

MECHANICS OF CORTICAL BONE

Exploring the Micro- and Nano-scale

by

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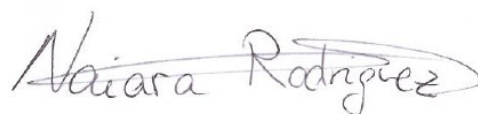
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This thesis was carried out between October 2011 and January 2015 under the supervision of Dr Sandra J. Shefelbine in the Department of Bioengineering at Imperial College London (UK). I was funded by the Basque Government's Predoctoral Scholarship "Doktoretza-aurreko Programa. PREDOC." (Spain).

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ABSTRACT

This PhD aimed at characterising mechanical, structural and compositional properties of cortical bone at the micro- and nano-scale employing various techniques applied to mouse models of ageing and disease.

Nanoindentation was used to probe bone at the micro-scale. Nanoindentation properties of the same control mouse bones were measured using a range of preparation, testing and analysis options. This was the first time that such systematic study was conducted. It was concluded that although the measured values were influenced by experimental and analysis choices, nanoindentation was capable of capturing relative trends in bone's mechanical properties.

Nanoindentation was then coupled to a poroelastic approach to measure age-related changes in mouse bone permeability. Permeability is key to understanding fluid flow in bone, which may indicate how bone cells sense changes in the mechanical environment. These first permeability measurements in mouse bone demonstrated that the permeability caused by fluid flowing through bone's lacunar-canalicular porosity decreases with age.

Porosity is expected to also affect bone's ability to resist fracture at the micro-scale. The influence of intra-cortical porosity on crack propagation was explored via extended finite element methods. A novel technique was suggested to propagate cracks through holes and applied to 2D models of the porosity of osteogenesis imperfecta mouse bone. Results showed that vascular canals affect crack propagation and might contribute to the brittleness of osteogenesis imperfecta bone.

Skeletal pathologies often cause alterations in bone's building blocks leading to deteriorated whole-bone toughness. Mineral properties of brittle and ductile mouse bone (models of osteogenesis imperfecta and rickets respectively) were evaluated. Results revealed that deviations in size, composition and organisation of bone mineral reduce bone's mechanical integrity both in brittle and ductile pathologic bone.

The outcomes of this thesis provide a deeper understanding of bone material, which is required for future improvements in treatments for skeletal diseases.

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NOMENCLATURE AND ABBREVIATIONS

NOMENCLATURE

a	contact radius
a_0	length of initial notch
A_c	contact area
c	diffusivity coefficient
C_i	creep function coefficients
Ca/P	Calcium to Phosphorous ratio
D	damage
E	Young's modulus
E_R	reduced modulus
E'	plane strain modulus
f	extent of viscosity (G_∞ / G_0)
G	shear modulus
G_c	fracture energy
G_0	zero-time shear modulus
G_∞	equilibrium shear modulus
h	displacement
h_e	elastic displacement
$h_{\max}$	maximum displacement
h_p	plastic displacement
h_v	viscous displacement
h_0	displacement in step conditions
h_∞	displacement in equilibrium
h^*	normalised displacement

H_{VEP}	hardness, resistance to plastic deformation
H_c	contact hardness, resistance to total deformation
J	creep function
k	intrinsic permeability
κ	Darcy's hydraulic permeability
K	bulk modulus
K_c	stress intensity factor
P	load
P_{max}	peak load
R	radius
S	stiffness
t	time
t_c	creep hold time
t_R	rise time
t^*	normalised time
α	Biot effective stress coefficient
$\alpha_1, \alpha_2, \alpha_3$	dimensionless geometry constant
δ	applied displacement
ϵ_{max}	maximum principal strain
η_Q	indentation viscosity
μ	dynamic viscosity
ν	Poisson's ratio
ν_u	undrained Poisson's ratio
τ_i	viscous-elastic time constant
σ_{max}	maximum principal stress
+/+	wild-type control
-/-	pathologic

ABBREVIATIONS

BSE SEM	Backscattered electrons scanning electron microscope
B6	C57BL/6 mouse
CT	Computed tomography
CZM	Cohesive zone model
FE	Finite element
FTIR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
OI	Osteogenesis imperfecta
Oim	Osteogenesis imperfecta mouse
OP	Oliver and Pharr analysis
PBS	Phosphate buffered saline
PMMA	Polymerised methyl methacrylate
R-curve	Crack growth resistance curve
SAXS	Small angle x-ray scattering
TCP	Tricalcium Phosphate
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
VEP	Viscoelastic-plastic analysis
VE	Viscoelastic analysis
Vol%	Volume percentage
XFEM	Extended finite element method
XRD	X-ray diffraction
2/3D	Two/three dimensional
3-pt bending	Three-point bending

1 INTRODUCTION AND OBJECTIVES

Bone is a hierarchical material. As such, the organisation of its constituents determines the mechanical properties of the whole bone. Bone's building blocks consist of organic matter (mainly collagen), mineral (hydroxyapatite), and water. Because of its highly hierarchical composite nature, bone exhibits a remarkable combination of mechanical properties, in particular, high strength and toughness. In addition, healthy bone has an exceptional ability to adapt its mass, shape and properties to changes in its mechanical environment.

However, aging and skeletal pathologies cause alterations in bone at different hierarchical levels and lead to the deterioration of bone's overall mechanical competence. Bone density and micro-architecture degrade with age, making bones weaker and more prone to fracture (Boyde and Kingsmill, 1998; Ferguson et al., 2003; McCalden et al., 1993; Zimmermann et al., 2011). In addition, there are skeletal pathologies which have a dramatic effect on whole-bone mechanics. Osteogenesis imperfecta (OI, brittle bone disease) is a genetic disorder of the collagen (molecular defect) that makes bones weak and very fragile (Shapiro et al., 2013). The Brittle Bone Society estimates that 1 person in 15,000 has OI in the UK, and there is no cure for this disease (<http://www.brittlebone.org/>).

The increased fracture risk associated with aged or OI bone was traditionally attributed to low bone mass. However, it has been shown that the increased risk of fracture cannot be fully explained by the decrease in bone mass, and that bone material, structural and compositional properties at different length-scales contribute to the degradation of whole-bone mechanics (Grynpas, 1993; Hui et al., 1988; Manolagas and Parfitt, 2010; McCalden et al., 1993; Ritchie et al., 2006). However, much is still unknown about how changes in the properties at the micro- and nano-scale relate to bone's mechanical integrity at the macro-scale.

The goal of this thesis is to characterise cortical bone at the micro- and nano-scale by combining experimental and computational techniques applied to mouse models of ageing and disease.

A full understanding of bone mechanics is essential to develop treatments for skeletal diseases. In addition, this might also contribute to a better design of implants and replacement materials.

1.1 Hypotheses and Thesis Outline

The comprehension of bone mechanics requires the interpretation of bone at multiple hierarchical levels. *Chapter 2* provides an introduction to cortical bone, its structure and mechanics, with special interest in mouse bone. The following chapters explore experimental and computational techniques to improve the understanding of bone mechanics.

Recent advances have enabled the adaptation of techniques previously used in Material Science to probe bone at different length-scales. One of these techniques is nanoindentation, which is widely used to measure micro-scale mechanical properties of bone. Since the pioneering work of Oliver and Pharr in 1992 (Oliver and Pharr, 1992), various approaches have been suggested to determine bone's elastic, plastic and viscous properties from nanoindentation data. However, the values of these properties vary widely among studies. Although efforts have been made to determine the influence of the sample preparation (Bembey et al., 2006a; Bushby et al., 2004), testing parameters (Paietta et al., 2011; Wu et al., 2011), or analysis models (Olesiak et al., 2009; Oyen and Cook, 2009), the reasons of the big discrepancy in nanoindentation values remain to be elucidated.

- I) The first hypothesis of this thesis is that, in nanoindentation, the chosen preparation, testing, and analysis protocols affect the measured material properties of bone.**

This hypothesis is tested in *Chapter 3* by evaluating and comparing the nanoindentation properties of the same healthy bones using a wide range of preparation, testing and analysis options.

Once a better understanding of nanoindentation in bone is achieved, the potential of this technique can be further exploited by analysing bone's poroelastic behaviour (Oyen, 2008), caused by fluid flowing through its porous network. In particular, interstitial fluid flow through bone's lacunar-canalicular network is thought to play a major role on how bone senses changes in the mechanical environment to drive mechano-adaptation (Weinbaum et al., 1994). This fluid flow is governed by lacunar-canalicular permeability, which is complicated to measure due to the small size of the pores and the interconnected multi-scale porosity found in bone. A study carried out by Li et al. in 1987 suggested that whole-bone permeability – including both lacunar-canalicular and vascular permeability – is significantly affected by age in osteonal cat bone (Li et al., 1987). However, it is still unclear if lacunar-canalicular permeability changes with age in non-osteonal mouse bone. In addition, although mouse models are often used to explore the mechano-adaptation mechanism of bone (Pereira and Shefelbine, 2013), there are no experimental estimates of the lacunar-permeability of mouse bone.

II) The second hypothesis is that the lacunar-canalicular permeability of mouse bone changes with age.

In *Chapter 4*, the effect of ageing in lacunar-canalicular permeability is explored by comparing the permeability values of young, skeletally mature and old mouse bone.

Characterising age-related changes in bone permeability will help in understanding the contribution of fluid flow through bone's porous network in bone mechano-adaptation. Due to ageing or skeletal pathologies, bone's mechano-adaptation capability deteriorates and it becomes more prone to fracture. Bone's micro-porosity might have an influence not only on the adaptation response, but also on the propagation of cracks at the micro-scale (Schneider et al., 2013; Voide et al., 2011). At the same time, evidence suggests that bones affected by osteogenesis imperfecta (brittle bones) exhibit altered intra-cortical porosity (Carriero et al., 2014a; Vardakastani et al., 2014).

III) The third hypothesis is that intra-cortical porosity affects crack propagation at the micro-scale and may contribute to bone brittleness.

Chapter 5 employs computational methods to assess the influence of intra-cortical porosity on micro-scale fracture mechanics using 2D models of the topology of osteogenesis imperfecta mouse bone porosity.

The study of intra-cortical porosity will provide insight into how cracks grow at the micro-scale. But at the nano-scale, crack initiation is governed by the composite nature of bone. The collagen and mineral act together to give bone its remarkable toughness (Gupta and Zioupos, 2008; Launey et al., 2010; Ritchie et al., 2009). In several skeletal pathologies which lead to alterations in bone toughness, such as osteogenesis imperfecta or rickets, bone mineral is altered (Camacho et al., 1996; Fratzl et al., 1996; Huesa et al., 2011; Miller et al., 2007a).

IV) The fourth hypothesis is that brittle and ductile bones have altered mineral properties at the nano-scale, which affect the mechanical integrity of bone at the micro-scale.

Chapter 6 investigates the mineral properties of brittle and ductile mouse cortical bone at the micro- and nano-scale to better understand the differences between bones which exhibit the two extremes of toughness.

Finally, conclusions are drawn in *Chapter 7*. The main outcomes of this thesis as well as contributions to the field of bone mechanics are discussed, and perspectives for future research are presented.

2 CORTICAL BONE BACKGROUND

This chapter provides a brief introduction to bone, its hierarchical structure and constituents, with particular interest in mouse cortical bone. The mechanics of cortical bone is investigated, describing bone's remarkable mechanical properties and selected experimental techniques that can be employed to characterise bone at its multiple length-scales. Finally, the mouse models of aging and disease used to study bone mechanics throughout this thesis are presented.

2.1 What is Bone?

Bone is a term that is used to represent the organ, as well as the material it is made of.

Bone is an organ. Bone is a tissue. Bones are made of bone.

Bones are the primary component of the skeleton. Mechanically, bones act as a framework to maintain the shape of the body, provide support to soft tissue, protect vital organs and facilitate movement in conjunction with muscles, tendons and ligaments. Within the bone, there are many different tissues, including bone tissue – osseous tissue-, bone marrow, periosteum, nerves, blood vessels and cartilage. Bone has a hierarchical and complex structure that leads to its extraordinary mechanical properties. In addition, bone is a dynamic material, as it adapts to external loads by changing its size, shape and structure in response to the mechanical environment.

2.1.1 Hierarchical Structure of Bone

Bone's hierarchical structure extends over multiple length-scales. These hierarchical levels can be distinguished as the whole bone (mm), tissue (μm) and collagen-mineral (nm) levels (Rho et al., 1998; Weiner and Wagner, 1998). Figure 2.1 provides a simplified representation of the hierarchical structure of mouse cortical bone. The mechanical competence of bone is strongly dependent on its complex hierarchical structure (Currey, 2003, 2012; Rho et al., 1998).

