu m$), the camera/optics combination selected for scanning limited the horizontal field of view ($FOV$) to 1.7 mm. The specimens were therefore machined into rectangular prismatic beams with a low speed diamond saw (IsoMet™ Low Speed Saw; Buehler, Lake Bluff, IL, USA) according to methods described for small pediatric bone specimens in previous work. Each specimen was machined by first gluing it to a wood mandrel and then making consecutive cuts adjacent to the periosteal surface, creating a slice with a thickness equal to the desired beam depth (i.e., 1 mm). This slice was then gripped along with an acrylic backing (to prevent bending of the slice during machining), and cut into beams having final dimensions of approximately $6 \times 1 \times 0.7$ mm.

C-3. Micro-Computed Tomography with a Synchrotron Light Source (SRµCT)

Beams were scanned in general accordance with the guidelines established in Chapter III for the SRµCT beamline at the Advanced Light Source (BL8.3.2; Berkeley, CA, USA). Each sample was air-dried overnight and fixed onto a sample holder using capillary sealing wax and a wax pen. The sample holder was gripped via a drill chuck assembly attached to a kinematic mount, which was then magnetically coupled to the rotational stage. For the current study, the high resolution X-ray microscope setup described in Chapter III (Optique Peter, Lentilly, France) was used in combination with the 50 µm thick LuAG scintillator, the 10× lens (Mitutoyo Corporation, Kawasaki, Japan), and the fast camera (pc.edge sCMOS camera, PCO-TECH Inc., Romulus, MI).
This X-ray microscope/lens/camera combination resulted in an object-to-scintillator distance of 7 mm, a FOV of $1.7 \times 1.4$ mm, and an isotropic imaged pixel size of 0.65 µm. Each scan consisted of 1025 radiographs collected over a continuous 180° rotation, at a monochromatic X-ray energy of 17 keV.

Pre-processing and tomographic reconstructions were performed using the beamline’s NormalizeStack832newnaming plugin for Fiji (a distribution of ImageJ v1.49f, National Institutes of Health, Bethesda, MD$^{237}$). The recommended settings from Chapter III were applied for noise filtering, phase retrieval, and ring removal. The noise filter level was set to 80%. The following values were used for ring removal: lower value = -100, upper value = 100, ring threshold=100, maximum ring size = 30 pixels, and minimum arc length = 30°. For phase retrieval, the real-part decrement ($\delta$) and the complex part ($\beta$) of the index of refraction were set as $2.5 \times 10^{-5}$ and $5 \times 10^{-10}$, respectively.

The plugin applied a commercial parallel-beam, filtered back projection reconstruction algorithm (Octopus v8.6, inCT, Ghent, Belgium). To conserve disk space, the datasets were converted to 16-bit signed image type using an automatically determined scaling factor in the plugin during the reconstruction process. The linear scaling equation was automatically embedded in the header file of each reconstructed slice, allowing for back-calculation of the original 32-bit real gray values, which were required for bone mineral density measurements. The final reconstructed 16-bit TIF stacks consisted of 2160 slices, with each slice having $2560 \times 2560$ pixels.
C-4. Bone Compositional Measurements

Monochromatic X-ray sources allow for direct calculation of bone compositional
measures from reconstructed gray values. Bone datasets were converted back to their
32-bit real values using the scaling equation contained in the header file. Each image
stack was cropped to remove all exterior voxels outside of the bone tissue, since these
were not part of the sample and therefore should not be counted in bone density
calculations. Gray values of all remaining voxels within the bone tissue (including
vascular canals, osteocyte lacunae, and resorption spaces) were then averaged, yielding a
mean linear attenuation coefficient ($\bar{\mu}_{BMD}$) for the entire stack. After segmenting out all
non-bone voxels from the tissue, a similar mean linear attenuation coefficient was
calculated for bone only ($\bar{\mu}_{TMD}$). Bone mineral parameters were then determined using
the following relationships:

$$\bar{\mu}_{BMD} = \left(\frac{\mu}{\rho}\right)_{\text{bone}} \times vBMD$$  \hspace{1cm} (Equation IV-1)

$$\bar{\mu}_{TMD} = \left(\frac{\mu}{\rho}\right)_{\text{bone}} \times vTMD$$  \hspace{1cm} (Equation IV-2)

where $(\mu/\rho)_{\text{bone}}$ is the X-ray mass attenuation coefficient of bone, $vBMD$ is the volumetric
bone mineral density, and $vTMD$ is the volumetric tissue mineral density. The value of
$\mu/\rho$ is a material constant that depends on photon energy. Reference tables from the
National Institute of Standards and Technology (NIST) list $\mu/\rho$ values for a variety of
elements, mixtures, and compounds (including bone). For an X-ray beam energy of 17
keV, $(\mu/\rho)_{\text{bone}}$ is 6.41 cm$^2$/g. The monochromator beam energy was calibrated at the
beginning of each beamtime shift using silver (Ag) and germanium (Ge) foils, which have known K edges at 25.514 keV and 11.103 keV, respectively.

C-5. Image Processing

Intracortical vascular porosity calculations were performed on 0.6 mm³ regions of interest (ROIs) within each 3D dataset via a semi-automated, customized script that called on several plugins in Fiji. To decrease processing time and computational load, ROIs were converted to 8-bit format and downsampled to a nominal voxel size of 1.3 × 1.3 × 1.3 μm³ using an accurate B-splines algorithm. Each ROI was smoothed with a 3D bilateral filter, which combines useful characteristics from both spatial and range filters to attenuate background noise while maintaining edges.

Datasets were segmented into different structural elements (i.e., bone, vascular porosity, and osteocyte lacunae) via a masking procedure. Because of the high signal-to-noise ratio and spatial resolution associated with SRµCT images, the bone structure had a clear bimodal histogram, indicating high contrast between bone tissue and the background (Figure IV-1). An initial bone mask was generated by blurring each image stack with a simple Gaussian low-pass filter (to remove all osteocyte lacunae), and thresholding the blurred stack via an IsoData algorithm on the stack histogram (Figure IV-2, panes b-d). The IsoData or iterative intermeans thresholding procedure can be summarized as follows: 1) compute the stack histogram (Figure IV-1); 2) separate pixels into either object (i.e., foreground) or background using an initial guess threshold between the two peaks in the histogram; 3) average the background pixel values; 4) average the foreground pixel values; 5) average the results from steps 3 and 4; 6)
increment the threshold guess and compare it to the result of step 5. This process is repeated until the threshold in step 6 is larger than the composite average from step 5.

An osteocyte lacunar mask was created from within the bone mask volume by thresholding the original image stack, inverting the result, and then performing the logical “AND” operation with the bone mask (Figure IV-2, panes d-f). Finally, a vascular porosity mask was produced by inverting the bone mask (Figure IV-2, pane g). The tissue volume ($TV$) and bone volume ($BV$) were determined from the bone mask as the total volume of the ROI and the total volume occupied by bone voxels (excluding osteocyte lacunae and vascular pore spaces), respectively. These quantities were used to determine the cortical bone volume fraction ($V_f$), as well as to normalize several other microstructural measurements outlined below.

Figure IV-1: Stack histogram of SRµCT bone image. The high spatial resolution and contrast allow for selection of a clear threshold value based on the location of the two peaks separating bone from background voxels.
C-6. 3D Osteocyte Lacunar Analysis

Individual osteocyte lacunae were labelled from the binary lacunar mask using the connected-components Particle Analyzer plugin in BoneJ$^{280}$, a set of open-source bone algorithms developed for Fiji. Particles having a volume $< 82 \mu m^3$ were considered as
noise and removed, while particles with a volume > 2000 µm³ were assumed to be canals. These lower and upper limits were invoked within the plugin, and they are based on prior laser scanning confocal microscopy²⁷⁴ and SRµCT¹³⁵–¹³⁷ studies of lacunar volume in human bone. Spurious particles generated from segmentation errors (e.g., ring artifacts and microdamage) were removed by imposing restrictions on the particles’ Euler number (χ) and the major/intermediate axis anisotropy (Lc.L1/Lc.L2).

The Euler number is a topological property used in connectivity analysis, and it is generally determined by subtracting the number of tunnels or handles in a continuous object, and adding the number of enclosed cavities²⁸¹,²⁸². By definition, any solid body that can be rigidly deformed into a solid sphere has χ = 1. Thus, all particles with χ ≠ 1 were removed. Moreover, the anisotropy of each particle was determined by calculating the ratio of its major (Lc.L1) and intermediate (Lc.L2) axes. Any particle having Lc.L1/Lc.L2 > 5 was removed, as highly prolate spheroids are commonly associated with ring artifacts in tomographic images¹³⁵. Although χ, Lc.L1, and Lc.L2 were calculated as a part of the particle labeling plugin above in Fiji, the actual restrictions were applied using Excel filters (Microsoft Excel® 2010, Microsoft Corporation, Redmond, WA). Finally, to avoid undue biasing of the results from partially truncated lacunae, any particle in contact with one of the six edges in the image stack volume was eliminated.

After labelling lacunar spaces, 3D algorithms within the Particle Analyzer plugin were used to analyze morphological descriptors for each lacunae, following terminology and methods that were previously established in the literature¹³⁵,²⁸⁰. The mean and total lacunar volumes were determined by voxel counting as the average volume of all lacunae
and the total volume of all lacunar spaces (Lc. TV), respectively. The mean lacunar surface (Lc. S) was calculated as the average surface area of the best-fit ellipsoid of each lacunae, while the total lacunar surface ratio was determined by summing all lacunar surfaces in the ROI (Lc. S/TV). The principal axes of the best-fit ellipsoids were determined via eigendecomposition, and the lengths of the resulting major, intermediate, and minor radii were used to calculate the average lacunar length (Lc. L1), width (Lc. L2), and depth (Lc. L3). The mean local thickness of all lacunae (Lc. Th) was determined using a maximum inscribed spheres algorithm. Several histomorphometric indices were also calculated from the above basic lacunar descriptors, including the lacunar porosity (Lc. TV/TV and Lc. TV/BV), lacunar number density (N.Lc/TV and N.Lc/BV), and the lacunar anisotropy ratios (Lc. L1/Lc. L2 and Lc. L1/Lc. L3). As a measure of goodness of fit, a volume ratio (τ) was calculated between the actual volume of each lacunae and the enclosed volume of the fitted ellipsoid.

The structure model index (Lc. SMI) is a shape descriptor that characterizes the lacunar space as either plate-like (Lc. SMI = 0), cylindrical (Lc. SMI = 3), or spherical (Lc. SMI = 4). The algorithm computes the average Lc. SMI for all lacunae in a 3D image stack by measuring local changes in the surface area of each lacuna via a mesh dilation procedure. Combinations of plate-like, cylindrical, and/or spherical elements in the lacunar structure are reflected in an Lc. SMI between 0-4, depending on the volume ratio of each element present.
C-7. 3D Vascular Porosity Analysis

Similar to osteocyte lacunae, intracortical canal spaces were individually labelled from the binary vascular porosity mask using the Particle Analyzer plugin\textsuperscript{237,280}. Small particles (< 2001 µm\textsuperscript{3}) were removed, as these have been shown to represent noise and other artifacts such as microdamage and ring artifacts\textsuperscript{135–137}. Morphological parameters describing vascular porosity in cortical bone were determined according to the nomenclature established by Parfitt et al\textsuperscript{122}, and extended to 3D by Cooper et al\textsuperscript{26}. The intracortical vascular porosity (Ca.V/TV) was calculated as the volume fraction of canals in the image ROI.