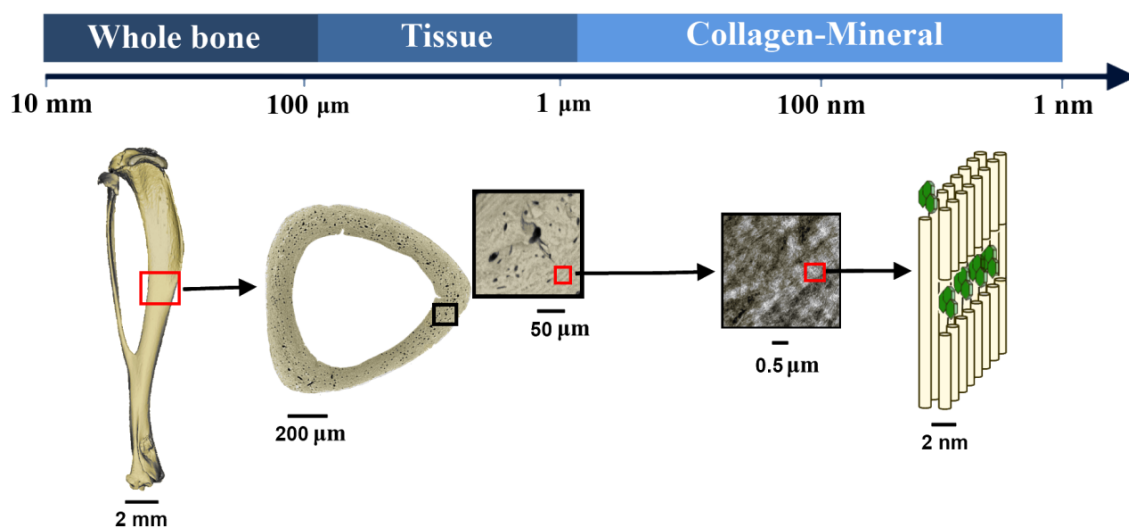


Figure 2.1: Schematic of the hierarchical structure of mouse cortical bone from the macro-scale (whole bone) to the nano-scale (collagen-mineral).

Macro-scale: whole bone level

At the whole bone level (mm), two different types of osseous tissues can be distinguished: cortical or compact bone; and trabecular or cancellous bone. In long bones (i.e. tibia and femur, Figure 2.2), dense cortical bone tissue surrounds the hollow mid-shaft region as well as the bony part of the outer shell of the epiphyses, and spongy trabecular bone fills in the regions close to the joint surfaces underneath the articular cartilage. Cortical bone is a dense, solid mass with microscopic pores (Section 2.1.2) and it comprises around 80% of bone mass. Cortical bone is responsible for the supportive and protective function of the skeleton. In contrast, trabecular or cancellous bone is made of a network of mostly interconnected plates and rods and it ensures strength at the end of the bone with minimal weight. This thesis focuses on the characterisation of cortical bone only, and thus trabecular bone will not be further considered.

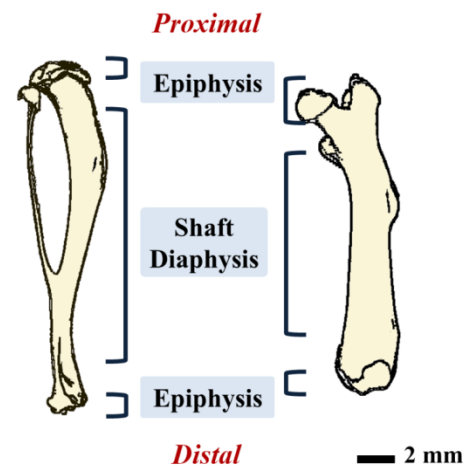


Figure 2.2: Schematic of mouse tibia (left) and femur (right).

Micro-scale: tissue level

At the microscopic scale, mammalian cortical bone can be structurally classified into woven and lamellar bone. Each of them contributes to bone's material heterogeneity and mechanical anisotropy. Woven bone is rapidly deposited during periods of rapid growth or rapid bone production (apposition) and it is poorly organised (Weiner and

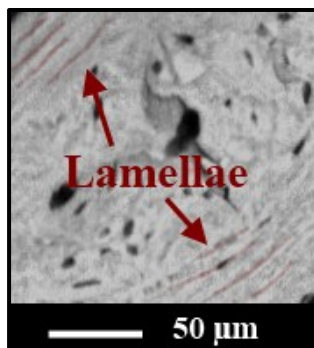


Figure 2.3: Circumferential lamellae in mouse bone.

Wager, 1998). Woven bone is later replaced by lamellar bone, which is laid down more slowly resulting in a more defined structure. Lamellar bone is built up of highly organised layers of lamellae (~3-7 μm thick) (Weiner et al., 1999). In human bone, lamellae wrap in concentric layers (3-8 lamellae) around a central vascular canal to form what is known as an osteon (200-250 μm in diameter) or a Harvesian system (Cooper et al., 1966). In contrast, mouse bones do not have osteons; instead,

lamellae are arranged around the circumference of the bone shaft (Figure 2.3). The comparison between osteonal and non-osteonal bone is represented in Figure 2.4.

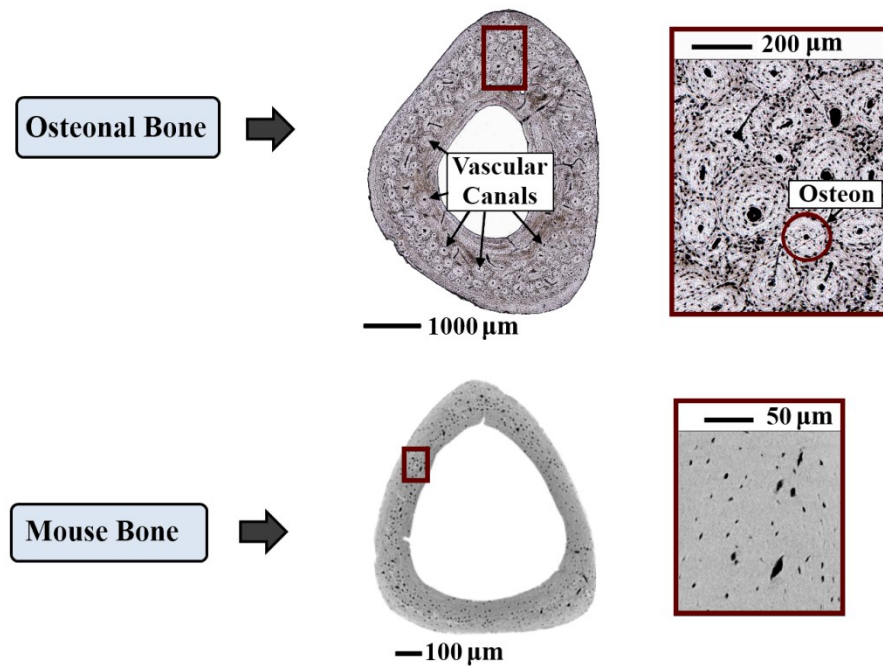


Figure 2.4: Osteonal vs. non-osteonal cortical bone sections. In osteonal bone lamellae wrap around the vascular canals (blood vessels), while in mouse bone there is no osteons. The figure of osteonal bone is the cross-section of bovine femur, adapted from (Gardinier et al., 2010) Copyright (2010) with permission of Elsevier.

Nano-scale: collagen-mineral level

Each lamella consists of mineralised collagen fibers arranged in roughly parallel arrays. The orientation of collagen fibers in adjacent lamellae is different, which results in a very strong structure. Mineralised collagen fibers are formed by collagen fibrils and hydroxyapatite nanocrystals, as represented in Figure 2.5. The mineral crystals are preferentially oriented parallel to the collagen fibrils, and arranged in a periodic manner along the fibrils (Rey et al., 2009).

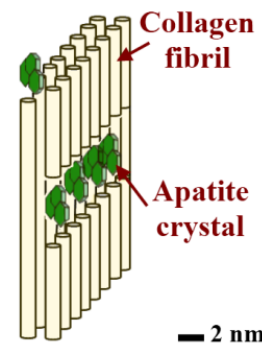


Figure 2.5: Schematic of mineralised collagen fiber.

Constituents of bone material

The constituent elements of bone material include apatite mineral, organic matter and water. Mature healthy bone consists of approximately 50 vol% mineral, 30 vol% organic matter and 20 vol% water (Gong et al., 1964). The

mineral phase of bone is composed of impure forms of hydroxyapatite (HA), which form nanometer-sized crystals. The hydroxyapatite in bone mineral deviates from the stoichiometric form of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ due to substitutions and vacancies (Boskey, 2003; Elliott, 2002; Fratzl et al., 2004; Glimcher, 2006; Rey et al., 2009). The organic phase is primarily composed of Type I collagen fibers (90%) and non-collagenous proteins. Collagen aligns and creates 3-dimensional frameworks into and onto which mineral crystals form. The third main component of bone is water, which resides on the surface of mineral crystals, within the crystals and between collagen fibers (Wilson et al., 2006). Bone combines the different mechanical properties of its components to result in a strong and tough biocomposite material.

2.1.2 Multi-scale Porosity in Bone

Porosity and bone fluid play an important structural and mechanical role in bone. Three interconnected levels of porosity can be distinguished in cortical bone: *vascular* porosity, *lacunar-canalicular* porosity and *collagen-apatite* porosity (Cowin, 1999; Cowin et al., 2009). The largest pores are associated with vascular porosity ($>10\text{ }\mu\text{m}$), which consists of canals that contain blood vessels (Figure 2.6). Lacunar-canalicular porosity consists of lacunar cavities ($\sim 1\text{--}10\text{ }\mu\text{m}$) and small canaliculi channels ($\sim 0.5\text{ }\mu\text{m}$). The smallest pore spaces ($\sim 10\text{ nm}$) exist between and within the collagen and mineral crystals, and these pores constitute the collagen-apatite porosity. Within these hierarchical pore spaces, interstitial fluid transports nutrients and wastes throughout the body. Evidence suggests that the interstitial fluid through the lacunar-canalicular network might play a major role in bone adaptation in response to the mechanical environment (Cardoso et al., 2013; Fritton and Weinbaum, 2009).

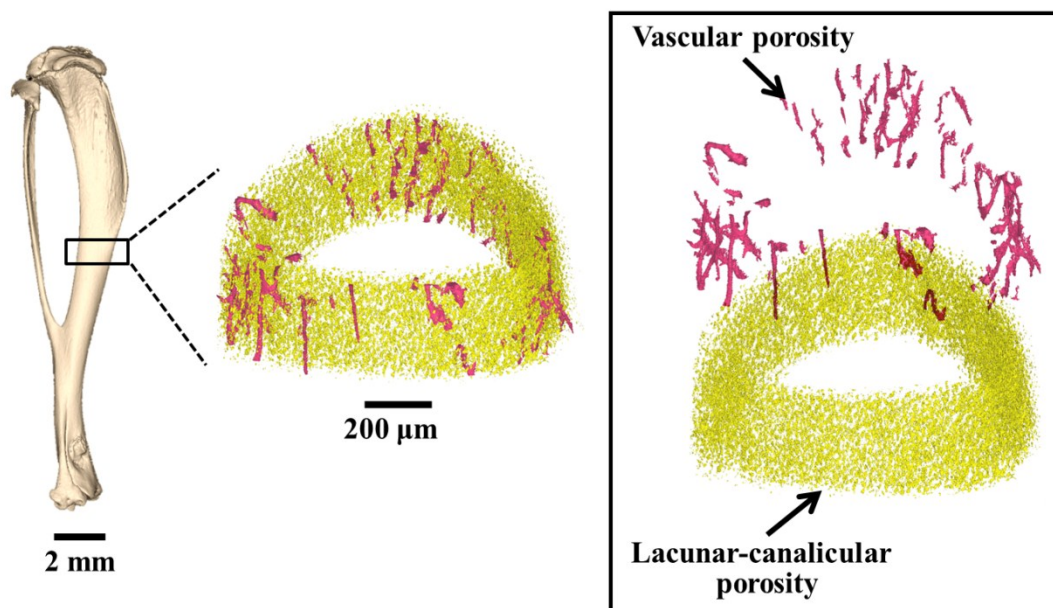


Figure 2.6: Multi-scale porosity of cortical bone of a mouse tibia from vascular (red big channels) to lacunar-canalicular (yellow dots and small channels) porosity.

2.2 Mechanics of Cortical Bone

There are a number of parameters that can be used to characterise bone's mechanical integrity. Mechanical properties of bone describe its behaviour under external loads and displacements. Figure 2.7 shows a representation of the load-deformation curves of cortical bone under tension and compression (Currey, 2002; Natali and Meroi, 1989).

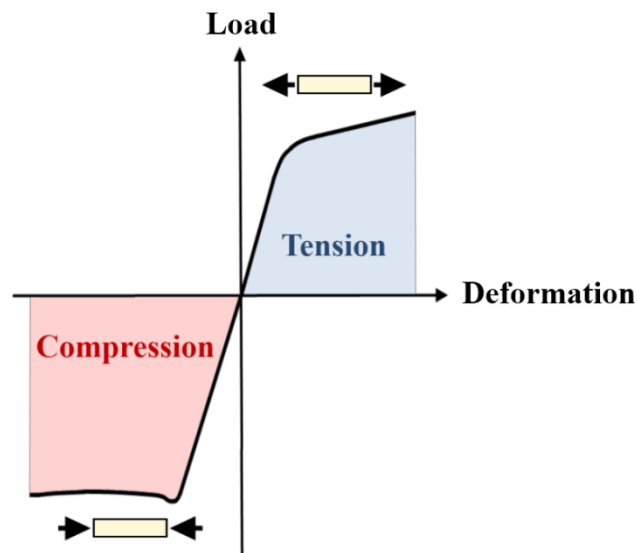


Figure 2.7: Schematic of load-deformation curves of cortical bone in tension and compression.

The load-deformation response can be translated to stresses (ratio of the load to the cross sectional area, in MPa) and strains (ratio of the deformation to the length, in %) in order to calculate the mechanical properties that are intrinsic to the material. The stress-strain curves of cortical bone show a linear elastic phase followed by a curved region where the material yields and then a plastic region followed by fracture, as represented in Figure 2.8. Properties such as stiffness, strength, ductility and toughness can be determined from these curves.

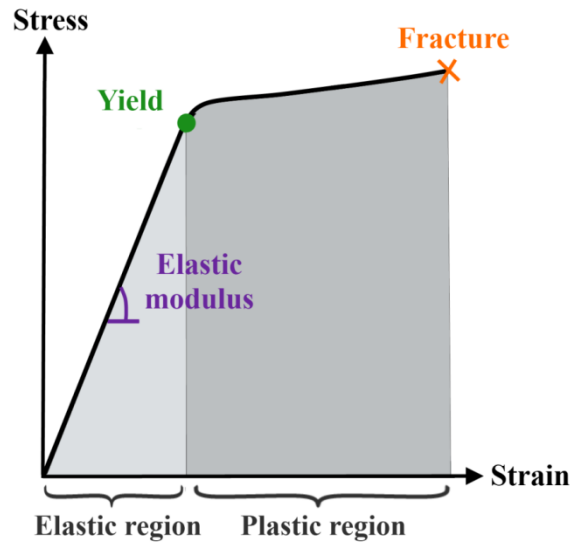


Figure 2.8: Schematic of a strain-stress curve of cortical bone in tension.

Stiffness is a measure of the force required to produce elastic deformation. It is related to the Young's or elastic modulus E , which is calculated as the initial slope of the linear elastic part of the stress-strain curve. Thus, the steeper the curve, the higher is the value of Young's modulus, and stiffer is the material.

Strength is a measure of the maximum stress that the material can withstand. It can be defined as the yield stress σ_y —maximum stress before the material starts deforming plastically— or by the maximum stress at the peak load before fracture, ultimate stress σ_u . The stronger the material, the higher stresses it can support.

Ductility is a measure of the maximum strain before fracture. It can be assessed by the % elongation of the sample or the relative change in the cross-sectional area.

Toughness is a measure of the resistance to fracture. It was traditionally described as the work to fracture W_f , defined by the area under the stress-strain curve. However, recent advances have introduced crack growth resistance curves (R-curves) (Ritchie et al., 2008) to capture not only the resistance of bone to crack initiation (stress intensity factor, K_c), but also to crack propagation.

It must be noted that bone is anisotropic, and thus, the stress-strain curves are dependent upon the direction of loading. In addition, bone's mechanical response is affected by the strain-rate, which reflects the time-dependent behaviour of bone. Such viscoelastic behaviour can be analysed through creep, which defines a gradual increase in strain under constant stress.

Bone exhibits a remarkable combination of mechanical properties, in particular high strength and toughness. Although mechanical properties vary depending on the testing mode, specimen, anatomical site and loading direction, most cortical bone has roughly similar mechanical properties (Currey, 2003). At the macro-scale, bone's elastic modulus spans 10-25 GPa (Liebschner, 2004; Reilly et al., 1974), about a third of metallic aluminium; the ultimate strength ranges between 40-300 MPa (Liebschner, 2004; Reilly and Burstein, 1975), comparable with alumina ceramics; and bone's fracture toughness is $\sim 3\text{-}10 \text{ MPa}\sqrt{\text{m}}$, around 3 to 10 times as big as silicon (Ritchie et al., 2009). Those mechanical properties are dependent upon the material, compositional and structural properties of bone at different length-scales.

2.2.1 Fracture Mechanisms of Bone

In 1920, Griffith suggested that materials start to fracture when the elastic energy dissipated by the growth of a crack exceeds the energy required to create a new surface. The more energy dissipation or toughening mechanisms exist, the higher is a material's ability to resist fracture. Bone is a composite material, and as such, its toughness values exceed those of its constituents, as exemplified in Figure 1.7 (Wegst et al., 2014).

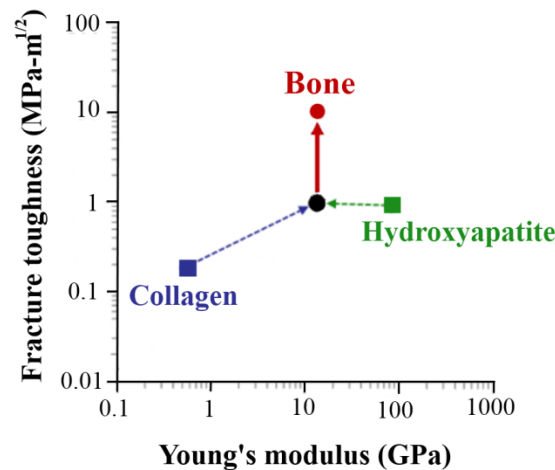


Figure 2.9: Bone's toughness for crack initiation (black circle) and crack growth (red circle) result in larger fracture toughness (red arrow) of bone than that of collagen (blue square) and hydroxyapatite (green square). Values from (Wegst et al., 2014).

Bone's resistance to fracture is influenced by inter-related aspects that act at different length-scales of its hierarchical levels (Gupta and Zioupos, 2008; Launey et al., 2010; Ritchie et al., 2009; Ural and Vashishth, 2014). Some of these aspects are summarised here (Bouxsein, 2005):

- *Overall bone composition*: relative ratios of contents of mineral, collagen, water, non-collagenous proteins (Nyman and Makowski, 2012; Nyman et al., 2005, 2006; Yeni et al., 1998).
- *Properties of its building blocks*: collagen cross-linking, crystal size and morphology, degree of mineralisation of the bone matrix (Boskey and Myers, 2004; Buehler, 2007; Gupta et al., 2013).
- *Bone morphology and micro-architecture*: bone size, shape, density, porosity, lamellar organisation (Balankin et al., 2011; Nobakhti et al., 2013; Tang and Vashishth, 2011; Yeni et al., 1997).

- *Amount of pre-existing micro-damage*: micro-cracks, crack lengths, crack density and location (Lucksanasombool et al., 2001; O'Brien et al., 2005; Robertson et al., 1978)
- *Previous and current loading*: strain rate, magnitude and direction of loading (Ural et al., 2011; Zimmermann et al., 2014)

Therefore, the understanding of bone's resistance to crack initiation and propagation requires the investigation of toughening mechanisms at all levels of bone's hierarchical structure (Buehler, 2007; Launey et al., 2010; Ritchie et al., 2006, 2009; Ural and Vashishth, 2014; Wang and Gupta, 2011). Previous studies have focused on the origin of cortical bone's fracture resistance and found that bone's toughness at the macro-scale displays a rising resistance-curve (R-curve) behaviour, where fracture resistance increases during the process of crack extension (Figure 2.10) in osteonal (Koester et al., 2008; Malik et al., 2003; Nalla et al., 2005) and non-osteonal (Carriero et al., 2014c) bone.

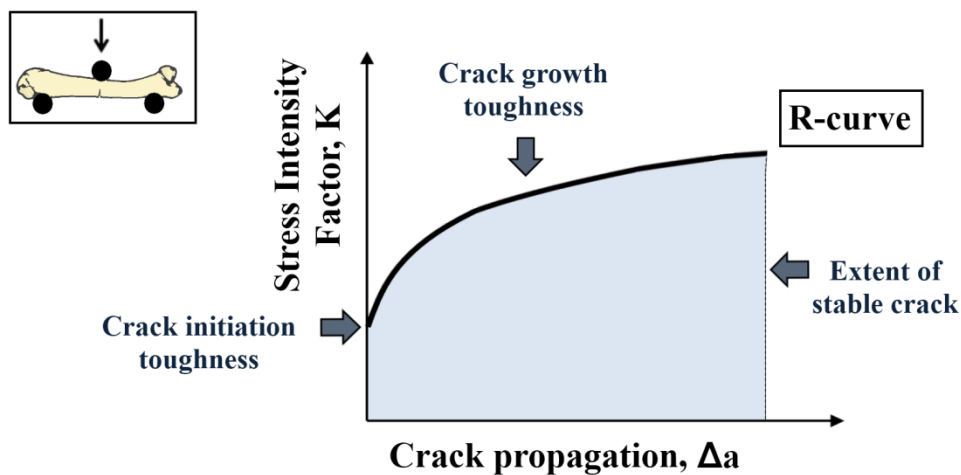


Figure 2.10: Schematic of rising resistance-curve (R-curve) in bone in a notched three-point bending setup. Bone has toughening mechanisms that inhibit crack initiation (crack initiation toughness) and propagation (crack growth toughness).

In order to describe this behaviour, the toughening mechanisms of cortical bone can be grouped as intrinsic toughening mechanisms that result primarily from plasticity, and extrinsic toughening mechanisms that act to reduce the local stresses/strains at the crack tip to resist crack growth (Koester et al., 2008; Launey et al., 2010; Ritchie et al., 2006).

At the nano-scale, *intrinsic toughening mechanisms* such as molecular uncoiling of the mineralised collagen fibrils and fibrillar sliding keep cracks from initiating and

growing. Intra-interfibrillar sliding is essential to promote plasticity by dissipating energy at the collagen-mineral level (Gupta et al., 2013; Jäger and Fratzl, 2000). The sliding of the mineral and collagen lead to the formation of local plastic zones which blunt the crack tip and reduce the driving force for cracking.

Bone's crack growth resistance, where fracture resistance increases with crack extension (Figure 2.10), reflects the presence of *extrinsic toughening mechanisms* that keep the crack from propagating too fast and too far at the micro-scale. These extrinsic mechanisms include crack deflection or twisting, and the formation of uncracked-ligament bridges or bridging of collagen fibers behind the crack tip. As micro-cracks form ahead of the crack tip, unbroken collagen fibrils bridge the gap of the crack. When the crack begins to grow, structural features such as lamellar organisation or intra-cortical porosity might deflect the crack path.

2.2.2 Multi-scale Characterisation Techniques

Bone's mechanical competence is determined by the mechanical, structural and compositional properties of its various hierarchical levels (Launey et al., 2010; Rho et al., 1998; Ritchie et al., 2009; Wang and Gupta, 2011). Therefore, multiple techniques are required to understand the nature of the contribution of each of the length-scales to the overall mechanical behaviour of bone. Some of the commonly used experimental techniques relevant to this thesis are summarised in Figure 2.11.

	Whole bone	Tissue	Collagen-Mineral
	10 mm	100 μ m	1 μ m
			100 nm
			1 nm
	Bulk bone	Lamellae/lacunae	Mineral crystals, collagen fibers
Mechanics	3-pt bending	Nanoindentation	SAXS/WAXD
Structure	micro-CT	nano-CT	XRD
Composition	Gravimetric	BSE-SEM	XRD after heating

Figure 2.11: Selected techniques for the assessment of bone's mechanical, structural and compositional properties at the whole bone, tissue and collagen-mineral levels.

Mechanics

Bending tests are useful for measuring the mechanical properties of long bones. In particular, notched 3 point bending (3-pt bending) can be used to characterise fracture toughness and crack propagation of mouse bones at the whole bone level (Carriero et al., 2014c; Ritchie et al., 2008). Nanoindentation is widely used to probe tissue mechanical properties of bone. The indentation data can be evaluated following elasto-plastic, viscoelastic, viscoelastic-plastic and poroelastic models in order to obtain the tissue elastic modulus, hardness, extent of viscosity and permeability (Oyen, 2010). The combination of small and wide angle X-ray scattering techniques (SAXS/WAXD) while performing in-situ uniaxial tensile tests provides information about the strain of the individual mineralised collagen fibril (from the SAXS spectra) and mineral crystals (from the WAXD diffraction pattern) compared to the macroscopic tissue strain

(Gupta et al., 2006). A representation of these three techniques is shown in Figure 2.12.