The canal surface (Ca.S) was measured by summing the areas of all triangles in the surface mesh as generated by the marching cubes method\textsuperscript{287}, where a resampling factor of three was used to reduce the number of triangles, thereby smoothing the mesh and reducing the computational burden. This sum was generalized as the canal surface to tissue volume ratio (Ca.S/TV). Canal connectivity density (Ca.ConnD), diameter (Ca.Dm), spacing (Ca.Sp), microstructural degree of anisotropy (DA), and traditional degree of anisotropy (tDA) were determined using the automated BoneJ plugin\textsuperscript{280}. Both DA and tDA describe the anisotropy of a structure in terms of how highly oriented it is within a volume, but each uses a different quantitative scale\textsuperscript{288}. For example, DA ranges from 0 in isotropic (i.e, randomly oriented) structures to 1 in anisotropic (i.e., highly oriented) structures. On the other hand, tDA ranges from 1-∞, where 1 indicates isotropy and ∞ indicates anisotropy.
C-8. Statistical Analysis

All statistical analyses were completed using an open-source software package (The R Project for Statistical Computing; www.r-project.org). Shapiro-Wilk normality tests were performed on all extracted imaging parameters for each of the three experimental groups (i.e., OI, healthy pediatric, and healthy adult bone). These tests revealed non-normal distributions in at least one of the three groups for most of the imaging parameters. A non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) was therefore used for comparison of the three group medians. Individual pairwise comparisons between the groups were then performed using a Mann-Whitney U test. For all analyses, significance was defined at a $P$-value less than 0.05 (i.e., $P < 0.05$).

D. Results

D-1. Tissue Level Results Describing Vascular Porosity

The SRµCT analysis of the bone specimens revealed the tissue level vascular porosity network, as well as the cellular level osteocyte lacunar characteristics of the OI, healthy pediatric, and healthy adult bone specimens (Figure IV-3). Box plots summarizing the tissue level microstructural results extracted from the tomography data are depicted in Figure IV-4. Cortical bone volume fraction ($V_f$) was 22% lower in patients with OI compared to both healthy groups ($P < 0.001$), and this translated to a six to ten-fold increase in vascular porosity ($Ca.V/TV; P < 0.001$) and a three-fold increase in the canal surface to tissue volume ratio ($Ca.S/TV; P < 0.001$). The canal connectivity
density was significantly different for all three groups, with OI and healthy pediatric bone showing the highest and lowest connectivity per tissue volume, respectively.

Figure IV-3. 3D tomographic assessment of cortical bone structure. (a to c) Visualizations from SRµCT images showing bone (grayscale), vascular porosity (red), and osteocyte lacunae (yellow) for (a) OI (donor 8, specimen 11, M, age 11 years, severe OI type III, femur), (b) healthy pediatric (HP, donor 25, specimen 32, M, age 1 year, femur), and (c) healthy adult bone (HA, donor 26, specimen 33, F, age 52 years, tibia), where the diaphyseal long bone axis is oriented vertically. (d to f) Measurement of vascular porosity showing a drastic increase in canal diameter (Ca.Dm) for (d) OI bone (Ca.Dm = 146 µm [117-171 µm]) compared to (e) HP bone (Ca.Dm = 31 µm [29-43 µm]; P < 0.001 OI vs. HP) and (f) HA bone (Ca.Dm = 49 µm [46-53 µm]; P < 0.001, OI vs. HA). (g to i) Tomographic images of osteocyte lacunae demonstrate a higher number density and intermediate lacunar volume (Lc.V) for (g) OI bone (Lc.V = 374 µm$^3$ [339-424 µm$^3$]) compared to (h) HP bone (Lc.V = 414 µm$^3$ [341-496 µm$^3$]) and (i) HA bone (Lc.V = 332 µm$^3$ [313-354 µm$^3$]; P = 0.036, OI vs. HA). All scalebars = 500 µm. Colorbars indicate relevant diameter and volume scales, respectively.
Figure IV-4. Comparison of vascular porosity parameters between OI, healthy pediatric (HP), and healthy adult (HA) groups. (a) Cortical bone volume fraction ($V_t$); (b) Vascular porosity ($Ca.V/TV$); (c) Canal surface to tissue volume ratio ($Ca.S/TV$); (d) Canal connectivity density ($Ca.ConnDn$); (e) Canal diameter ($Ca.Dm$); (f) Canal separation ($Ca.Sp$); (g) Degree of anisotropy ($DA$); (h) Traditional degree of anisotropy ($tDA$). Box plots depict minimum, first quartile, median, third quartile, and maximum. For group comparisons: Solid lines = $P < 0.05$, OI vs. HP; Dashed lines = $P < 0.05$, OI vs. HA; Dotted lines = $P < 0.05$, HP vs. HA.

Canals were also three to five times larger in OI cortical bone ($Ca.Dm = 146 \mu m$ [117-171 \mu m]) compared to healthy adult ($P < 0.001$) and pediatric ($P < 0.001$) tissue, respectively. OI vascular porosity appeared highly heterogeneous in size and spacing (Figure IV-3 (d-f)), especially compared to the more tightly packed pore network seen in healthy peers. For the control populations, vascular canals were 58% larger and two times further apart in adults than in children. For all groups, Haversian systems were
visible and had an obvious preferential orientation in the direction of the diaphyseal long bone axis (Figure IV-3 (a-f)). This observation was also noted quantitatively (Figure IV-4, (g-h)), as the median normalized degree of anisotropy was over 0.8 in OI cortical bone and over 0.9 in the control groups. However, both DA and tDA were significantly reduced in individuals with OI compared to healthy adults.

D-2. Cellular Level Results Describing Osteocyte Lacunar Porosity

Cellular lacunae were densely embedded within bone regions as evidenced by SRµCT visualizations for all three groups (Figure IV-3 (a-c) and (g-i)). Box plots summarizing the quantitative osteocyte lacunar properties are shown in Figure IV-5. The first five parameters describe the overall lacunar porosity (Figure IV-5 (a-e)), while the remaining metrics summarize the median shape and volume of individual lacunar spaces. Lacunar porosity accounted for 1.9% [1.5-2.3%] of the total bone volume and 1.3% [1.1-1.7%] of the total tissue volume in OI specimens. This translated to significant increases in \( Lc.TV/BV \) and \( Lc.TV/TV \) for OI cortical bone compared to healthy adults \( (P < 0.001) \), as well as a similar increase in \( Lc.TV/BV \) compared to healthy pediatric bone \( (P = 0.006) \). In general, both properties describing lacunar porosity were elevated in young individuals vs. adults. OI cortical bone specimens contained 60% more osteocytes than healthy bone (e.g., Figure IV-3 (g-i)) for a given tissue volume \( (N.Lc/TV; P < 0.05) \) and twice as many for a given bone volume \( (N.Lc/BV; P < 0.001) \).

The median volume of each lacuna (i.e., \( Lc.V \)) was higher in OI cortical bone compared to adults \( (P = 0.036) \), but was not significantly different from other healthy children \( (P = 0.350; \) see Figure IV-3 (g-i)). The \( \tau \) ratio for each group was above 93%,
indicating that the osteocyte lacunar spaces were well-represented by the best-fit ellipsoid determined using the marching cubes method. The median surface area of the lacunae was largest in healthy pediatric bone ($P < 0.05$), and was associated with an increase in the length of the major lacunar axis ($Lc.L1; P < 0.001$). Lacunar geometries for each group were as follows: 15.6 µm [14.3-17.0 µm] × 9.3 µm [8.9-9.8 µm] × 5.6 µm [5.3-5.9 µm] for OI cortical bone, 20.9 µm [17.9-21.3 µm] × 9.8 µm [9.6-10.12 µm] × 5.2 µm [5.0-5.4 µm] for healthy pediatric bone, and 16.0 µm [15.4-16.3 µm] × 8.5 µm [7.9-8.7 µm] × 5.2 µm [5.1-5.5 µm] for healthy adult bone. Thus, lacunar width (i.e., $Lc.L2$) was 10-15% larger in young vs. adult bone ($P < 0.001$), but was not significantly different between the pediatric groups ($P = 0.144$). Lacunar depth (i.e., $Lc.L3$) was approximately 8% larger in the OI group compared to both control groups ($P < 0.05$). Similarly, the median lacunar thickness (i.e., $Lc.Th$) was 13% greater in OI versus healthy pediatric bone ($P = 0.004$). As shown in Figure IV-6, OI lacunae appeared more spherical than healthy lacunae. This observation corresponded with significant reductions for OI lacunae vs. controls in both the in-plane ($P < 0.001$) and out-of-plane ($P < 0.05$) anisotropy ratios. On the other hand, healthy pediatric lacunae were more prolate than either OI ($P < 0.001$) or healthy adult ($P < 0.001$) lacunae, and thus had an $Lc.SMI$ value close to an ideal rod (i.e., $SMI_{rod} = 3$).
Figure IV-5. Comparison of lacunar parameters between OI, healthy pediatric (HP), and healthy adult (HA) groups. (a-o) Box plots depict minimum, first quartile, median, third quartile, and maximum. Solid lines = $P < 0.05$, OI vs. HP; Dashed lines = $P < 0.05$, OI vs. HA; Dotted lines = $P < 0.05$, HP vs. HA.
Figure IV-7. Comparison of bone mineral density parameters between OI, healthy pediatric (HP), and healthy adult (HA) groups. (a) $vBMD$ (g/cm$^3$); (a) $vTMD$ (g/cm$^3$). Solid lines = $P < 0.05$, OI vs. HP; Dashed lines = $P < 0.05$, OI vs. HA; Dotted lines = $P < 0.05$, HP vs. HA.

D-3. Bone Mineralization Results

Bone mineral density calculations are summarized in Figure IV-7. Differences in $vBMD$ were highly significant ($P < 0.001$) among the three groups, where healthy adult bone showed the highest $vBMD$ and OI cortical bone the lowest. Reductions in $vBMD$ of 18% and 28%, respectively, were noted for OI cortical bone compared to healthy pediatric and healthy adult specimens. In contrast, $vTMD$ was not significantly different ($P = 0.068$) between healthy adult and OI bone. However, the OI ($P = 0.017$) and healthy adult ($P < 0.001$) groups showed a 10% and 14% increase, respectively, in $vTMD$ over healthy pediatric bone.
E. Discussion

The purpose of this study was to use SRµCT imaging to investigate microstructural and mineral properties in long bone diaphyseal specimens obtained from children and adolescents with OI. To better understand these properties in the context of healthy bone development and homeostasis, control specimens were also analyzed in both young individuals and adults. At the tissue level, OI cortical bone was characterized by drastically increased vascular porosity \( \text{Ca.V/TV} \), connectivity \( \text{Ca.ConnD} \), and canal diameter \( \text{Ca.Dm} \). Altered bone mineral parameters were also reported \( \text{vBMD} \) and \( \text{vTMD} \), as they have previously been associated with mechanical properties\(^{96,289}\). At the cellular level, abnormalities were noted including increased lacunar porosity \( \text{Lc.TV/TV} \) and density \( \text{N.Lc/TV} \). Building on previous work\(^{128,269}\), this is the first study to report 3D osteocyte lacunar descriptors in human OI cortical bone.