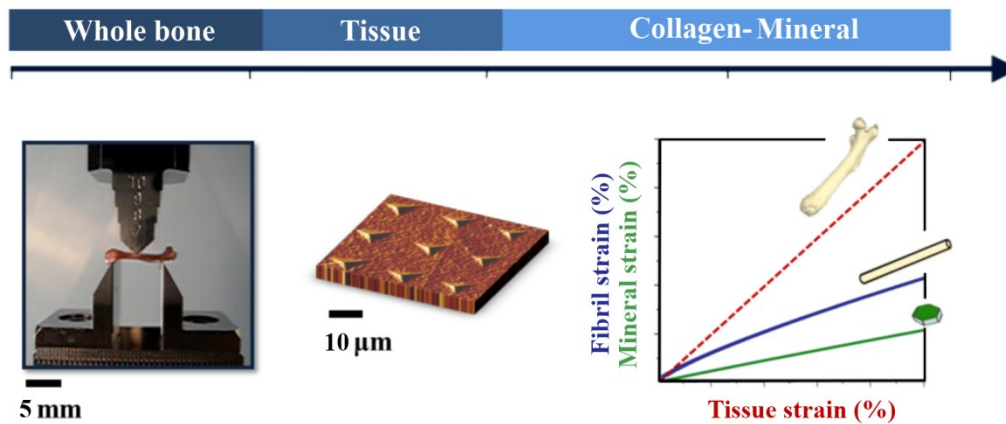


Figure 2.12: Representation of techniques to measure bone mechanics (from left to right): 3-pt bending setup of mouse femur; indents on bone tissue made with a nanoindenter; contributions of fibril (SAXS) and mineral (WAXD) strain to bone tissue strain.

Structure

The 3D architecture of bone can be assessed by tomography techniques. Micro-computed tomography (micro-CT, with a resolution of 5-10 μm) is used to image whole mouse bone and determine bone size and shape (Miller et al., 2007b). Nano-CT (with a resolution of 500-750 nm) provides information about intra-cortical micro-porosity and it can be used to measure the dimensions and geometry of lacunar and vascular pores (Carriero et al., 2014a). X-ray diffraction (XRD) is a well-established technique for probing the average apatite crystal size (in the c-direction) in bone (Ziv and Weiner, 1994). These techniques are represented in Figure 2.13.

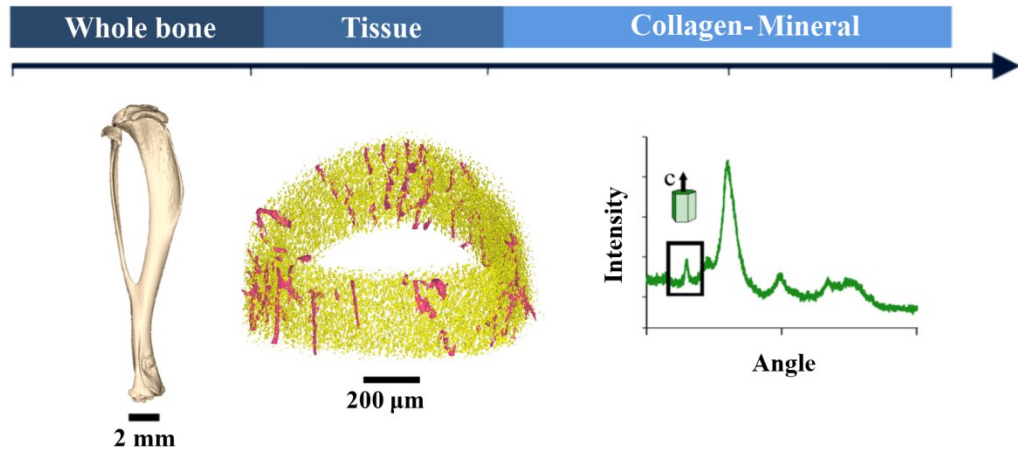


Figure 2.13: Representation of techniques to characterise bone structure (from left to right): micro-CT image of mouse tibia; lacunar pores (yellow) and vascular channels (red) captured by nano-CT; mineral crystal size along the c-axis measured at the highlighted peak of the XRD spectra.

Composition

Thermogravimetric analysis (TGA) monitors the loss of bone mass with temperature, from which the fractions of water, organic matter and inorganic content are inferred. TGA is used to calculate the bulk mineral to matrix ratio of the whole bone (Peters et al., 2000). A major advantage of this technique is its relative simplicity to obtain quantitative information; however, it cannot characterise the spatial distribution of the mineral within the bone or the quality of the mineral. Backscattered electron scanning electron microscopy (BSE SEM) provides information about the extent of mineralisation of the bone matrix, thus giving insight into the distribution of the mineral (Roschger et al., 1998). The crystallinity of the mineral increases with temperature; XRD after thermal treatment reveals the phases present on the bone. It can be used to estimate the fractions of β -tricalcium phosphate and hydroxyapatite in bone, from which differences in the Ca/P ratio can be inferred (Raynaud et al., 2002). Figure 2.14 summarises these three techniques to measure composition at different hierarchical levels.

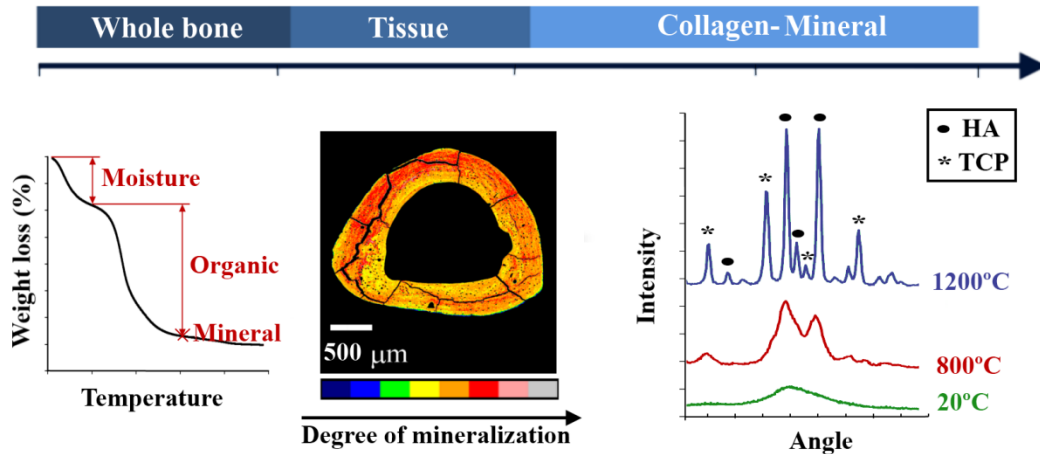


Figure 2.14: Representation of techniques to measure bone composition (from left to right): moisture, organic, and mineral fractions (weight %) of bone measured by TGA; BSE SEM scan of a cross section of a mouse tibia from non-mineralised (black) to highly mineralised (white); XRD spectra of bone at room temperature, heated to 800°C and 1200°C, with the peaks corresponding to hydroxyapatite (HA) and β -tricalcium phosphate (TCP) specified.

Experimental techniques are often complemented by computational studies to gain a greater understanding of bone's mechanical behaviour. One of the advantages of modelling techniques is their ability to explore the sole influence of individual bone features on bone mechanics. As such, Extended Finite Element Methods (XFEM) can be used to assess the influence of micro-structure on crack formation and growth (Yazid et al., 2009).

Those are some of the selected techniques that will be mentioned or used during this thesis. More extended reviews of current techniques to probe bone at different levels can be found elsewhere (Cardoso et al., 2013; Casanova et al., 2014; Donnelly, 2011; Paris, 2008; Schneider et al., 2010).

2.3 Pre-clinical Studies on Mouse Bone

Mouse models are widely used in pre-clinical bone research (Elefteriou and Yang, 2011). Mice offer numerous benefits thanks to their availability, fast reproduction rate, rapid bone growth, low cost and small size. Though mouse bone is slightly different in structure to human bone (Figure 2.4), the fundamental insights gained are anticipated to be translatable to human conditions.

Through transgenic and knockout models, mice provide an excellent platform to investigate skeletal pathologies. Different strains of inbred mice can be used to create knock-out models. An inbred strain is defined as a strain in which sibling matings have been performed for over 20 generations (Stephenson, 1997). Knockout of a particular gene can result in very different phenotypes depending on the initial strain used, which is why it is important to create a wild-type control for each knock-out (Carleton et al., 2008; Syberg et al., 2012).

In this thesis, the bone characterisation techniques described in the previous section are used to explore the micro- and nano-scale properties of mouse models that display different behaviour at the whole bone level. A brief description of these models is shown here:

C57BL/6

Standard C57BL/6 mouse, also called “black-6” (B6), are the most used mouse strain in research. B6 are extendedly used for targeted mutant or knock-out mice; and thus, they are often considered as controls. They are very stable genetically, which makes them suitable for comparing or testing bone characterisation techniques. In addition, B6 mice are widely used as a model of age-related bone loss in humans (Jilka, 2013).

Altered Collagen: Oim

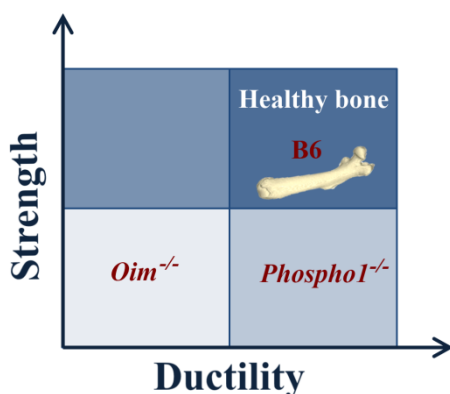
Osteogenesis imperfecta murine B6C3FE-a/aCol1a2oim/oim (*oim*^{-/-}) model replicate the moderate to severe condition of osteogenesis imperfecta (OI) in humans (Chipman et al., 1993). Osteogenesis imperfecta, also called brittle bone disease, affects 1 in 15,000 births in the UK and it is characterised by high skeletal fragility (<http://www.brittlebone.org/>). OI is primarily caused by a

genetic mutation in type I collagen (Forlino et al., 2011). The *oim* mouse model of OI is commonly used to investigate therapies that could later be translated to the human condition (Guillot et al., 2008; McCarthy et al., 2002; Mehrotra et al., 2010; Rao et al., 2008; Vanleene and Shefelbine, 2013; Vanleene et al., 2010). It is also an excellent model of the implications of molecular defects on whole bone integrity. The decreased strength and toughness found in *oim*^{-/-} mice are attributed to deteriorated bone material properties (Bart et al., 2014; Camacho et al., 1996; Grabner et al., 2001; Mehta et al., 2009; Miller et al., 2007a; Vanleene et al., 2012). The wild-type control for this *oim*^{-/-} model is B6C3Fe-a/a-+/+ (*oim*^{+/+}).

Altered mineralisation: *Phospho1*

Phospho1-R74X null mutant (*Phospho1*^{-/-}) mice lack a phosphatase that is required for the generation of inorganic phosphate for bone mineralisation (MacRae et al., 2010; Millán, 2013). Hence, *Phospho1*^{-/-} mice are a model of inactive production of phosphate in bone and can be used to study skeletal diseases associated with altered mineralisation, such as rickets in children and osteomalacia in adults (Yadav et al., 2011, 2014). *Phospho1*^{-/-} mice have ductile bones at the macroscopic scale (Huesa et al., 2011). Their wild-type controls are termed *Phospho1*^{+/+}.

The overall mechanical responses of these models belong to different quadrants of strength and ductility (Figure 2.15): B6 mice are strong and ductile, but their properties



deteriorate with age; *oim*^{-/-} are weak and brittle; while *Phospho1*^{-/-} are ductile (Carriero et al., 2014d). Therefore, they provide an excellent platform to explore a range of mechanical properties and structural defects at the micro- and nano-scale.

Figure 2.15: Healthy bone is strong and ductile.

2.4 Summary

This chapter has presented a review of cortical bone, its mechanics and the mouse models used in this thesis. In summary, bone is a hierarchical material which has remarkable mechanical properties. These properties depend on the mechanical, structural and compositional properties of bone at multiple scales. In the recent years several techniques have been developed or improved to probe bone at the micro- and nano-scale. Many of these techniques have been applied to mouse models of disease and aging in order to gain an insight into how different skeletal pathologies might affect bone at its different hierarchical levels. This understanding is vital to develop preventive or therapeutic treatments to target skeletal diseases, as well as to design replacement materials.

A widely used technique to characterise mechanical properties of bone at the micro-scale is nanoindentation. However, there is not one defined protocol to be followed in the nanoindentation study of bone, which has led to substantial differences in the nanoindentation properties measured by different research-groups. In Chapter 3 the mechanical properties of B6 wild-type bones are evaluated and compared using a wide range of testing and analysis options to better understand the reasons of the differences in the nanoindentation properties of bone.

3 NANOINDENTATION TO MEASURE TISSUE MECHANICS

The research described in this chapter has been published in: N. Rodriguez-Florez, M.L. Oyen, S.J. Shefelbine, 2013, Insight into differences in nanoindentation properties of bone, Journal of the Mechanical Behavior of Biomedical Materials 18, 90-99. Copyright (2013) with permission of Elsevier.

Nanoindentation provides the means to determine tissue level (μm) mechanical properties of bone. Unlike whole bone mechanical testing, the analysis of material properties at the micro-scale is not affected by the size and shape of the bone, allowing for local material characterisation in a non-destructive manner. This becomes especially advantageous in the study of pathologic bones, where altered geometry complicates the measurement of material properties.

Initially, nanoindentation was limited to the measurement of Young's modulus and hardness following an elasto-plastic analysis on dry bone. The continuing advancements of nanoindentation with applications on biomaterials have enabled the incorporation of viscoelastic, viscoelastic-plastic and poroelastic frameworks to measure properties of dry and hydrated bone. Despite the rapid growth of nanoindentation in bone tissue research, significant differences are found in results from different studies. However, a direct comparison across various studies is complicated due to the differences in the bone samples, and testing and analysis protocols.

The goal of this chapter is to evaluate and compare mechanical properties of the same bones using a wide range of preparation, testing and analysis methods in order to determine how these methods affect the measured material properties.

3.1 Nanoindentation Background

Nanoindentation is a measurement technique to determine the mechanical response of materials at the micro-scale. In nanoindentation a probe is brought into contact with a polished flat surface, pushed into the material, and retracted, while the load (P), displacement (h) and time (t) are monitored (Figure 3.1). Based on the P - h - t curves, multiple analytical models exist to extract mechanical properties depending on the deformation modes of the indented material.

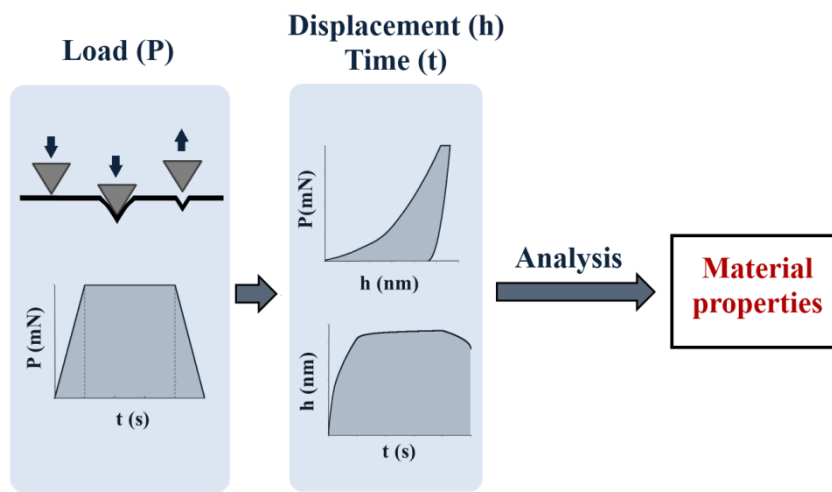


Figure 3.1: Schematic of load-control nanoindentation: a load (P) is applied while the load-displacement (P - h) and displacement-time (h - t) are recorded. Different data analysis methods can be used to obtain material properties.

A series of experimental and analytical options must be considered prior to testing, depending on the material to be tested and the properties to be measured.

Experimental considerations

- *Probe geometry:* The most important experimental selection is that of indenter probe geometry, as this determines the induced deformation modes. The two most common tips in the testing of bone are the three-sided pyramid, called Berkovich, and the spherical tip (Figure 3.2). Berkovich has a well-defined geometry and thus, the contact area, A_c , can be easily calculated. However, being a sharp tip, with a radius of curvature ~ 150 nm (similar size to a lamella), the transition from elastic to plastic behaviour happens almost instantaneously. In contrast, a spherical tip with a large radius allows an extended elastic-plastic transition. In general, sharp indenter tips are recommended for elasto-plastic

analysis; while spherical tips are used for elastic, viscoelastic and poroelastic analysis (Oyen, 2010).

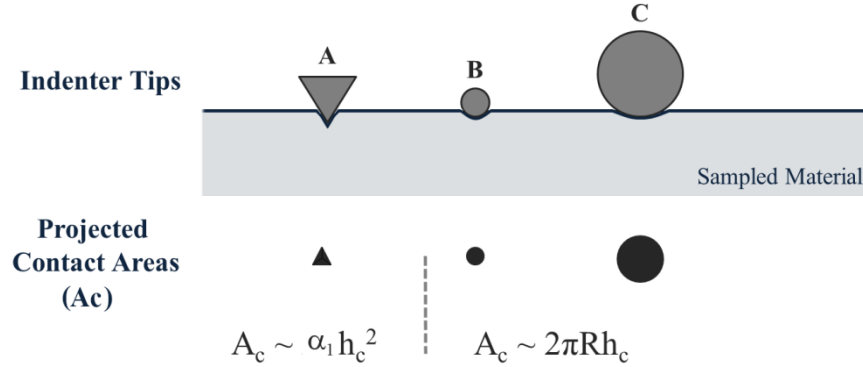


Figure 3.2: Schematic of Berkovich (A) and small (B) and big (C) sphere tips and their projected areas, A_c . h_c is the contact depth, α_1 is a geometric constant, and R the sphere radius.

- *Sample preparation:* The sample collection and storage methods must be chosen. In the case of small specimens, such as mouse bones, samples are often embedded in a resin to facilitate sample handling and surface polishing. Samples might be dry, hydrated prior to testing, or fully submerged in fluid while testing.
- *Testing mode:* Most nanoindenters operate in a mode of loading that is controlled by the applied load (load-control), although some indenters can also apply a displacement (displacement-control). For elasto-plastic materials a pyramidal loading profile is usually employed; while, in time-dependent materials a trapezoidal protocol is often used to exhaust the creep prior to unloading (Briscoe et al., 1998) (Figure 3.3).

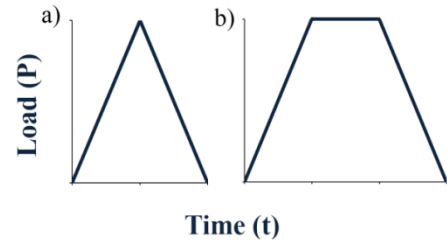


Figure 3.3: Pyramidal (a) and trapezoidal (b) loading profiles.

Data analysis choices

Historically, results of bone nanoindentation have been analysed using an elasto-plastic approach. However, the application of nanoindentation on time-dependent materials has led to new analysis techniques that either suppress the viscous behaviour or incorporate a time-dependency into the analysis. The different analysis models considered in this thesis are further discussed in the following section.

3.2 Nanoindentation Data Analysis Models

Bone is a time-dependent material; hence, nanoindentation on bone might induce elastic, plastic and viscous deformations. Various analytical and numerical models have been developed and adapted to determine bone's tissue level mechanical properties according to the induced deformation mode (Isaksson et al., 2010; Menčík et al., 2009; Olesiak et al., 2009; Oyen, 2006a). The method that is built into most commercial indentation systems is the Oliver – Pharr (OP) method (Oliver and Pharr, 1992, 2004) to extract elasto-plastic properties, neglecting any contribution from time-dependent deformation. As described in the previous section, large spherical indenters may be used to maintain small indentation strains thus preventing yielding and plastic deformation, allowing for viscoelastic (VE) analysis (Oyen, 2005, 2006a, 2007). Indentations on bone with sharp probes result in plastic deformation; therefore, a viscoelastic-plastic (VEP) approach has been used for Berkovich indentations (Olesiak et al., 2009; Oyen and Cook, 2003). Those three data analysis models (OP, VE and VEP) are considered in this chapter.

Oliver-Pharr (OP)

In the commonly used Oliver-Pharr approach (Oliver and Pharr, 1992, 2004) the elastic modulus is calculated from the unloading curve based on the assumption that the unloading response is purely elastic. Due to the time-dependent behaviour of bone, the unloading is viscoelastic but an attempt is made to limit the contribution of viscoelasticity by introducing a creep hold at peak load (Briscoe et al., 1998; Chudoba and Richter, 2001; Feng and Ngan, 2002), as shown in Figure 3.3b.

The general relationship for the unloading portion of the load-displacement curve (P - h) is derived according to the following power-law expression (Sneddon, 1965):

$$P = n(h - h_f)^m \quad [3.1]$$

where P is the indenter load, h is the indentation depth, h_f represents the final displacement after the indenter is fully unloaded (Figure 3.4), and n and m are power law fitting constants. The values of n , m and h_f are determined from regression analysis of the experimental data.

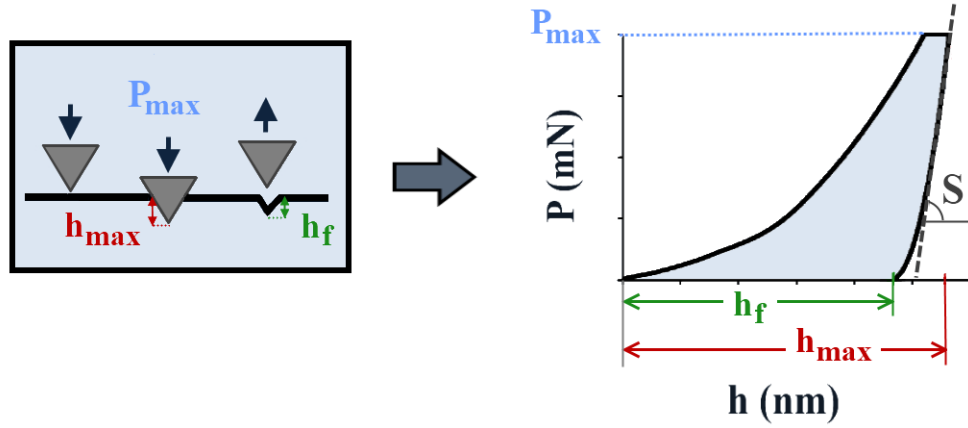


Figure 3.4: Schematic of load-control indentation and the induced load-displacement (P - h) data. P_{max} is the maximum load, which is kept during the creep hold; h_{max} is the maximum displacement induced; and h_f is the final indentation depth after unloading.

The stiffness at peak load S is calculated as the slope of the unloading P - h curve by differentiating Eq. 3.1. In the current study, 80% of the unloading curve has been used to obtain the slope, as the last 20% is often influenced by surface roughness. The obtained stiffness is used to estimate the contact depth h_c at maximum load according to:

$$h_c = h_{max} - \lambda \frac{P_{max}}{S} \quad [3.2]$$

where λ is a constant which depends on the indenter probe geometry – λ is accepted to be 0.75 for Berkovich and spherical indenter tips (Oliver and Pharr, 2004). The contact depth is used to estimate the contact area (A_c) via a calibration function. These two parameters are used to compute the reduced modulus:

$$E_R = \frac{S\sqrt{\pi}}{2\sqrt{A_c}} \quad [3.3]$$

The reduced modulus is a series combination of indenter (E_i , ν_i) and sample (E_s , ν_s) Young's moduli and Poisson's ratios.

$$\frac{1}{E_R} = \frac{(1 - \nu_i^2)}{E_i} + \frac{(1 - \nu_s^2)}{E_s} \quad [3.4]$$

Since bone is far less stiff than the diamond tip with ($E_s < 30$ GPa) the reduced modulus can be considered as the plane strain modulus of the material (Olesiak et al., 2009).

$$E_R \approx \frac{E_s}{(1 - \nu_s^2)} = E' \quad [3.5]$$

Although the reduced modulus, and thus the plane strain modulus, is directly identified by the indentation test, the value of Poisson's ratio must be assumed in order to compute the value of the elastic modulus.

The contact hardness, or the mean supported contact stress, is defined as the peak load divided by the contact area.

$$H_c = P_{max}/A_c \quad [3.6]$$

The contact hardness reflects the resistance to penetration of a hard indenter regardless whether the deformation is elastic, plastic, viscous, or due to crushing fracture. Therefore, it is not a material property.

Viscoelastic Analysis (VE)

Negligible plastic deformation occurs with spherical indenter tips provided that the indentation strain is smaller than the yield strain, allowing for the use of viscoelastic analysis (Oyen, 2005, 2006a, 2007). The maximum indentation strain ε_{max} in spherical indentation can be defined as:

$$\varepsilon_{max} \sim 0.2 \sqrt{\frac{h_{max}}{R}} \quad [3.7]$$

In the viscoelastic method, a linear viscoelastic response and a non-decreasing contact area are assumed.

For many viscoelastic problems, analytical expressions governing the deformation of a material can be determined by solving the Boltzmann hereditary integral for the applied loading condition. The time-displacement data $h(t)$ from spherical indentation is fitted to the viscoelastic Boltzmann integral equation:

$$h^{3/2}(t) = \frac{3}{8\sqrt{R}} \int J(t-u) \frac{dP(u)}{du} du \quad [3.8]$$

where R is the radius of the tip; P is the applied load; u is the dummy variable of integration for time; and $J(t)$ is the material creep function. The creep function can be mathematically represented by the generalised standard linear solid model shown in Figure 3.5.

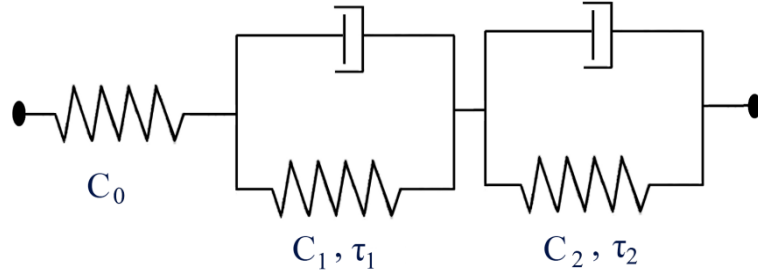


Figure 3.5: Rheological model for VE during creep hold.