To date, there is very little data describing the internal, 3D microstructural properties of pediatric OI bone. Most clinical studies on this topic have relied on histological assessment of iliac crest biopsies\(^{21–23}\). The most extensive of these studies was by Rauch et al\(^{21}\), who analyzed 70 iliac biopsies from pediatric OI patients and reported significantly reduced cortical wall thickness for various degrees of OI severity. The lagging cortical thickness was attributed to a defect in the modeling process, which is responsible for increasing bone mass and size during development\(^{290}\). It is unclear how well histological studies of bone structure translate to other anatomical sites such as the long bones, which are the most common fracture locations in OI patients.
One early study used clinical CT to examine pediatric OI cortical bone at the femoral diaphysis and noted a decreased cross-sectional area compared to healthy peers. Another reported a similar finding at the proximal radius using pQCT. Recent advances in clinical systems such as high-resolution pQCT (HR-pQCT) have enabled analysis of cortical and trabecular compartments at distal skeletal sites such as the radius and tibia. Indeed a recent HR-pQCT study comparing adult subjects with mild OI type I to age-matched controls reported significant decreases in trabecular number and cortical bone area at the ultradistal tibia. However, even HR-pQCT systems are not sensitive enough to capture finer cortical bone details such as small vasculature and osteocyte lacunae.

In the current study, the use of SRμCT allowed for simultaneous analysis of cellular and tissue level properties describing bone structure, as well as bone mineral characteristics assessed using monochromatic X-rays. OI cortical bone displayed substantial vascular porosity (accounting for nearly 24% of the total tissue volume) that was enlarged and highly connected compared to healthy pediatric and healthy adult bone. For the control groups, the porosity values measured in the current study were within the range of those typically seen in normal pediatric and adult bone (e.g. 2-10%). Both healthy groups showed similar $V_f$, $Ca.V/TV$, $Ca.S/TV$, and anisotropy ($DA$ and $tDA$). Vascular canals in adult bone showed a slight increase in $Ca.Dm$ and $Ca.Sp$. Taken together with the $Ca.V/TV$ results, these findings imply a reduced number of canals in middle-aged adult bone, signifying lower bone turnover in adulthood compared to the rapid growth experienced in children. This trend seems to reverse with advanced aging, as vascular porosity has been reported to rise in elderly bone.
and this behavior has been associated with elevated osteonal density and reduced canal diameter as a result of increased bone turnover\textsuperscript{83,297}. This shift fits in the context of bone loss associated with osteoporosis and other age-related conditions of osteopenia.

Prior biochemical immunoassay studies on young and adult OI bone have also revealed elevated bone turnover markers\textsuperscript{21}, especially in more severe forms of the disorder such as OI types III and IV\textsuperscript{104}. However, elevated bone turnover seems to have much different effects in OI cortical bone compared to elderly individuals. In the current study, \textit{Ca.V/TV}, \textit{Ca.S/TV}, \textit{Ca.ConnD}, and \textit{Ca.Dm} were all elevated for OI tissue compared to healthy bone. Apart from our preliminary work\textsuperscript{269}, no other studies have reported these parameters in 3D for human OI cortical bone.

A recent electron microscopy study noted altered bone remodeling units in diaphyseal OI bone sections that appeared as enlarged, flattened canals which the authors described as “drifting osteons”\textsuperscript{298}. This phenomenon has been observed previously in human and baboon cortical bone\textsuperscript{299}, where the authors suggest that OI bone porosity may be partially realigning in response to bone deformity. This concept would help to explain the reduced degree of anisotropy found in the current study, as osteons could be “drifting” away from the predominant orientation, causing a more random (isotropic) structure. Moreover, the current connectivity results are in agreement with an animal model of the disorder (i.e., \textit{oim} mouse model of severe OI type III)\textsuperscript{300}. However, caution should be used in interpreting microstructural data in mice, since murine bone does not contain osteons\textsuperscript{180}. 
It is useful to place the vascular porosity results in context with other bone pathologies. Recent work by Busse et al\textsuperscript{172} found high vascular porosity (\textgreater ;15\%) in vitamin D deficient bone that was also accompanied by an increase in \textit{Ca.Dm} vs. healthy adults. It is noteworthy that the median \textit{Ca.Dm} of vitamin D deficient bone in that study (\approx 125 \, \mu m) was similar to the value observed for OI cortical bone specimens in the current work (146 \, \mu m [117-171 \, \mu m]). Future mechanical testing studies are warranted to investigate how the altered microstructural anisotropy and vascular porosity in OI cortical bone affect bone material properties. For example, several linear and power law relationships have been developed describing the negative impact of cortical porosity on elastic modulus and yield stress in bone\textsuperscript{145,291,301,302}.

At the cellular level, OI cortical bone showed high osteocyte lacunar porosity compared to both healthy groups, and this finding was explained primarily by an increase in the lacunar density per unit volume. The osteocyte lacunar properties of both healthy groups were within the ranges typically reported for normal bone\textsuperscript{135–138,275,303,304}. However, these studies show high variability for lacunar descriptors owing to a number of experimental factors including donor age, sampling site, image resolution, and segmentation strategy. For the current study, the segmentation strategy introduced by Dong et al\textsuperscript{135} was followed, since it incorporated sophisticated topological features of the lacunae in the design. Presented in this context, the current lacunar results showed very good agreement in all parameters describing adult human bone.

The role of osteocyte lacunar properties in bone adaptation and disease is still poorly understood. For example, Mullender et al\textsuperscript{305} found a decrease in areal lacunar
density for osteoporotic females compared to adult controls. A similar osteoporosis study by McCreadie et al\textsuperscript{274} found no significant difference in lacunar size or shape compared to healthy controls. However recent work on vitamin D deficiency has shown an increase in lacunar volume but no change in lacunar density for pathological vs. normal bone\textsuperscript{172}.

In the current study, elevated osteocyte lacunar density is likely related to the recruitment of bone remodeling cells, consistent with the increase in bone turnover. Lacunar depth is also increased in OI cortical bone compared to both control groups, and this resulted in a more spherical lacunar shape. These findings are in agreement with a recent oim study\textsuperscript{300}, which attributed the increased lacunar density and more spherical shape to a more disorganized microlamellar structure\textsuperscript{306} as well as increased bone turnover\textsuperscript{105}, which does not allow the osteocytes sufficient time to adapt to mechanical loading (i.e., to orient themselves along the principal bone axis). Thus, osteocyte lacunar properties may provide insight on the internal loading environment in bone abnormalities such as OI. Along with other structural features (e.g., osteocyte canaliculi, vasculature, muscle insertions, etc.), osteocyte lacunae have been implicated as potential sites of stress concentration and microcrack formation\textsuperscript{148–154}. Future complementary mechanical testing studies are needed to determine the role of osteocyte lacunae, if any, in the mechanical properties of OI bone.
F. Conclusions

This study presented novel data on the 3D microstructural properties of OI cortical bone at the cellular and tissue level. SRµCT was used to analyze the quantity, geometry, and distribution of vascular pores and osteocyte lacunae in diaphyseal long bone specimens from individuals with OI, as well as healthy peers and adults. OI cortical bone displayed a vast, highly connected vascular porosity network that was also more isotropic compared to controls. Similar analyses on OI cellular characteristics revealed high lacunar porosity and lacunar number density, which was also accompanied by an increase in lacunar depth and a corresponding shift towards a more spherical shape.
V. STRENGTH, FRACTURE TOUGHNESS, AND ANISOTROPY ARE ASSOCIATED WITH INTRACORTICAL VASCULAR POROSITY WITHIN LONG BONES OF CHILDREN WITH OSTEOGENESIS IMPERFECTA

A. Abstract

Normal cortical bone is transversely isotropic, meaning its mechanical properties are the same in two directions (i.e., circumferential and radial) but significantly different in the longitudinal direction. The purpose of this study was to examine the mechanical isotropy of OI cortical bone in pediatric patients. Longitudinal and circumferential beams were machined and mechanical properties including elastic modulus ($E_f$), yield strength ($\sigma_y$), ultimate strength ($\sigma_{f,max}$), crack-initiation toughness ($K_{J,o}$), and crack-growth toughness ($dK_J/d\Delta a$) were measured in three-point bending. Due to the reduced cortical wall thickness of human OI bone specimens, it was not possible to machine beams in the radial direction. Intracortical vascular porosity ($Ca.V/TV$), osteocyte lacunar density ($N.Lc/TV$), and volumetric tissue mineral density ($\nuTMD$) were also determined using SR\(\mu\)CT. In contrast with previous claims that OI cortical bone displays isotropic mechanical properties, the mean values for $E_f$, $\sigma_y$, and $\sigma_{f,max}$ were approximately three times higher for the longitudinal vs. circumferential beams ($P$-value < 0.001). Similar to healthy bone, these mechanical properties were negatively correlated with $Ca.V/TV$ in the longitudinal but not the circumferential direction. Despite comparable $K_{J,o}$ values for both orientations, the longitudinal beams showed over five times greater resistance to crack growth (i.e., $dK_J/d\Delta a$). None of the mechanical properties in either direction were significantly correlated with $N.Lc/TV$ or $\nuTMD$, suggesting that vascular porosity may be a key contributor to OI fragility. Note: a version of this chapter has been published as a journal article in Bone$^{307}$. 
B. Introduction

The mechanisms responsible for bone fragility in OI remain poorly understood but are likely related to a characteristic decrease in overall bone mass. Children and adolescents with OI tend to have low areal bone mineral density (\(aBMD\)) which can be attributed to decreased bone size and/or volumetric bone mineral density (\(vBMD\)). Studies using quantitative backscattering scanning electron microscopy (qBSEM) have also noted sparse regions of cortical and trabecular bone with high cellularity and hypermineralized bone matrix in individuals with OI compared to age-matched controls. Histomorphometric studies on iliac crest bone biopsies from children with OI have reported decreases in cortical wall thickness, trabecular thickness, and trabecular bone volume fraction. These findings have been offered as proof of a defect in OI bone modeling and remodeling, the two processes by which bone growth, repair, and adaptation occur.

As shown in Chapter IV, intracortical vascular porosity was found to be over six times higher in OI compared to healthy bone, with OI canals showing a larger, more disorganized (i.e., more isotropic) structure. The extent to which this extreme porosity affects the mechanical properties of the bone is currently poorly understood. As mentioned in previous chapters, a few groups have used nanoindentation to measure the elastic modulus and hardness of pediatric OI cortical bone. These properties were shown to be higher in OI patients compared to age-matched controls, with mild OI type I bone showing significantly increased properties compared to severe OI type III. Interestingly, no significant difference in modulus was observed between indents taken parallel versus perpendicular to the long bone axis, prompting speculation that OI
cortical bone may exhibit more isotropic material properties than normal bone\textsuperscript{188,196}. However, bone is a complex hierarchical tissue, and it is unclear how well these microscopic observations hold true at larger length scales in OI bone.

In a recent pilot study performed in preparation for the current work\textsuperscript{19}, two osteotomy specimens from long bone diaphyses of children with OI were tested in three-bending, and their ultimate strength was lower than values typically seen in healthy pediatric bone\textsuperscript{59}. Visual inspection of the OI cortical bone specimens revealed noticeable porosity near the periosteal surface, a region that is normally occupied by dense cortical bone. An additional aim of the current study is therefore to investigate possible relationships between microstructural characteristics (e.g., vascular and osteocyte lacunar porosity) and mechanical properties of diaphyseal bone in a young OI population.

The specific objectives of this study were: 1) to measure the flexural properties (i.e., elastic modulus, yield strength, ultimate strength, crack-initiation toughness, and crack-growth toughness) of cortical tissue from the long bone diaphyses of children with OI; 2) to assess mechanical isotropy within OI cortical bone tissue by comparing the material properties of specimens oriented in either the longitudinal or circumferential directions relative to the long bone axis; and 3) to explore relationships among the flexural properties and the intracortical vascular porosity ($Ca.V/TV$), osteocyte lacunar density ($N.Lc/TV$), and volumetric tissue mineral density ($vTMD$).
C. Methods

C-1. Bone Specimens

A total of 12 cortical osteotomy bone specimens were obtained from 9 children diagnosed with mild to severe OI during routine surgeries at Shriners Hospitals – Chicago, with informed consent/assent from the donors and under an IRB-approved protocol (Rush University Medical Center #10101309, Marquette University #HR-2167, BUA#205 Lawrence Berkeley National Lab). Patient characteristics are shown below in Table V-1. All samples were wrapped in saline-soaked gauze and fresh-frozen at -80°C prior to mechanical testing\textsuperscript{189}.

Table V-1. OI donor and specimen descriptions.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Specimen</th>
<th>Phenotype (Genotype)\textsuperscript{d}</th>
<th>Age</th>
<th>Gender</th>
<th>Anatomic site</th>
<th>Bisphosphonates (#treatments)</th>
<th>#Beams tested in bending (imaged)\textsuperscript{e}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 F</td>
<td></td>
<td>Tibia</td>
<td>Pamidronate (&gt;3)</td>
<td>6 (2) 2 (0)</td>
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<tr>
<td>1</td>
<td>1</td>
<td>I (I)</td>
<td>11</td>
<td>F</td>
<td>Femur</td>
<td>Pamidronate (1)</td>
<td>3 (2) 0 (0)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>I</td>
<td>9 M</td>
<td>M</td>
<td>Tibia</td>
<td>None</td>
<td>3 (1) 1 (1)</td>
</tr>
<tr>
<td>3</td>
<td>3\textsuperscript{a}</td>
<td>IV (IV)</td>
<td>11</td>
<td>M</td>
<td>Humerus</td>
<td>Pamidronate (1)</td>
<td>9 (1) 3 (1)</td>
</tr>
<tr>
<td>4</td>
<td>4\textsuperscript{a}</td>
<td>IV (IV)</td>
<td>8</td>
<td>F</td>
<td>Tibia</td>
<td>Pamidronate (2)</td>
<td>3 (1) 1 (1)</td>
</tr>
<tr>
<td>5</td>
<td>5 IV (IV)</td>
<td>14 M</td>
<td>14</td>
<td>M</td>
<td>Tibia</td>
<td>Pamidronate (&gt;3)</td>
<td>2 (1) 1 (1)</td>
</tr>
<tr>
<td>6\textsuperscript{b}</td>
<td>III</td>
<td>8 F</td>
<td>6</td>
<td>F</td>
<td>Femur</td>
<td>Pamidronate (&gt;3)</td>
<td>4 (1) 2 (1)</td>
</tr>
<tr>
<td>7\textsuperscript{b}</td>
<td>III</td>
<td>9 F</td>
<td>9</td>
<td>F</td>
<td>Tibia</td>
<td>Pamidronate (&gt;3)</td>
<td>3 (1) 2 (1)</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>III</td>
<td>6</td>
<td>F</td>
<td>Tibia</td>
<td>Pamidronate (1)</td>
<td>0 (0) 2 (1)</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>III</td>
<td>16</td>
<td>M</td>
<td>Tibia</td>
<td>None</td>
<td>1 (1) 1 (1)</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>III (VIII)</td>
<td>16</td>
<td>M</td>
<td>Tibia</td>
<td>Alendronate (3 yrs)</td>
<td>6 (1) 3 (1)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Specimens 3 and 4 were obtained from contralateral tibiae of the same donor within a two-year interval. The donor received two courses of bisphosphonates treatment between donations.

\textsuperscript{b}Specimens 6 and 7 were obtained simultaneously from contralateral tibiae during an elective surgery.

\textsuperscript{c}Specimens 8 and 9 were obtained simultaneously from contralateral femora during an elective surgery.

\textsuperscript{d}OI types are based on Sillence clinical classification\textsuperscript{1}. Genotype, if known, is indicated in parentheses.

\textsuperscript{e}L = longitudinal; C = circumferential

Bone specimens were then machined into a total of 65 rectangular prismatic beams with a low-speed diamond saw (IsoMet Low Speed Saw, Buehler, Lake Bluff, IL) and a 0.3 mm-thick wafering blade (Series 15HC Diamond, Buehler, Lake Bluff, IL).
Details of the machining process are outlined in Chapter IV. Final beam dimensions were defined to be approximately 5-6 mm in length, 1 mm in width ($w$), and 700 µm in depth ($d$). These dimensions were determined based on the size of the smallest specimens. As part of a validation study, similar sized beams machined from acrylic and bovine bone produced errors of between 3-13% for elastic modulus and ultimate strength$^{19}$. A digital micrometer (Model 293-340, Mitutoyo Corporation, Japan) was used to measure the average values for $w$ (1005 ± 46 µm) and $d$ (668 ± 60 µm). Each beam was machined such that its long axis was either parallel (i.e., longitudinal orientation; 44 beams) or perpendicular (i.e., circumferential orientation; 23 beams) to the long axis of the bone (Table V-1).

C-2. Flexural Testing – Three-Point Bending

A total of 40 longitudinal beams and 19 circumferential beams were tested to failure in three-point bending using a test frame and jig developed specifically for characterizing small bone specimens (Figure V-1)$^{19}$. The loading nose and supports consisted of 1/16-inch (1.59 mm) diameter stainless steel dowel pins, which were fixed with cyanoacrylate into machined grooves within an aluminum platen. A span length ($s$) of 4 mm (actual measurement 3.973 mm) was selected between the lower supports since it was the maximum distance that could accommodate the size of the osteotomy specimens collected for this study. The bending jig was secured to an electromechanical testing frame (Model 3345, Instron®, Norwood, MA, USA) with a 50-N load cell (Model 2519-102, Instron®, Norwood, MA, USA). An external linear variable differential
transformer (LVDT; Model 2601, Instron®, Norwood, MA, USA) was used to determine the beam deflection at mid-span during the test.

Flexural loading was controlled using the instrument’s built-in Bluehill 2 software (Instron®, Norwood, MA, USA). Each bending test consisted of five cycles of preconditioning (0.05-1.0 N for longitudinal beams, and 0.05-0.5 N for circumferential beams) with a constant crosshead displacement rate of 0.2 mm/min, followed by a ramp to failure at a constant deflection rate of 2.0 mm/min. This displacement rate corresponded to a tensile surface strain rate of approximately 0.009 s⁻¹ at mid-span. Load and deflection data were collected simultaneously at a sampling rate of 100 Hz. Samples were kept hydrated throughout the bending test by placing a small drop of saline below the beam (on the tensile side) that was held in place via surface tension. The duration of each test was approximately two minutes.
Flexural stress ($\sigma_f$) and strain ($\varepsilon_f$) at mid-span were calculated on the tensile surface from the load/deflection data using the following solutions from linear elastic beam theory:\cite{140,313}:

\[ \sigma_f = \frac{3Ps}{2wd^2} \]  \hspace{1cm} \text{Equation V-1}

\[ \varepsilon_f = \frac{6dD}{s^2} \]  \hspace{1cm} \text{Equation V-2}

where $P$ is the applied load, $s$ is the span length, $w$ is the beam width, $d$ is the beam depth, and $D$ is the beam deflection at mid-span (as measured by the LVDT). The stress-strain data was used in conjunction with a custom Matlab script (R2012a, Mathworks, Natick, MA, USA) to determine the elastic modulus ($E_f$), yield strength ($\sigma_y$), and ultimate strength ($\sigma_{f,\text{max}}$) for each beam. The properties $E_f$ and $\sigma_y$ were computed using an iterative approach, where an initial estimate of $E_f$ was calculated as the slope between two points that were selected manually from the linear portion of the stress-strain data. An initial value for $\sigma_y$ was then calculated using the initial value of $E_f$, along with a 0.2% strain offset method\cite{126}. A new estimate for $E_f$ was selected as the slope between one-third and two-thirds of the previous estimate of $\sigma_y$, and this new $E_f$ value was used to compute a new $\sigma_y$ value using the same offset method. This process was iterated until convergence, which occurred within 10 iterations.

C-3. \textit{In Situ} Fracture Toughness Testing

A subset of 3 longitudinal beams and 3 transverse beams were also prepared for fracture toughness testing. The machined beams were pre-cracked at mid-span on the
bottom surface using an irrigated razor blade notching system coupled to a micrometer that was used to set the appropriate notch depth. The initial notch tip was sharpened by applying a 1-µm diamond solution to the razor blade, resulting in an initial crack length \( (a) \) of approximately 0.35 mm (actual measurement = 0.374 mm ± 0.111 mm). To remove surface flaws, all of the notched samples were hand-polished using progressively finer 800 and 1200 grit silicon carbide (SiC) paper, followed by micro-polishing with a 0.05 µm diamond suspension (Micropolish® B; Beuhler, Lake Bluff, IL, USA).

Prior to testing, the samples were soaked in saline for at least 12 hours to ensure tissue hydration. Notched beams were tested \textit{in situ} in a Hitachi S-4300SE/N (Hitachi America, Pleasanton, CA) variable pressure environmental scanning electron microscope (ESEM) at a vacuum of 35 Pa under three-point bending loading conditions using a Gatan Microtest 2 kN bending stage (Gatan, Abington, UK) equipped with a 150-N load cell. Flexural tests were performed at a displacement rate of 0.55 µm/s, while one side of the sample surface was periodically imaged in electron back-scattering mode to visualize any potential toughening mechanisms during crack growth. Fracture toughness calculations were carried out in general accordance with American Society for Testing and Materials (ASTM) Standard E1820 for single-edge notched bend specimens, which uses elastic-plastic fracture mechanics, or the “\( J \)-integral approach”, to account for the role of plastic deformation\textsuperscript{169}.