As such, $J(t)$ is defined as a function of the creep coefficients C_0 , C_i and the material time constants τ_i :

$$J(t) = C_0 - \sum_{i=1}^2 C_i \exp(-t / \tau_i) \quad [3.9]$$

In the current study, two Kelvin-Voigt bodies (Figure 3.5), and therefore two time constants (τ_1 , τ_2) have been used to represent bone creep during the hold period, as incrementing the number of time constants did not improve the fitting of the curve. It must be noted that rheological models are only a useful tool to describe the viscoelastic behaviour of the material, but do not necessarily provide a real physical insight which can be related to the underlying micro-structure.

The solution of Eq. 3.8 for the holding period ($P = P_{max}$, $dP/dt = 0$) results in the following expression:

$$h^{3/2}(t) = \frac{3}{8\sqrt{R}} P_{max} \left[C_0 - \sum_i^2 C_i \exp(-t / \tau_i) RCF_i \right] \quad [3.10]$$

where the radius of the sphere, R , and the peak load, P_{max} , are test parameters; and C_0 , C_i and τ_i are the fitting parameters. The dimensionless ramp correction factor, RCF_i , accounts for the fact that the loading is not instantaneous (rising time, $t_R > 0$) and it is given by (Oyen, 2007):

$$RCF_i = \frac{\tau_i}{t_R} [\exp(t_R/\tau_i) - 1] \quad [3.11]$$

From the obtained creep coefficients, the zero-time (instantaneous) G_0 and infinite-time G_∞ shear moduli for the incompressible ($\nu = 0.5$) case can be computed as:

$$G_0 = \frac{1}{2J(0)} = \frac{1}{2(C_0 - \sum C_i)} \quad [3.12]$$

$$G_\infty = \frac{1}{2J(\infty)} = \frac{1}{2C_0} \quad [3.13]$$

The ratio of these two extremes $f = G_\infty/G_0$ gives a compact idea of the extent of the time-dependent deformation in a material (Oyen, 2006a): $f=1$ signifies a perfectly elastic material (no creep) and $f=0$ a perfectly viscous material ($J(0) = 0$). Since in bone $\nu = 0.3$, the calculated incompressible ($\nu = 0.5$) zero-time shear modulus G_0^I must be translated to G_0^ν via (Oyen, 2005):

$$G_0^\nu = 2G_0^I(1 - \nu) \quad [3.14]$$

The plane strain modulus is obtained from the incompressible instantaneous shear modulus (Bembey et al., 2006a):

$$E' = \frac{2G_0^I}{1 - \nu} \quad [3.15]$$

Viscoelastic-Plastic Analysis (VEP)

Berkovich tips result in plastic deformations, thus, a viscoelastic-plastic analysis is appropriate (Olesiak et al., 2009). This method combines viscous, elastic and plastic quadratic elements in series (Figure 3.6) to model the full response of time-dependent materials (Oyen and Cook, 2003).

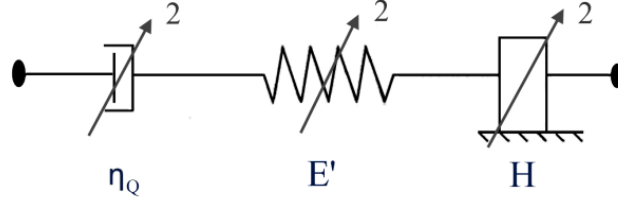


Figure 3.6: Rheological quadratic model for VEP while loading, proposed by (Oyen and Cook, 2003).

The loads on the viscous element P_v , elastic spring P_e and plastic dashpot P_p corresponding to Figure 3.6 are defined as:

$$P_v = \alpha_3 \eta_Q \left(\frac{dh_v}{dt} \right)^2 \quad [3.16]$$

$$P_e = \alpha_2 E' (h_e)^2 \quad [3.17]$$

$$P_p = \alpha_1 H_{VEP} (h_p)^2 \quad [3.18]$$

where h_v , h_e and h_p correspond to the viscous, elastic and plastic displacements; $\alpha_1, \alpha_2, \alpha_3$ are dimensionless geometric constants; η_Q is the viscous coefficient; E' refers to the plane strain modulus; and H_{VEP} is the hardness (resistance to plastic deformation). The dimensionless geometric constants for a perfect Berkovich tip are $\alpha_1 = 24.5$, $\alpha_2 = \alpha_3 = 4.4$ (Oyen and Cook, 2003).

The total displacement is equivalent to the sum of the displacements of each of the elements:

$$h = h_v + h_e + h_p \quad [3.19]$$

Therefore, the constitutive differential equation for the VEP model for Berkovich indentations is given by:

$$\begin{aligned} \frac{dh}{dt} &= \frac{dh_v}{dt} + \frac{dh_e}{dt} + \frac{dh_p}{dt} \\ &= \frac{P^{1/2}}{(\alpha_3 \eta_Q)^{1/2}} + \frac{1}{P^{1/2}} \frac{dP}{dt} \frac{1}{2(\alpha_2 E')^{1/2}} + \frac{1}{P^{1/2}} \frac{dP}{dt} \frac{1}{2(\alpha_1 H_{VEP})^{1/2}} \end{aligned} \quad [3.20]$$

Using a trapezoidal loading function, the full VEP displacement-time (h - t) response is defined by Eq. 3.21-3.23 (Olesiak et al., 2009): the loading is considered to have a viscous-elastic-plastic behaviour (h^{LOAD}), the holding period is defined by a viscous response (h^{CREEP}) and the unloading is viscoelastic (h^{UNLOAD}) (Figure 3.7).

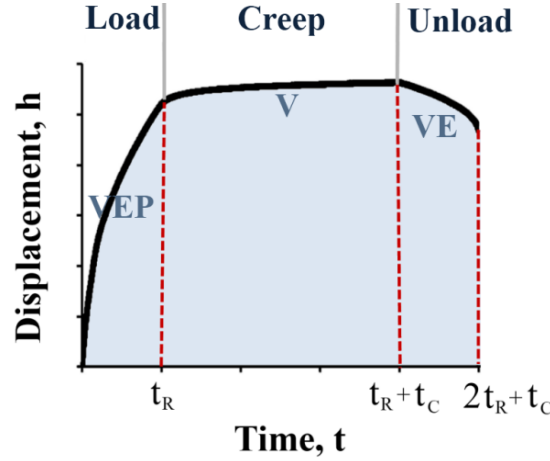


Figure 3.7: Displacement-time curve caused by a trapezoidal loading profile: loading during rising time t_R is viscous-elastic-plastic (VEP); creep hold during t_c is viscous (V) and unloading until $2t_R + t_c$ is viscoelastic (VE).

$$h^{LOAD}(t) = (kt)^{1/2} \left(\frac{2t}{3(\alpha_3 \eta_Q)^{1/2}} + \frac{1}{(\alpha_2 E')^{1/2}} + \frac{1}{(\alpha_1 H_{VEP})^{1/2}} \right) \quad t < t_R \quad [3.21]$$

$$h^{CREEP}(t) = \frac{(kt_R)^{1/2}}{(\alpha_3 \eta_Q)^{1/2}} (t - t_R) + h^{LOAD}(t_R) \quad t_R < t < t_R + t_c \quad [3.22]$$

$$h^{UNLOAD}(t) = (kt)^{1/2} \left(\frac{t_R^{3/2} - (2t_R + t_c - t)^{3/2}}{3/2(\alpha_3 \eta_Q)^{1/2}} + \frac{(2t_R + t_c - t)^{1/2} - t_R^{1/2}}{(\alpha_2 E')^{1/2}} \right) + h^{CREEP}(t_R + t_c) \quad t > t_R + t_c \quad [3.23]$$

The times t_R , t_c stand for the rising and holding time respectively; k is the loading rate ($k = P_{max}/t_R$). Fitting the displacement time (h - t) curve to the full VEP solution allows for the direct extraction of the indentation viscosity (η_Q), plane strain modulus (E') and hardness (H_{VEP} , resistance to plastic deformation).

In the VEP model a linear creep rate is assumed for the entire hold period. However, this is an approximation, and therefore in the current study only the steady-state creep was used to estimate the viscosity term:

$$h^{CREEP}(t) = \frac{(P_{max})^{1/2}}{(\alpha_3 \eta_Q)^{1/2}} (t - t_1) + h(t_1) \quad [3.24]$$

where t_l was defined as $t_l = t_R + t_c/6$ to only consider the last 5/6 of the holding period and obtain a better fit of the curve.

As the objective of this chapter was to compare different models to extract material properties, two extra parameters were estimated from the VEP analysis. The viscous time constant for VEP, τ_Q , was defined for comparison with the viscoelastic time constants (τ_l , τ_2) as (Oyen and Cook, 2003):

$$\tau_Q = \left(\frac{\alpha_3 \eta_Q}{\alpha_2 E'} \right)^{1/2} \quad [3.25]$$

This time constant from the VEP model represents the characteristic time scale of the material associated with the viscous-elastic-plastic response to indentation. In addition, the contact hardness (H_c , resistance to all components of deformation) was calculated for comparison purposes with the Oliver-Pharr hardness (Oyen, 2006b). In order to compute the contact area A_c , the contact deformation h_c was considered to be a combination of independent viscous h_v , elastic h_e , and plastic h_p deformations (Ferguson, 2009).

$$H_c = \frac{P_{max}}{A_c} = \frac{P_{max}}{\alpha_1 h_c^2} = \frac{P_{max}}{\alpha_1 (h_v + h_e + h_p)^2} \quad [3.26]$$

Each of these deformations can be defined as:

$$h_v = \sqrt{\frac{P_{max}}{\alpha_3 \eta_Q}} \left(\frac{2}{3} t_R + t_c \right) \quad [3.27]$$

$$h_e = \sqrt{\frac{P_{max}}{\alpha_2 E'}} \quad [3.28]$$

$$h_p = \sqrt{\frac{P_{max}}{\alpha_1 H_{VEP}}} \quad [3.29]$$

Substituting the deformations h_v , h_e and h_p into Eq. 3.26 leads to the following expression of the contact hardness:

$$H_C = \frac{1}{\alpha_1 \left((2t_R/3)(\alpha_3 \eta_Q)^{-1/2} + (\alpha_2 E')^{-1/2} + (\alpha_1 H_{VEP})^{-1/2} \right)^2} \quad [3.30]$$

3.3 Nanoindentation of Bone: Systematic Analysis of Testing and Analysis Options

Nanoindentation has been extensively applied to bone tissue research (Guo and Goldstein, 2000; Haque, 2003; Lewis and Nyman, 2008; Oyen, 2010; Rho et al., 1997; Zysset et al., 1999). All three approaches described in the previous section, elastoplastic, viscoelastic, and viscoelastic-plastic, have been used to determine bone's mechanical response. However, the values of the plane strain modulus obtained from different studies vary significantly.

In indentation of dry bone, where OP analysis was used, an average modulus of 30.8 GPa was measured with a Berkovich tip (Chang et al., 2011), while a modulus of 18.1 GPa was found with a spherical tip (Bushby et al., 2004). The viscoelastic approach in wet bone, using spherical indentation, gave moduli as small as 2 GPa (Oyen et al., 2012). Values of 24.78 GPa were obtained in dry bone, employing Berkovich indentation and using the VEP model (Olesiak et al., 2009). Since the differences in the sample itself (bone type and animal), sample preparation, hydration state, and test parameters complicate direct comparisons across the various studies, these discrepancies in values could not be compared directly.

Previous studies have analysed mechanical properties of bone in different hydration states (Bembey et al., 2006a; Bushby et al., 2004; Ferguson, 2009; Hoffler et al., 2005); following several testing protocols (Bembey et al., 2006a; Isaksson et al., 2010; Oliver and Pharr, 1992; Oyen, 2007; Oyen and Cook, 2003; Zhang et al., 2008); and using indenters of different size/shape (Bushby et al., 2004; Paietta et al., 2011). However, none of these experiments have explored the influence of both testing and analysis methods on the mechanical properties of the same samples.

In this chapter, the tibiae of genetically identical mice are indented both wet and dry, and after different embedding processes. Both spherical and Berkovich indenter probes are utilised, and data analysis is considered within the OP, VE and VEP frameworks. Thus, for the first time, direct comparisons can be made of mechanical properties of bone measured by nanoindentation after following different testing and analysis protocols.

3.3.1 Materials and Methods

The outline of the materials and methods is shown below in Figure 3.8.