As discussed in Chapter I, the two-valued \( J \)-integral method has been suggested as a truer estimate of bone toughness than the single-valued linear elastic fracture mechanics approach because it incorporates the stress intensity associated with both
crack initiation and crack growth prior to fracture\textsuperscript{160,170,171}. The $J$-integral can be determined from the load-displacement curve as the sum of elastic ($J_{el}$) and plastic ($J_{pl}$) components as follows:

$$J = J_{el} + J_{pl}$$  \text{Equation V-3}

$$J_{el} = \frac{K^2}{E/(1 - \nu_{bone}^2)}$$  \text{Equation V-4}

$$J_{pl} = \frac{\eta A_{pl}}{db}$$  \text{Equation V-5}

For the elastic component ($J_{el}$), $E$ is the elastic modulus, $\nu_{bone}$ is Poisson’s ratio ($\approx 0.3$), and $K$ is the linear elastic stress intensity calculated as:

$$K = \frac{P_s}{dw^{3/2}} \times f\left(\frac{a}{w}\right)$$  \text{Equation V-6}

where $P$ is the applied load, $s$ is the span length, $d$ is the beam depth, $w$ is the beam width, $a$ is the crack length, and $f(a/w)$ is a geometry-dependent function provided in ASTM Standard E1820\textsuperscript{160}. For the plastic component ($J_{pl}$), $\eta$ is a geometric factor equal to 1.9 for single-edge notched beams in three-point bending, $A_{pl}$ is the plastic area under the load-displacement curve, and $b$ is the uncracked ligament width (i.e., $b = w - a$).

By convention, the toughness of biological materials such as bone is typically reported in terms of $K$ rather than $J$\textsuperscript{160}. Effective stress intensities were therefore back-calculated using the standard $J$-$K$ equivalence relationship:
\[ K_J = \sqrt{J \times E/(1 - \nu_{\text{bone}}^2)} \]  

Equation V-7

The value of \( K_J \) varies with the beam depth \( d \) unless the following plane strain conditions apply\(^{169}\):

\[ d > \frac{10 \times J}{\sigma_y} \]  

Equation V-8

\[ b_o > \frac{10 \times J}{\sigma_y} \]  

Equation V-9

where \( \sigma_y \) is the yield strength and \( b_o \) is the initial uncracked ligament width. When these conditions are met, \( K_J \) is said to be “size-independent” and can be referred to as the plane strain fracture toughness\(^{314}\). For a given notched beam, the values for \( E \) and \( \sigma_y \) in the preceding discussion were approximated as the mean elastic modulus and yield strength calculated during three-point bending tests of the other un-notched beams from the same specimen. The criteria in Equations V-8 and V-9 are most stringent when \( J \) is high and \( \sigma_y \) is low. Using the maximum \( J \) and minimum \( \sigma_y \) values from the results, it was estimated that a minimum \( d \) and \( b_o \) of 0.5 mm were required for plane strain conditions to apply, which was satisfied by the current setup (i.e, \( d \approx 0.6 \) mm after polishing, \( b_o \approx 0.7 \) mm after notching).

Recalling the discussion from Chapter I, there is typically a period of stable growth after the onset of cracking, when the bone can sustain additional loading and the fracture resistance increases due to toughening mechanisms such as microcracking\(^{156–158}\), crack deflection\(^{83,159–161}\), and uncracked ligament bridging\(^{163,165,167,168}\). It is therefore useful to visualize how \( K_J \) changes as a function of crack extension (\( \Delta a \)) using a crack-
resistance or R-curve\textsuperscript{160}. For each beam orientation, an R-curve was generated by fitting a line to the plot of $K_J$ vs. $\Delta a$, where the y-intercept and slope correspond to the crack-initiation toughness ($K_{J,o}$) and crack-growth toughness ($dK_J/d\Delta a$), respectively.

C-4. SR\mum CT Imaging

After mechanical testing, 21 beams were imaged on the SR\mum CT beamline at the ALS using the following setup: 17 keV photon energy, 50 $\mu$m thick LuAG scintillator, high resolution X-ray microscope setup, 10× lens (Mitutoyo Corporation, Kawasaki, Japan), and the fast camera (pc.o.edge sCMOS camera, PCO-TECH Inc., Romulus, MI). The imaging setup and methods have been described in detail in Chapters III and IV. The final reconstructed 16-bit grayscale datasets were composed of 2160 slices each having $2560 \times 2560$ pixels (imaged pixel size = 0.65 $\mu$m). For each beam, parameters describing bone microstructure (i.e., intracortical vascular porosity, $Ca.V/TV$; osteocyte lacunar density, $N.Lc/TV$) and mineralization (i.e., volumetric tissue mineral density, $\nu TMD$) were calculated within a 0.6 mm$^3$ rectangular prismatic region of interest (ROI). Visual inspection of the SR\mum CT scans revealed that microdamage was present within a region extending approximately 0.25 mm on either side of the fracture plane from mechanical testing. The ROI for microstructural calculations was selected at least 0.5 mm away from this fracture site, thereby excluding any bias introduced from the fracture and its associated microdamage. 3D visualizations of the bone and intracortical porosity spaces were generated in Avizo 7.1 software (Visualization Sciences Group, FEI, Mérignac, France).
C-5. Statistical Analysis

Mechanical properties were compared between the longitudinal and circumferential beams using a linear mixed model analysis with random specimen effect. Simple linear regression analyses were also used to explore possible relationships among the mechanical, microstructural, and mineralization properties for each group (i.e., longitudinal vs. circumferential beam orientation). Power law relationships were investigated between the cortical bone volume fraction \( V_f \) and the flexural properties, as such relationships have often been reported in healthy bone\textsuperscript{301,315–319}. For all analyses, significance was defined as a \( P \)-value of less than 0.05.

D. Results

D-1. Flexural Three-Point Bending Results

Representative load/displacement curves for each beam orientation group are displayed in Figure V-2 (a) below. Longitudinal beams were able to sustain a significantly larger applied load than circumferential beams. As shown in Table V-2, mean anisotropy ratios \( L/C \) for \( E_f \), \( \sigma_y \), and \( \sigma_{f,max} \) were 2.8, 3.0, and 3.1, respectively, for longitudinal vs. circumferential beams.
Table V-2. Mechanical, microstructural, and mineralization properties of OI cortical bone specimens oriented longitudinally or circumferentially with respect to the diaphyseal axis. All values are listed as mean (standard error).

<table>
<thead>
<tr>
<th></th>
<th>Longitudinal orientation, L</th>
<th>Circumferential orientation, C</th>
<th>Anisotropy ratio, L/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flexural mechanical properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastic modulus, $E$ (GPa)</td>
<td>4.4 (0.4)</td>
<td>1.6 (0.4)*</td>
<td>2.8</td>
</tr>
<tr>
<td>Yield strength, $\sigma_y$ (MPa)</td>
<td>61.4 (5.3)</td>
<td>20.8 (6.0)*</td>
<td>3.0</td>
</tr>
<tr>
<td>Ultimate strength, $\sigma_{f,max}$ (MPa)</td>
<td>83.0 (7.8)</td>
<td>26.5 (8.6)*</td>
<td>3.1</td>
</tr>
<tr>
<td>Microstructural and mineralization properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracortical vascular porosity, Ca.V/TV (%)</td>
<td>20.0 (11.1)</td>
<td>21.4 (9.6)</td>
<td>-</td>
</tr>
<tr>
<td>Osteocyte lacunar density, $N.Lc/TV$ (mm$^{-3}$)</td>
<td>35523 (12452)</td>
<td>35812 (14493)</td>
<td>-</td>
</tr>
<tr>
<td>Volumetric tissue mineral density, $vTMD$ (g/cm$^3$)</td>
<td>1.60 (0.14)</td>
<td>1.67 (0.14)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Denotes $P < 0.001$ between longitudinal and circumferential properties.

D-2. Fracture Toughness Results – $R$-Curves

$R$-curves characterizing the growing cracks in both beam orientations are plotted in Figure V-2 (b), which presents the stress intensity ($K_I$) in terms of the stable crack extension $\Delta a$. Results for the two beam orientations showed a similar crack-initiation toughness ($K_{I,o} = 0.5$ MPa·m$^{1/2}$ for both orientations). However, analysis of the slope of
the $R$-curve indicated that longitudinal beams exhibited a near six-fold increase in the crack-growth toughness ($dK_J/dAa; L/C = 5.8$) compared to circumferential beams.

The latter was also visible in real-time ESEM images of the final crack paths after fracture toughness testing (Figure V-3). In longitudinal beams, local crack deflections ran for tens of microns at an angle of approximately 10-60° away from the prevailing growth direction, acting as a toughening mechanism to increase $K_J$. Evidence of additional toughening via energy absorption was also apparent from the formation of microcracks in highly mineralized interstitial bone regions. In contrast, circumferential beams, whose vascular porosity was oriented in the plane of crack growth, displayed minimal deflections and resistance to fracture (Figure V-3 (b)).

### D-3. SRµCT Results

Table V-2 summarizes the microstructural and mineralization properties calculated from SRµCT images. The amount of intracortical vascular porosity, $Ca.V/TV$,
in the OI bone specimens varied between 3-42%, with a mean value of 21% ± 10%. This corresponded to a mean cortical bone volume fraction, $V_f$, of 0.79 ± 0.10. As expected, there was no significant difference in $Ca.V/TV$ between the longitudinal (20% ± 11%) and circumferential (21% ± 10%) beam orientations. Mean values for $N.Lc/TV$ and $vTMD$ were 35661 mm$^{-3}$ ± 13118 mm$^{-3}$ and 1.63 g/cm$^3$ ± 0.14 g/cm$^3$, respectively, and neither parameter differed with beam orientation. Figure V-4 shows representative 3D visualizations of the ROIs within longitudinal and circumferential beams from specimen 9, which was collected from the femoral mid-diaphysis of a 3 year-old female with severe OI type III (donor 6). As expected, the primary orientation of osteons was parallel to the beam axis for longitudinal beams and perpendicular to the beam axis for circumferential ones. Although both displayed high vascular porosity, the longitudinally oriented beam had a much higher ultimate strength than the circumferential one ($\sigma_{f,\text{max}} = 78$ MPa vs. 15 MPa, respectively).

D-4. Relationships Among Mechanical and Microstructural Properties

Table V-3 summarizes the linear correlations between mechanical properties from flexural tests and microstructural/mineralization parameters from SRµCT imaging. For both beam orientations, strong positive linear relationships were observed among $E_f$, $\sigma_y$, and $\sigma_{f,\text{max}}$, with the highest correlation occurring between $\sigma_y$ and $\sigma_{f,\text{max}}$. Highly significant ($P \leq 0.004$), negative correlations were also found between $Ca.V/TV$ and $E_f$, $\sigma_y$, and $\sigma_{f,\text{max}}$ in the longitudinal direction. Similar relationships were not significant ($P > 0.1$) for the circumferential beams.
Figure V-4. 3D tomographic assessment of OI cortical bone. (Top panels) 3D renderings show bone (grayscale) and intracortical vascular porosity (red) for longitudinal (left; \( Ca.V/TV = 16\% \)) and circumferential (right; \( Ca.V/TV = 21\% \)) beams from the femoral mid-diaphysis of a 3 year-old female with severe OI type III (donor 6 – see Table V-1 for additional donor and specimen descriptions). Osteocyte lacunae are visible as small, ellipsoidal spaces. (Bottom panels) Idealized 3D drawings of longitudinal (left) and circumferential (right) beams, along with tomographic slices corresponding to partial cuts through the ROI as indicated by blue dashed boxes. The predominant orientation of osteons is illustrated by black arrows. All scalebars represent 0.5 mm.
Table V-3. Pearson’s correlation coefficients ($R$) and slopes for linear regressions between the mechanical and microstructural/mineralization properties. Bolded values denote statistical significance.