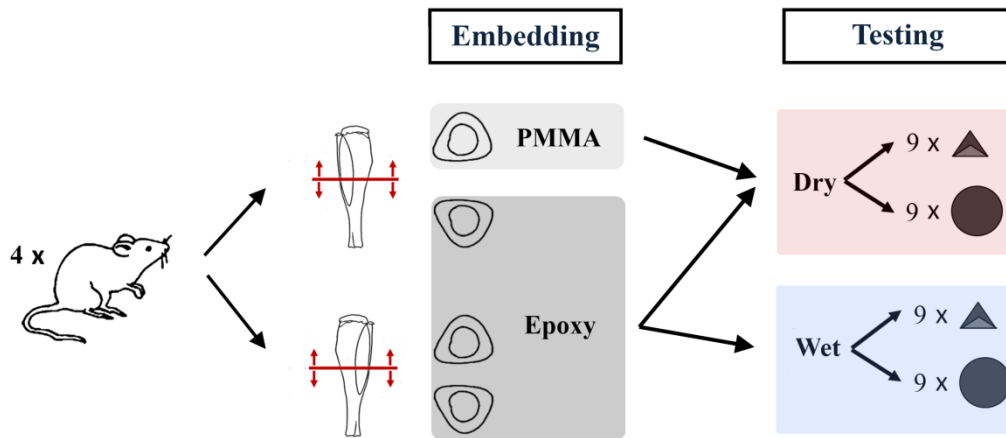


Figure 3.8: Outline of methods. Tibiae of four B6 mice were cut in half. From each mouse, one half-tibia was embedded in PMMA and the other three halves were embedded in epoxy resin. PMMA samples were tested in dry conditions and the epoxy samples were tested in dry and wet conditions. For each condition, nine indents were made with pyramidal and sphere indenter tips.

Specimen Preparation

Tibiae from four 9 week-old female C57BL/6 (B6) mice were cleaned of soft tissue. One tibia from each mouse was cut transversally at the mid-diaphysis using a low speed diamond saw (Isomet, Buehler GmbH, Germany). Half of one tibia from each mouse (four halves) were fixed in 70% ethanol for 48 hours, dehydrated in a series of increasing concentrations of ethanol (80, 90 and 100% for 24, 24 and 72 hours respectively), and changed to a xylene solution (48 hours). The bones were then infiltrated in pure methyl methacrylate (MMA + α -azo-iso-butyronitrile, VRW, UK) under vacuum for 24 hours. The MMA was changed for fresh MMA and infiltrated for a further 24 hours. The four half tibiae were kept in a vacuum chamber and they were let to polymerise at room temperature.

The rest of the tibiae (one whole and one half from each mouse) were kept frozen at -20°C in phosphate buffered saline (PBS) gauze. Tibiae were thawed and dried in air for an hour, embedded in low viscosity epoxy resin (EPOTHIN; Buehler, Lake Bluff, IL, USA), and allowed to cure at room temperature for 24 hours. No vacuum chamber was used, in order to minimise the infiltration of the epoxy in the bone. The whole tibiae were also sectioned transversally at the mid-diaphysis in order to have 12 specimens (3 from each mouse) embedded in epoxy resin.

All cross-sections were polished using increasing grades of carbide papers (from P600 to P1200) and finally with diamond slurry of 3, 1, 0.25 and 0.05 μm particle size. The samples were cleaned ultrasonically with distilled water between each polishing step.

Nanoindentation tests

Nanoindentation studies were conducted on the tibia mid-diaphyseal cross-sections using the TI700 UBI (Hysitron, Minneapolis, MN, USA). A maximum load of 8 mN was applied longitudinally at a constant loading rate of 0.8 mNs^{-1} following a holding time of 30 s (Figure 3.9). Nine indents were made in each specimen for each condition. A minimum spacing of three times the contact radius ($a_{\text{max}} \sim 5 \mu\text{m}$) was left between indents to avoid interference between adjacent indents (Paietta et al., 2011).

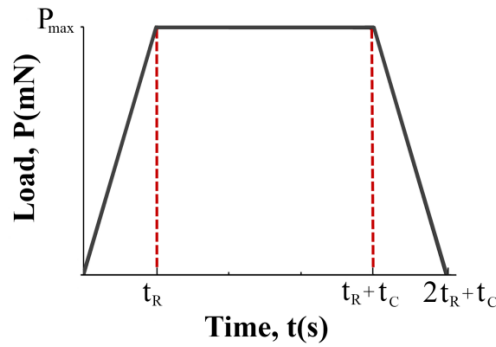


Figure 3.9: Trapezoidal load profile: loading to the peak load ($P_{\text{max}} = 8 \text{ mN}$) during rise time ($t_R = 10 \text{ s}$) with a creep hold ($t_C = 30 \text{ s}$).

Tests were first performed on dry PMMA-infiltrated and epoxy-embedded samples using a Berkovich diamond tip. Then, the epoxy-embedded samples were re-polished and rehydrated in distilled water overnight. A second set of indentations with the same load protocol was carried out on the rehydrated samples. Care was taken to prevent sample drying, which was identified by observing a reduction in the displacement. When the maximum displacement dropped to similar displacements as the ones recorded in the dry samples ($\sim 400 \text{ nm}$ in spherical indents and $\sim 700 \text{ nm}$ in Berkovich indents), samples were considered to be dry. Hence, they were removed from the stage and re-hydrated again. The same dry-wet procedure was followed for testing with a $55 \mu\text{m}$ radius spherical tip. This sphere size was chosen so that the contact areas were relatively small ($A_c \sim 55\text{--}80 \mu\text{m}^2$), for comparison with the Berkovich results ($A_c \sim 10\text{--}35 \mu\text{m}^2$), but sufficiently large to avoid plasticity during indentation. The elastic behaviour (with no plasticity) was verified by observing the load-displacement curves on a logarithmic scale (Oyen, 2010), as explained later in the discussion.

3.3.2 Data Analysis

The trapezoidal loading profile resulted in load-displacement ($P-h$) and displacement-time ($h-t$) curves, such the ones shown in Figure 3.10.

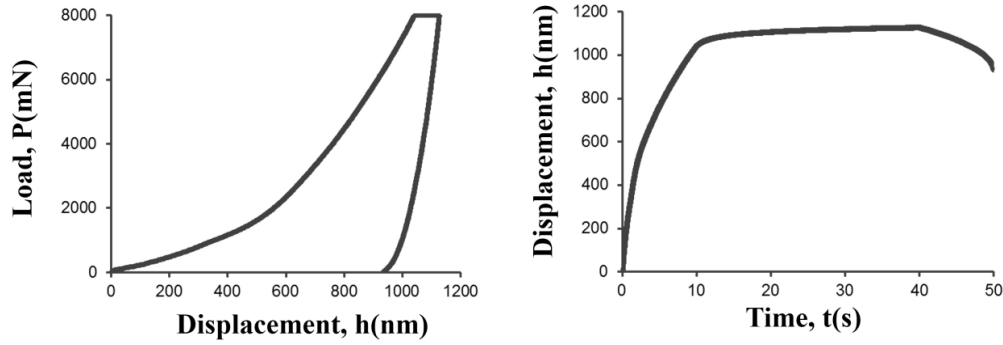


Figure 3.10: $P-h-t$ curves obtained on a rehydrated sample using a Berkovich indenter probe.

Analysis models

As previously discussed in Section 3.2, three models were used to extract bone's mechanical properties from the $P-h-t$ curves: Oliver-Pharr, viscoelastic and viscoelastic-plastic (Figure 3.11).

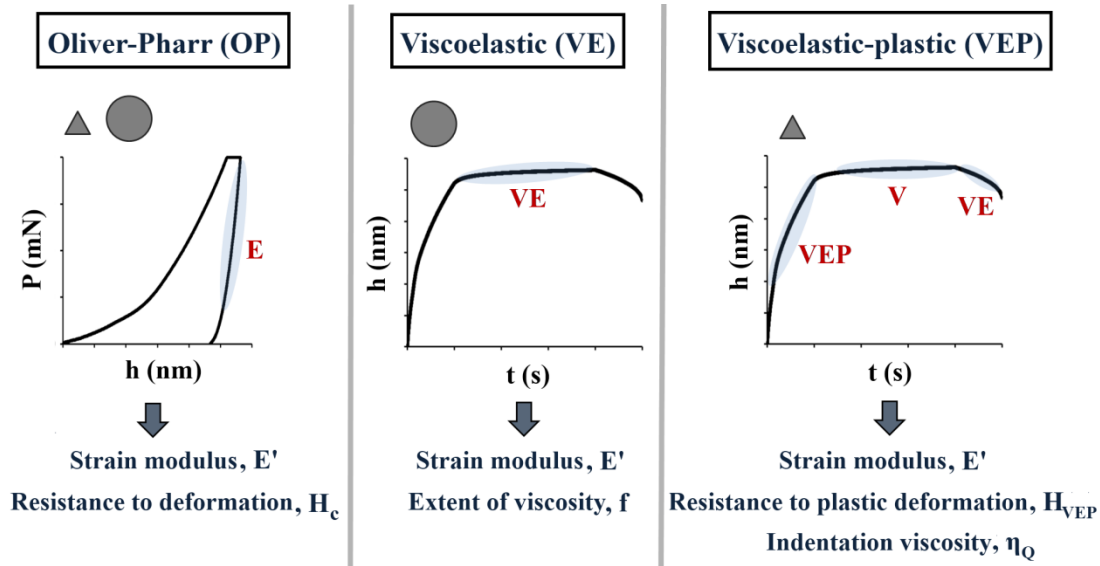


Figure 3.11: The three data analysis models compared in this chapter. The Oliver-Pharr method was used in Berkovich and spherical indentations, the viscoelastic analysis in spherical indentations and the viscoelastic-plastic approach in Berkovich indentations. The common material property inferred from the three models is the plane strain modulus.

Oliver-Pharr analysis was employed in Berkovich and spherical indentations. The unloading curve, which was considered to have an elastic response, was used to

calculate the plane strain modulus E' and the resistance to deformation H_c . These properties were obtained directly from the nanoindenter software.

The *viscoelastic* approach was used for spherical indentations. The creep hold period was evaluated to obtain E' and the extent of viscosity f . The nonlinear least-square curve-fit function (`lsqnonlin`) in MATLAB (Mathworks, Natick, MA) was used to perform this routine. The viscoelastic fitting of the creep hold started with an initial guess, which was iterated until the best fit was achieved (default tolerance options were used).

Berkovich indentations were analysed via the *viscoelastic-plastic* model to obtain E' , the resistance to plastic deformation H_{VEP} , and the indentation viscosity η_Q . In this approach, the loading was considered to have a viscous-elastic-plastic (VEP) response, the creep was considered to have a viscous (V) behaviour and the unloading viscous-elastic (VE). Taking this into account, a 3-step process was developed in MATLAB to extract the mechanical properties: i) η_Q was calculated by fitting the holding period (Eq. 3.22); ii) knowing the indentation viscosity, E' was obtained from the unloading curve (Eq. 3.23); iii) finally, while these two parameters were held constant, the loading curve was used to compute H_{VEP} (Eq. 3.21).

Deformation partitioning

The displacements caused by the indentation can be defined as viscous h_v , elastic h_e , and plastic h_p deformations.

From the OP model, the plastic (h_{p_OP}) deformation was equal to the displacement at zero load at the end of the test, assuming that the unloading curve was purely elastic. However, if the viscous behaviour had not been exhausted during the holding period, this would only be an approximation. The elastic deformation (h_{e_OP}) was defined as the difference between the maximum and final deformations.

$$h_{p_OP} = h(t_{max}) = h_{final} \quad [3.31]$$

$$h_{e_OP} = h_{max} - h_{p_OP} \quad [3.32]$$

For spherical indentations with large radius, only elastic and viscous deformations are present. In the VE analysis, the displacement is defined as a function of the shear modulus.

$$h^{3/2}(t) = \frac{3}{16\sqrt{R}} \frac{P_{max}}{G(t)} \quad [3.33]$$

Hence, the displacement associated with the equilibrium modulus was considered to be the elastic displacement (h_{e_VE}), while the viscous deformation (h_{v_VE}) was estimated as the difference between the elastic displacement and the displacement associated with the instantaneous modulus.

$$h_{e_VE} = \left(\frac{3}{16\sqrt{R}} \frac{P_{max}}{G_{\infty}} \right)^{2/3} \quad [3.34]$$

$$h_{v_VE} = h_{e_VE} - \left(\frac{3}{16\sqrt{R}} \frac{P_{max}}{G_0} \right)^{2/3} \quad [3.35]$$

In sharp Berkovich indentations, the VEP model allowed for the partitioning of the indentation response into independent elastic (h_{e_VEP}), plastic (h_{p_VEP}) and viscous (h_{v_VEP}) deformation components (Ferguson, 2009), as shown in Eq. 3.27-3.29.

Statistical evaluation

Mean values and standard deviations of the mechanical properties of each specimen were computed. Normality tests were carried out between these means using Shapiro-Wilk test. Dependent t-test was used to compare normally distributed data sets; Wilcoxon signed-rank test was used for non-parametric data. A difference was considered significant when $p < 0.05$. Statistical analysis was performed using SPSS (v. 20, SPSS Inc., Chicago, IL).