<table>
<thead>
<tr>
<th>Flexural mechanical property</th>
<th>Factor</th>
<th>Slope</th>
<th>$R$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longitudinal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_f$ (GPa)</td>
<td>$Ca.V/TV$ (%)</td>
<td>-0.15</td>
<td>-0.86</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>6.21 E-5</td>
<td>0.40</td>
<td>0.220</td>
<td></td>
</tr>
<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>4.98</td>
<td>0.35</td>
<td>0.285</td>
<td></td>
</tr>
<tr>
<td>$\sigma_y$ (MPa)</td>
<td>$E_f$ (GPa)</td>
<td>13.28</td>
<td>0.95</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>$Ca.V/TV$ (%)</td>
<td>-1.90</td>
<td>-0.78</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>6.37 E-4</td>
<td>0.30</td>
<td>0.377</td>
<td></td>
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<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>49.9</td>
<td>0.25</td>
<td>0.450</td>
<td></td>
</tr>
<tr>
<td>$\sigma_{f,max}$ (MPa)</td>
<td>$E_f$ (GPa)</td>
<td>17.47</td>
<td>0.90</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>$\sigma_y$ (MPa)</td>
<td>1.34</td>
<td>0.97</td>
<td>$&lt; 0.001$</td>
<td></td>
</tr>
<tr>
<td>$Ca.V/TV$ (%)</td>
<td>-2.58</td>
<td>-0.77</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>4.92 E-4</td>
<td>0.16</td>
<td>0.629</td>
<td></td>
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<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>11.0</td>
<td>0.04</td>
<td>0.905</td>
<td></td>
</tr>
<tr>
<td>Circumferential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_f$ (GPa)</td>
<td>$Ca.V/TV$ (%)</td>
<td>-0.06</td>
<td>-0.53</td>
<td>0.110</td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>2.92 E-5</td>
<td>0.38</td>
<td>0.276</td>
<td></td>
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<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>3.68</td>
<td>0.47</td>
<td>0.167</td>
<td></td>
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<tr>
<td>$\sigma_y$ (MPa)</td>
<td>$E_f$ (GPa)</td>
<td>9.29</td>
<td>0.91</td>
<td>$&lt; 0.001$</td>
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<tr>
<td>$Ca.V/TV$ (%)</td>
<td>-0.59</td>
<td>-0.51</td>
<td>0.126</td>
<td></td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>4.83 E-5</td>
<td>0.06</td>
<td>0.864</td>
<td></td>
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<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>16.9</td>
<td>0.21</td>
<td>0.551</td>
<td></td>
</tr>
<tr>
<td>$\sigma_{f,max}$ (MPa)</td>
<td>$E_f$ (GPa)</td>
<td>11.00</td>
<td>0.86</td>
<td>$0.001$</td>
</tr>
<tr>
<td>$\sigma_y$ (MPa)</td>
<td>1.24</td>
<td>0.99</td>
<td>$&lt; 0.001$</td>
<td></td>
</tr>
<tr>
<td>$Ca.V/TV$ (%)</td>
<td>-0.68</td>
<td>-0.47</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>$N.Lc/TV$ (mm$^3$)</td>
<td>-4.51 E-6</td>
<td>0.00</td>
<td>0.990</td>
<td></td>
</tr>
<tr>
<td>$vTMD$ (g/cm$^3$)</td>
<td>14.4</td>
<td>0.15</td>
<td>0.687</td>
<td></td>
</tr>
</tbody>
</table>

None of the mechanical properties were associated with $N.Lc/TV$ ($P \geq 0.22$) or $vTMD$ ($P \geq 0.167$) in either beam orientation. Power law relationships between each longitudinal mechanical property and $V_f$ are also presented in Table V-4. Although $E_f$, $\sigma_y$, and $\sigma_{f,max}$ were all well-associated with $V_f$ using the power law relationships, the greatest fit occurred between $E_f$ and $V_f$ ($R^2 = 0.8$).
Table V-4. Power law relationships between longitudinal mechanical properties and $V_f$.

<table>
<thead>
<tr>
<th>Flexural mechanical property</th>
<th>Power law relationship $\text{Property} = a \times V_f^b$</th>
<th>$a$</th>
<th>$b$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_f$ (GPa)</td>
<td>$E_f = a \times V_f^b$</td>
<td>8.67</td>
<td>2.93</td>
<td>0.80</td>
</tr>
<tr>
<td>$\sigma_y$ (MPa)</td>
<td>$\sigma_y = a \times V_f^b$</td>
<td>117.4</td>
<td>2.76</td>
<td>0.68</td>
</tr>
<tr>
<td>$\sigma_{f,\text{max}}$ (MPa)</td>
<td>$\sigma_{f,\text{max}} = a \times V_f^b$</td>
<td>162.1</td>
<td>2.83</td>
<td>0.66</td>
</tr>
</tbody>
</table>

E. Discussion

The purpose of this study was to examine the isotropy of pediatric OI cortical bone in terms of its flexural mechanical properties (i.e., $E_f$, $\sigma_y$, $\sigma_{f,\text{max}}$, $K_{J,0}$, and $dK_J/d\Delta a$). Building upon previous efforts\textsuperscript{19,128,181,269}, this study presents the first data describing the strength and fracture toughness of bone tissue in young individuals with OI. Similar to healthy bone\textsuperscript{146,188,320–323}, OI cortical bone exhibits anisotropic material behavior including increased elastic modulus, yield strength, ultimate strength, and crack-growth toughness in the longitudinal vs. circumferential direction. In contrast with healthy bone, SRµCT imaging revealed abnormally high vascular porosity in OI bone within regions that are normally occupied by dense cortical bone. Moreover, there was also a strong negative relationship between $\text{Ca.V/TV}$ and $E_f$, $\sigma_y$, $\sigma_{f,\text{max}}$ in these specimens.

The mean results for $E_f$ (i.e., 1.6-4.4 GPa) are lower than those reported in Chapter II, as well as in other nanoindentation studies (i.e., 13-25 GPa) on specimens from patients with mild to severe OI\textsuperscript{20,54–56,181}. This observation is not surprising, since nanoindentation tests are performed at a much smaller scale, within lamellar bone regions only, and excluding the effects of void spaces such as Haversian canals, Volkmann’s canals, and resorption cavities. Nanoindentation also requires multiple tissue processing steps including dehydration, fixation, and impregnation with embedding media.
Dehydration of bone samples has been shown to increase modulus measurements determined by nanoindentation\textsuperscript{185,187,198}. Results from one of the aforementioned nanoindentation studies suggested that OI bone has more isotropic mechanical properties than normal tissue, which is transversely isotropic\textsuperscript{54,140,146,173–175}. However, the current work indicated clear anisotropy in machined mini-beams, with longitudinal mechanical properties being three to six times larger than circumferential properties. This observation points to the role of bone microstructure, and specifically vascular porosity orientation, in the anisotropic mechanical behavior of OI bone.

Strong positive linear relationships were noted between $E_f$ and both strength measures. This observation is similar to previous studies on both humans\textsuperscript{62} and other species\textsuperscript{324}. To the author’s knowledge, the current study is the first to characterize bone tissue strength in individuals with OI. In general, current values for modulus (0.2-8.5 GPa), yield strength (4-118 MPa), and ultimate strength (6-176 MPa) were on the low end of the range of values previously reported for healthy pediatric bone undergoing either tensile or flexural loading (i.e., 1-32 GPa for $E$, 31-150 MPa for $\sigma_y$, and 35-207 MPa for $\sigma_{\text{max}}$)\textsuperscript{57–62}. Interestingly, the two longitudinal OI beams having the lowest porosity (i.e., 3% and 8%) had comparable mechanical properties to the abovementioned healthy ranges. This finding supports the idea that the overall reduced mechanical properties seen in OI specimens are likely related to the high mean degree of vascular porosity\textsuperscript{291,292,301,325}.

Bending tests are a common tool in characterizing the material properties of bone samples\textsuperscript{10,11,19,58,142,212,213,322}, and they are often more practical when specimen size is
limited. Despite their popularity, there are noteworthy limitations associated with three-point bending tests. For example, the bending moment ($M$) is not uniform but varies linearly with the distance from the lower supports (Figure V-5). As a result, the peak moment occurs at mid-span in the plane of the loading nose, and the stress distribution varies linearly from zero at the neutral axis to a maximum value at the outer surface. Bone is stronger in compression than in tension, so fracture initiation is more likely to occur on the bottom (i.e., tensile) surface of the beam at mid-span (Figure V-5). In contrast, for tensile testing the stress distribution is more uniform and fracture may initiate within regions of relative weakness including void spaces and/or around pre-existing micro-damage. Bending tests also rely on beam theory, which assumes linear elastic material behavior and negligible shear deformation, to calculate strains within the specimen. In materials such as bone that exhibit post-yield deformation, this assumption is not valid beyond the yield point and results in an overestimation of $\sigma_{f,max}$ compared with values obtained from a tensile test.

According to ASTM standard C674, the minimum recommended span length (e.g., distance between lower supports) to beam depth ($s/d$) ratio for three-point bending tests is 8 when determining the flexural properties of ceramic specimens having a rectangular cross-section. For bone, $E_f$ results have been reported to decrease with specimen widths and depths less than approximately 500 µm, and also for $s/d$ ratios less than 15. For the current study, a span of 4 mm was chosen to accommodate the smallest osteotomy specimens. To meet the $s/d$ ratio specifications outlined above, this span would require a beam depth of 267-500 µm. Based in part on the work by Choi et al., a beam depth of approximately 650 µm (actual measurement 668 µm ± 60 µm) was
selected. A preliminary validation study using similar beam dimensions resulted in errors for \( E_f \) of between 3-13% for acrylic and bovine bone\(^{19,325,330}\).

![Diagram of three-point bending test](image)

**Figure V-5. Shear and moment diagrams for three-point bending test.** The applied load \( (P) \) results in reaction forces \((P/2)\) at both supports. The neutral axis of the cross-section extends into the page along the dash-dotted line. The neutral axis experiences no bending stresses (or strains) and serves as a transition between compression and tension within the beam. The vertical shear force \((V)\) can be plotted along the span length \((s)\), where the point loads cause sudden changes in \(V\). Moving from left to right, the cumulative area under \(V\) can be used to determine the bending moment \((M)\). Thus, positive shear forces to the left of \(P\) cause the moment to increase, while negative shear forces to the right of \(P\) cause the moment to decrease.

These results suggest that the relatively low \(s/d\) ratio of 6.2 may have introduced errors due to shear deformation in the specimens\(^{329}\). It is possible to estimate the effect of this error using a branch of mechanics called Timoshenko beam theory\(^{331}\). For three-
point bending, the deformation measured at mid-span \( (D_{total}) \) can be broken down into the sum of deformations due to bending \( (D_{bend}) \) and shear \( (D_{shear}) \) as follows:

\[
D_{total} = D_{bend} + D_{shear}
\]

Equation V-10

The Timoshenko elastic modulus \( (E_T) \) can be determined for a beam with a rectangular cross-section using the following equations:

\[
E_T = \frac{P s^3}{4 w d^3} \times \frac{1}{D_{total} - D_{shear}}
\]

Equation V-11

\[
D_{shear} = \frac{P s}{4 w d G T}
\]

Equation V-12

where \( P \) is the applied load, \( s \) is the span length, \( w \) is the beam width, \( d \) is the beam depth, \( G \) is the shear modulus (i.e., the shear equivalent of elastic modulus), and \( T \) is the Timoshenko shear correction factor \( (T = 0.833 \) for a rectangular cross-section\)\(^{332,333} \). The value of \( G \) can be estimated as:

\[
G = \frac{\bar{E}}{2(1 + \nu_{bone})}
\]

Equation V-13

where \( \bar{E} \) is the mean elastic modulus for a given beam orientation in Table V-2, and \( \nu_{bone} \) is again Poisson’s ratio \( (\approx 0.3) \). Using these equations, \( E_T \) was determined for a representative longitudinal and circumferential beam, and the results were compared to the respective uncorrected \( E_f \) values. These calculations revealed that the uncorrected \( E_f \) values underestimated the true elastic modulus by 10% \( (\approx 0.5 \text{ GPa}) \) in the longitudinal beam and 14% \( (\approx 0.3 \text{ GPa}) \) in the circumferential one.
Moreover, one can also compare the maximum bending stress \( \sigma_{\text{max}} \), which occurs on the outer surface at mid-span, to the maximum shear stress \( \tau_{\text{max}} \), which occurs at the neutral axis, using the following relationships from beam theory\textsuperscript{140,313}:

\[
\sigma_{\text{max}} = \frac{6M_{\text{max}}}{wd^2} = \frac{3Ps}{2wd^2} \quad \text{Equation V-14}
\]

\[
\tau_{\text{max}} = \frac{3V_{\text{max}}}{2wd} = \frac{3P}{4wd} \quad \text{Equation V-15}
\]

where \( M_{\text{max}} \) is the maximum bending moment and \( V_{\text{max}} \) is the maximum vertical shear force on the cross-section (Figure V-5). Applying these equations to the same example beams from above, the ratios of \( \sigma_{\text{max}} \) to \( \tau_{\text{max}} \) were approximately 13 and 11 in the longitudinal and circumferential beam orientations, respectively. Thus, despite a relatively small \( s/d \) ratio of 6.2, the shear stresses remain small with respect to the stresses due to bending. Nevertheless, a corrected ultimate stress \( \sigma_T \) can be calculated from Timoshenko beam theory\textsuperscript{332} according to:

\[
\sigma_T = \frac{3Ps}{2d^2} - 0.266 \frac{2P}{d} \quad \text{Equation V-16}
\]

Using the same beams from before, \( \sigma_T \) was 2\% (or \( \approx 1 \text{ MPa} \)) higher than \( \sigma_{f,\text{max}} \) for the longitudinal beam and 4\% (or \( \approx 1 \text{ MPa} \)) lower than \( \sigma_{f,\text{max}} \) for the circumferential beam. Notice that the absolute differences between elastic moduli (i.e., \( E_T - E_f \)) and ultimate strengths (i.e., \( \sigma_T - \sigma_{f,\text{max}} \)) when accounting for the effect of shear deformation are generally well-within the standard errors for each property (Table V-2).
Using SRµCT, substantial and heterogeneous intracortical vascular porosity was observed in the OI bone beams ($Ca.V/TV = 3-42\%$). In most cases, the amount of vascular porosity was drastically higher than normal values reported for children and young adults (e.g., $Ca.V/TV = 3-7\%$)\textsuperscript{222,269,291–293,295}. It is unlikely that this calculation was significantly affected by small microstructural flaws (e.g., microdamage). As mentioned previously, porosity was measured in the beams at a minimum distance of 0.5 mm away from the damage region surrounding the fracture plane.

Moreover, microcracks in cortical bone typically account for less than 0.5\% of the total bone volume and have an ellipsoidal 3D shape, with a length and width on the order of 100 µm and a thickness of approximately 1 µm\textsuperscript{153,334–336}. Animal models representing OI (i.e., Mov13, oim/+, and Brtl/+ mice) suggest that OI cortical bone may have an increased susceptibility to microdamage accumulation compared to healthy tissue\textsuperscript{177–179}. Indeed, ESEM images during fracture toughness tests revealed microcracking in interstitial bone regions, which have a higher local degree of mineralization. Similar to other recent studies, a minimum size threshold of approximately 2000 µm was used to eliminate microdamage and other artifacts when segmenting vascular porosity\textsuperscript{135–137,274}.

Low overall bone mass is a hallmark of OI and has been noted previously in both cortical and trabecular bone at a variety of skeletal sites including the hip, pelvis, and long bones\textsuperscript{21,127,183,298,310,337,338}. Diaphyseal long bone regions are well-suited for studying secondary remodeling and lamellar organization in OI cortical bone. Indeed, a recent SEM study of femoral and tibial osteotomies observed a higher percentage of non-ossified vascular/resorption area in OI bone compared to controls\textsuperscript{298}. The same study
also reported abnormal cortical remodeling characterized by wide, flattened resorption spaces formed by “drifting osteons”.

Relationships were also examined among the flexural mechanical properties and microstructural parameters assessed via SRµCT such as Ca.V/TV, Vf, N.Lc/TV, and vTMD. Similar to previous studies, significant linear and power law relationships were observed between the longitudinal material properties and bone porosity (i.e., Ca.V/TV and Vf)\textsuperscript{145,301,315–319}. These relationships are usually presented as “property = a × Vf\textsuperscript{b}”, with b ranging between 2 and 3. In the current study, longitudinal bone properties followed a similar power law with b exponents of 2.93, 2.76, and 2.83 for E\textsubscript{f}, \sigma\textsubscript{y}, and \sigma\textsubscript{f,max}, respectively. These relationships indicate that high intracortical porosity (i.e., low Vf) may be an important contributor to the increased bone fragility of young individuals with OI. Indeed, large porous regions within the cortex likely act as local stress-risers that cause fracture to occur at lower levels of nominal stress\textsuperscript{148}.

Although the crack-initiation toughness (\(K_{I,o}\)) was similar for both specimen orientations, the crack-growth toughness (\(dK_{I}/d\Delta a\)) of longitudinal beams was nearly six times higher than in the circumferential orientation. Local toughening via small crack deflections and interstitial microcracking was observed in longitudinal beams, but not in circumferential ones. A recent murine study on severe OI reported similar fracture patterns for bone tested in the longitudinal orientation\textsuperscript{176}. Other studies on bone pathology and aging have reported reduced fracture toughness (both \(K_{I,o}\) and \(dK_{I}/d\Delta a\)) that has been attributed to changes in the osteonal pore network such as increased density and/or diameter of canals\textsuperscript{83,172}. Future SRµCT work is needed to examine whether other
toughening mechanisms are present in 3D for OI cortical bone. For example, uncracked ligament bridging has been reported to be the dominant toughening mechanism in healthy bone\textsuperscript{153,160,162–168}, whereby small load-bearing segments help to reduce the driving force for further cracking.

The density of osteocyte lacunae ($N.Lc/TV$) was not significantly associated with bone mechanical properties in either beam orientation. A wide range of values has been reported for $N.Lc/TV$ owing to a number of experimental factors including donor age, local anatomical sampling site, image resolution, and segmentation strategy\textsuperscript{135–138,275,303,304}. Inter-study comparison of $N.Lc/TV$ calculations is therefore difficult; nevertheless our results are within the range previously observed in young adult bone (e.g., 23,000-90,000 lacunae/mm$^3$)\textsuperscript{136–138}. One recent SRµCT study on the oim mouse model noted a significant increase in lacunar density compared to wild-type controls\textsuperscript{300}. Cracks propagate freely through osteocyte lacunae\textsuperscript{153,154}, so changes in the density or geometry of lacunae could contribute to bone fragility in OI. Osteocyte lacunae are one of a number of structural features (e.g., osteocyte canaliculi, vasculature, muscle insertions, etc.) that can act as potential sites of localized stress concentration and microdamage accumulation\textsuperscript{148–154}. Thus the absence of a relationship between $N.Lc/TV$ and the flexural properties in the current study suggests a deemphasized role of osteocyte lacunae in the mechanical properties of OI cortical bone.

The 12 specimens included in this study were donated by a group of 9 children and adolescents who were diagnosed with phenotypes ranging from mild to severe OI. All bone samples were collected as a byproduct of corrective intramedullary rodding.
procedures, however a third of these were also in the vicinity of a recent fracture. Namely, specimens 1 and 2 came from a combination rodding and fracture repair surgery. Specimen 3 was near a site of fracture non-union, while specimen 5 was close to a healed fracture site. It is therefore possible that residual microdamage accumulation near the fracture sites may have compromised the mechanical properties in these 4 specimens. Nevertheless, visual inspection confirmed that there was no apparent fracture callus within these specimens, and further manual examination of the high resolution SRµCT images did not reveal the presence of microcracks aside from those produced near the fracture plane of the three-point bending tests. Indeed, longitudinal flexural properties from specimens 2 and 3 were among the highest of all beams tested.

In the current study, all donors except two had previously been treated with bisphosphonates anti-resorptive therapy, and most received multiple rounds of treatment. Thus, it was not possible to assess the treatment effect of bisphosphonates on the flexural mechanical properties. Moreover, it is unlikely that there was an association between the high vascular porosity in the current OI specimens and these drugs, as other researchers have noted reduced cortical porosity in postmenopausal and osteoporotic women$^{339,340}$. Recent work has suggested that long-term bisphosphonates treatment may be associated with an increased risk of developing osteonecrosis in the jaw$^{111,112}$, as well as atypical femoral fractures$^{113,114}$. Thus, it is possible that a prior history of treatment may have contributed to the reduced material properties observed in the current study. Future work is warranted to investigate this potentially undesirable effect.
F. Conclusions

This study examined the mechanical, microstructural, and compositional properties in cortical bone specimens from children and adolescents diagnosed with mild to severe OI. Flexural bone mechanical properties including elastic modulus, yield strength, ultimate strength, and crack-growth toughness were found to be anisotropic and greater along the longitudinal diaphyseal axis than in the circumferential orientation. Similar to Chapter IV, high intracortical vascular porosity was noted in OI bone. This porosity was strongly and negatively correlated with mechanical behavior in the longitudinal direction. These observations indicate that the presence of elevated vascular porosity may be an important contributor to OI bone fragility.
VI. SUMMARY AND CONCLUSIONS

A. Summary

Osteogenesis imperfecta (OI) is a complex, genetic disorder of bone fragility. OI is often accompanied by varying degrees of long bone deformity that can require surgical intervention as a normal part of clinical care. Small tissue specimens retrieved from these corrective osteotomy procedures are an invaluable asset in evaluating the biomechanical properties of OI cortical bone. A detailed understanding of these properties is vital in the development of potential treatment strategies, as well as in the assessment of fracture risk during various activities. However, to date there is very little data describing OI cortical bone mechanical properties and microstructure in humans. Histomorphometric and clinical bone density measurements are convenient for tracking basic OI properties over time, but they offer limited information on the distribution and quality of bone material. Other lab-based techniques such as materials testing and 3D high resolution imaging are well-suited for evaluating OI properties at its many hierarchical levels.

The purpose of this dissertation was to investigate the mechanical properties, microstructure, and mineral characteristics of diaphyseal long bone in children and adolescents with OI. The first part of the study (Chapter II) used nanoindentation to evaluate material-level differences in elastic modulus and hardness in the longitudinal direction (i.e., parallel to the primary long bone axis) for mild vs. severe OI. The second part of the study (Chapter III) outlined the synchrotron radiation micro-computed tomography (SRμCT) program at the Advanced Light Source (ALS), suggesting a set of
setup and image processing parameters for bone microstructural analysis based on empirical observations. The third part of the study (Chapter IV) analyzed the structure of vascular pores and bone cellular spaces in OI, as well as in healthy bone populations. Because elevated bone fragility is often attributed to changes in the total amount and local degree of mineralization, the above SRµCT system was also used to examine volumetric bone mineral density and tissue mineral density. Finally, the last part of the study (Chapter V) combined mechanical testing (i.e., three-point flexural testing and notched fracture toughness testing) and imaging (i.e., SRµCT and environmental scanning electron microscopy (ESEM)) to evaluate the anisotropy of OI cortical bone oriented parallel vs. perpendicular to the primary long bone axis.

B. Key Findings

On the basis of the mechanical testing and imaging results presented in this dissertation, there is significant evidence to support both hypotheses outlined in Chapter I. Namely, nanoindentation (Chapter II) and three-point bending (Chapter V) studies showed that bone mechanical properties such as elastic modulus, flexural yield strength, ultimate strength, and crack-growth toughness vary with OI clinical severity and local microstructure (Hypothesis 1). Moreover, SRµCT imaging methods developed specifically for bone (Chapter III) revealed that there are significant changes in bone density for OI vs. healthy tissue, with accompanying alterations in the degree and morphology of vascular pores and cellular spaces (Chapter IV; Hypothesis 2).

Chapter II is the first study to compare nanoindentation properties in patients with mild OI type I and severe OI type III. Previous indentation work has reported a lower
longitudinal elastic modulus and hardness for healthy vs. severe OI cortical bone\textsuperscript{20}. Based on this finding, one might suspect the elastic modulus and hardness of mild OI type I bone to fall between that of healthy and severe OI cortical bone. However, this was not the case in the current study, as both properties were 7-8\% greater in mild vs. severe OI.

It has been speculated that nanoindentation modulus and hardness are directly related to the degree of local mineralization\textsuperscript{205–208}. Previous quantitative backscattering imaging (qBSEM) work has noted hypermineralization in OI bone compared to controls, yet the highest levels were seen in the more severe phenotypes\textsuperscript{183,184}. Other characteristics of the mineral (e.g., crystal size, shape, packing density, etc.)\textsuperscript{4,6,38,80,209} and the collagen fibril (e.g., fibrillar diameter, orientation, cross-linking, etc.)\textsuperscript{76–79,81,82,176,210} have also been shown to be affected in OI. Indeed recent simulation studies on individual collagen molecules have suggested an inverse relationship between the elastic modulus and the severity of the mutation\textsuperscript{81,82}. There is likely some combination of effects occurring in OI such that the increased mineralization of the matrix causes increases in elastic modulus and hardness compared to controls, while differences in the collagen environment reduce these mechanical properties in OI type III vs. I. Future work is warranted to determine what relationships, if any, exist between the small-scale fibrillar/mineral characteristics and the mechanical properties of the various OI types.

Chapter II is also the first study to investigate the spatial distribution of indentation properties in OI cortical bone. Previous work in healthy bone has reported 8-25\% higher elastic modulus and hardness values for interstitial vs. osteonal bone, where
the former has an older tissue age and thus a higher degree of mineralization\textsuperscript{165–168,208,212,214,215}. The current study found an 11-13\% increase in longitudinal elastic modulus and hardness for interstitial vs. osteonal regions within OI bone, suggesting that mineralization kinetics are affected similarly in both regions. In this way, spatial differences in nanoindentation modulus and hardness are maintained despite a net increase in matrix mineralization. Future combined nanoindentation and qBSEM work is needed to confirm this phenomenon around indents in regions of varying tissue age.

Chapter III described the SR\(\mu\)CT beamline at the ALS, and introduced a robust Fourier Ring Correlation (FRC) method for quantifying 2D and 3D resolution in X-ray tomographic images. Using a variety of common setup parameters, it was shown that increasing the number of projections and the camera exposure time can improve image resolution by three-fold and reduce noise by up to 30\%. These trends were consistent with results seen in cone-beam CT\textsuperscript{265–267}. One of the main advantages of SR\(\mu\)CT is that it can achieve significant improvements in spatial and compositional resolution compared to clinical and lab-based \(\mu\)CT. However, due to constant upgrades in system components, it is imperative to continuously monitor resolution to ensure image quality and data reliability across multiple user shifts. Strategies such as the FRC method should be integrated into the data processing and reconstruction process, so that users can receive real-time feedback on their experiments. This idea could be increasingly important as the ALS shifts towards a more automated data collection, processing, and retrieval pipeline through supercomputing initiatives such as the National Energy Research Scientific Computing Center (NERSC).
Chapter IV is the first study to quantify and compare the characteristics of the vascular and lacunar pore spaces in OI, healthy pediatric, and healthy adult cortical bone. Using the SRµCT system described in Chapter III, it was shown that vascular pores account for over 25% of the total tissue volume in OI cortical bone. This value was approximately six times higher than that of the healthy populations. The mean diameter of the canals was also three to five times larger in OI bone compared to healthy pediatric and adult populations, respectively. These findings likely speak to an imbalance or alteration in the normal bone remodeling process in children with OI. Indeed, prior biochemical immunoassay studies have revealed elevated bone turnover markers in OI.

Further evidence of abnormal OI cortical bone metabolism was observed in Chapter IV via osteocyte lacunar results. The current study showed a 60% increase in the density of osteocyte lacunae for a given tissue volume in OI cortical bone compared to healthy populations. The difference was even starker when normalized for the effect of vascular porosity. Incredibly, for the same volume of bone material, there were nearly twice as many osteocyte lacunae in OI vs. healthy bone. Previous studies have suggested that osteocytes may impose an inhibitory signal on osteoblasts, which in turn reduces the rate of infilling during osteon formation and increases the diameter of Haversian canals. Indeed, prior OI work has shown a decreased mineral formation rate despite increased bone turnover. This behavior could explain, at least in part, the elevated vascular porosity and canal diameter seen in OI cortical bone.
The physiological purpose of high osteocyte density in OI cortical bone is a key area for future work, and could be related to the mechanosensory role of these cells\textsuperscript{343,344}. It is possible that a high initial density of osteocytes, combined with additional recruitment of osteoblasts into the osteocyte lineage, could aid in detecting microdamage in the weakened OI bone material. Finally, osteocyte morphology is thought to adapt according to mechanical loading\textsuperscript{136,274,300}. Thus the more spherically shaped OI lacunae found in the current study likely reflect altered stress/strain patterns within the tissue.

Chapter V is the first study to investigate the flexural properties of human OI cortical bone. On the basis of one nanoindentation study, it had been assumed that bone mechanical properties such as elastic modulus, yield strength, and ultimate strength are more isotropic in OI than in healthy bone\textsuperscript{54}. However, it is known that microstructural characteristics such as vascular porosity (which indentation experiments largely neglect) and mineral content have a strong effect on the above mechanical properties in normal bone\textsuperscript{145,301,315–319}. The current three-point bending study found that the elastic modulus, yield strength, and ultimate strength of young OI cortical bone are nearly three times higher in the longitudinal vs. circumferential orientation. Additional toughness testing of notched samples revealed a six-fold increase in the crack-growth toughness for the longitudinal vs. circumferential orientation.

Subsequent SR\mu CT imaging experiments in Chapter V showed that the above mechanical properties of OI bone are highly dependent on the amount and orientation of vascular porosity. Indeed, significant correlations were reported between elastic modulus, yield strength, ultimate strength, and the vascular porosity in the longitudinal
direction. Similarly, ESEM images collected during fracture toughness tests showed evidence of energy dissipating “toughening mechanisms” such as crack deflection and interstitial microcracking in the longitudinal direction. Such behavior was not observed in the circumferential direction, where the main orientation of vascular pores is perpendicular to the line of action of the bending stress, thereby generating large stress concentrations that severely weaken the bone. Additional work is needed to determine how these stress concentrations, which should be more pronounced in OI cortical bone, affect the flexural properties compared to healthy bone.

C. Future Directions

There may be a clinical shift away from corrective osteotomy procedures in OI patients with limb deformity. Furthermore, even with the improved resolution of peripheral clinical CT scanners (e.g., HR-pQCT), there are obvious radiation dosage concerns, making human studies on OI cortical bone biomechanics increasingly difficult. Thus, there is a need for better clinical diagnostic tools to evaluate OI cortical bone quality. One simple but elegant approach has recently been introduced based on microindentation technology. The instrument monitors the progressive increase in penetration depth experienced by an indenter tip during cyclic testing. The diameter of the tip is small enough (i.e., on the order of a normal hypodermic needle) to be applied through the skin after application of a local anesthetic. However, the resulting indentations are also large enough to generate small cracks or microfractures that can be used to evaluate fracture resistance.
Previous nanoindentation techniques have already been used to evaluate fracture toughness in bone biopsies embedded in epoxy resin\textsuperscript{214,351,352}. However, the current microindentation device may be more clinically relevant because it can be safely administered \textit{in vivo} without requiring a biopsy or causing pain (other than the minimal discomfort of local anesthesia) to the patient. This device is already being used to identify differences in bone quality between healthy individuals and those suffering from hip fractures\textsuperscript{346}, atypical femoral fractures\textsuperscript{109}, and type II diabetes\textsuperscript{347}. Similar minimally invasive tools have great potential in OI, where they could be used for routine clinical assessment, surgical planning/evaluation, and fracture risk prediction.

The cracks caused by the indentation device have been shown to display similar characteristics in 2D to those measured via traditional three-point bending experiments (e.g., Chapter V)\textsuperscript{346}. It is therefore possible that the 3D structure of such microfractures could shed additional light on microdamage accumulation in bone. To this end, a recent pilot study using this technique was performed on the anterior mid-diaphyseal region of a cadaveric tibia, and the resulting indents were analyzed using SR\textmu CT at the ALS. Interestingly, microdamage was clearly visible in 3D around the indentation wake (Figure VI-1), and the amount of microcracking was correlated with the mechanical properties\textsuperscript{353}. Additional preclinical studies (e.g., in animal models) have the potential to uncover differences in damage accumulation in OI and evaluate new treatments for the disorder.
Figure VI-1. SRμCT images of a new minimally invasive bone diagnostic tool. Multiple views of a microindentation (blue) on the native bone surface of a human tibia show microdamage accumulation (green) within the indent wake. Vascular canals (red) are also shown for reference.
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