3.3.3 Results

Table 3.1 summarises the mean values of the mechanical properties obtained from this study. From the VEP analysis, the plane strain modulus (E'), hardness (H_{VEP}), contact hardness (H_c), indentation viscosity (η_0) and time constant (τ_I) were measured. The OP method gave the reduced modulus (E_R , which in this case is equal to E') and the contact hardness (H_c). From the VE approach the plane strain modulus (E') and the extent of viscosity (f) were calculated together with the time constants (τ_1, τ_2).

Table 3.1: Means and standard deviations (in brackets) of B6 cortical tibiae mechanical properties according to the probe geometry and data analysis method. E' is the plane strain modulus; H_{VEP} is hardness, resistance to plastic deformation; H_c is contact hardness, resistance to deformation; η_Q is indentation viscosity; f represents the elastic fraction (viscous, $0 \leq f \leq 1$, elastic); and τ_1, τ_2 are viscoelastic time constants (one time constant for VEP and two for VE).

		<i>Berkovich</i>		<i>Sphere</i>	
		VEP	OP	VE	OP
<i>Dry pmma</i>	E' (GPa)	36.4 (9.0)	22.9 (3.7)	7.2 (2.6)	15.4 (3.7)
	H_{VEP} (GPa)	2.57 (0.40)			
	H_c (GPa)	0.93 (0.06)	0.93 (0.07)		0.17 (0.05)
	η_Q ($\times 10^{15}$) (Pa s²)	2.96 (1.86)			
	$f = G_\infty/G_0$			0.63 (0.04)	
	τ_1, τ_2 (s)	277.2 (64.7)		2.0 (0.8) 19.6 (14.6)	
<i>Dry epoxy</i>	E' (GPa)	33.7 (6.4)	20.1 (3.9)	6.6 (2.0)	11.6 (1.7)
	H_{VEP} (GPa)	1.91 (0.56)			
	H_c (GPa)	0.75 (0.16)	0.74 (0.19)		0.15 (0.05)
	η_Q ($\times 10^{15}$) (Pa s²)	2.53 (1.62)			
	$f = G_\infty/G_0$			0.54 (0.13)	
	τ_1, τ_2 (s)	252.0 (73.4)		2.0 (0.7) 18.8 (11.5)	
<i>Wet epoxy</i>	E' (GPa)	27.5 (6.5)	11.5 (2.0)	4.1 (1.4)	9.2 (2.4)
	H_{VEP} (GPa)	0.47 (0.11)			
	H_c (GPa)	0.26 (0.04)	0.23 (0.03)		0.10 (0.04)
	η_Q ($\times 10^{15}$) (Pa s²)	0.50 (0.28)			
	$f = G_\infty/G_0$			0.51 (0.08)	
	τ_1, τ_2 (s)	133.3 (39.0)		2.0 (0.6) 17.3 (9.0)	

The mean values of plane strain moduli, which is one parameter comparable across all models, vary dramatically depending on the test method (Figure 3.12) ($p < 0.001$). Berkovich indentations on epoxy-embedded dry bones analysed by VEP measured a mean plane strain modulus of 33.7 GPa; while rehydrating the same bones and indenting them with a spherical tip and utilising the VE analysis, the mean modulus value was nearly an order of magnitude smaller, at 4.1 GPa.

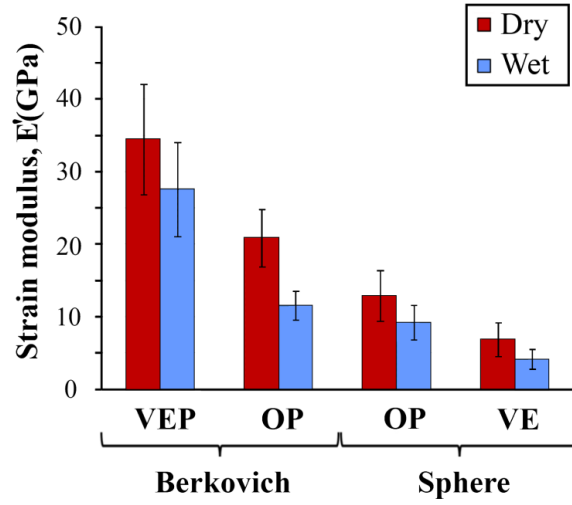


Figure 3.12: Mean and standard deviations of plane strain moduli of epoxy-embedded bone for Berkovich indentations analysed by VEP and OP, and spherical indentations modelled with OP and VE in dry and rehydrated conditions.

Hydration state

Plane strain modulus was significantly greater in dry specimens than in rehydrated specimens in all the cases ($p = 0.035$ for VEP; $p < 0.001$ for OP indented by Berkovich; $p = 0.006$ for OP indented by sphere; $p < 0.001$ for VE). The VEP model showed that the hardness and the viscosity term were also significantly higher in dry specimens ($H_{VEP_dry} = 1.91 \pm 0.56$ GPa; $\eta_{Q,dry} = 2.53 \pm 1.62 \times 10^{15}$ Pa s²) than in their wet counterparts ($H_{VEP_wet} = 0.47 \pm 0.11$ GPa; $\eta_{Q,wet} = 0.50 \pm 0.28 \times 10^{15}$ Pa s²) ($p < 0.001$ for H_{VEP} ; and $p = 0.003$ for η_Q).

Indenter probe geometry

In each of the hydration states, Berkovich indentations yielded bigger plane strain moduli ($E'_{dry} = 27.6 \pm 9.1$ GPa, $E'_{wet} = 19.5 \pm 9.4$ GPa) than spherical indentations ($E'_{dry} = 9.8 \pm 3.9$ GPa, $E'_{wet} = 6.4 \pm 3.1$ GPa) ($p < 0.001$ for dry indentations, and $p = 0.002$ for wet indentations). Even when the same OP approach was employed to analyse the data, the plane strain moduli were bigger in Berkovich indentations than in spherical indentations ($p < 0.001$ for dry indentations, and $p = 0.01$ for wet indentations), as shown in Table 3.1.

Embedding medium

No significant differences were found between the plane strain moduli and viscosity values of dry epoxy-embedded and PMMA-infiltrated samples across the models ($p > 0.3$ for E' , $p = 0.233$ for η_0 , and $p = 0.071$ for f). However, the VEP model showed that the hardness of bone was larger for PMMA-infiltrated samples ($H_{VEP_PMMA} = 2.57 \pm 0.40$ GPa), where the pores were filled with resin, than for epoxy embedded ones ($H_{VEP_epoxy} = 1.91 \pm 0.56$ GPa), where there was no resin in the pores ($p = 0.026$). In both cases, the material indented was bone and pores were avoided.

Deformation partitioning

The mean elastic h_e , plastic h_p and viscous h_v deformations and deformation fractions h_e/h_{max} , h_p/h_{max} , h_v/h_{max} for each condition are summarised in Figure 3.13. The total deformation increased from dry to rehydrated conditions both in Berkovich ($h_{max_dry} = 686 \pm 75$ nm, $h_{max_wet} = 1276 \pm 175$ nm; $p = 0.002$) and spherical indentations ($h_{max_dry} = 377 \pm 100$ nm, $h_{max_wet} = 555 \pm 157$ nm; $p = 0.001$). However the deformation partitioning depended on the method used to analyse the same data.

In the VEP approach, elastic, plastic and viscous deformations increased significantly from dry to wet conditions ($p = 0.025$ for h_e ; $p = 0.002$ for h_p ; $p < 0.001$ for h_v). All deformation fractions also varied according to the hydration state ($p < 0.001$ for h_e/h_{max} and h_p/h_{max} ; $p = 0.038$ for h_v/h_{max}).

In the OP method, elastic deformations did not change with hydration state (not in Berkovich indentations nor in spherical indentations). However, plastic deformation increased significantly from dry to rehydrated bone ($p = 0.002$ in Berkovich indentations; $p = 0.001$ in spherical indentations). Both deformation fractions also changed significantly with the hydration state ($p < 0.001$ in Berkovich indentations, $p = 0.041$ in spherical indentations).

The VE model exhibited the highest elastic and viscous deformations, showing a significant increase in both deformations from dry to wet conditions ($p < 0.001$ for h_e , $p = 0.002$ for h_v). In contrast, the values of elastic and viscous fractions analysed by VE did not depend on the hydration state.

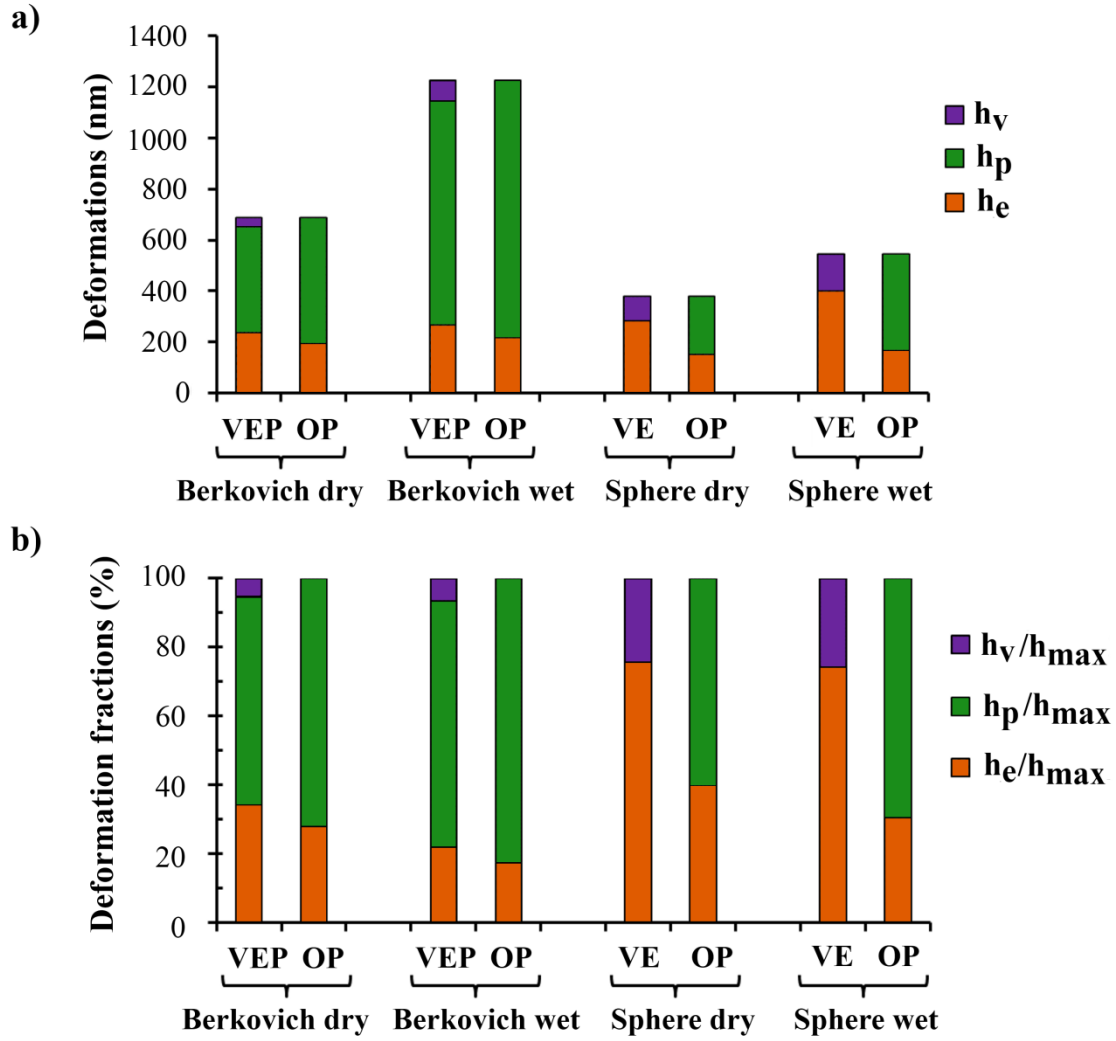


Figure 3.13: Viscous h_v , plastic h_p and elastic h_e average deformations (a) and mean deformation fractions (b) of Berkovich and spherical indentations in dry and rehydrated conditions.

3.3.4 Discussion

In this chapter, systematic investigations of the effect of a wide range of indentation testing methodological options were considered for indentation of the same bone samples. Results show that the measured mechanical properties depend on the hydration state of the samples, the probe geometry and the model used to analyse the data.

The measured plane strain moduli are comparable to the wide range of values found in literature (Figure 3.14). In Berkovich indentations, plane strain modulus of 31 GPa was measured for B6 mice femur embedded in epoxy (Chang et al., 2011). Moduli of 22 GPa were found in NTG mice femur submerged in water (Lopez Franco et al.,

2011). Bushby et al. (2004) had modulus of 18 GPa for equine bone embedded in PMMA indented using a spherical tip. Spherical indentations on fully rehydrated equine bone gave modulus as small as 2 GPa (Oyen et al., 2012) after analysing the data using a viscoelastic approach. Until now, these discrepancies in values were considered to be mainly the result of the differences between the bones. However, this study demonstrates that different methods give dramatically different results even on the same bone.

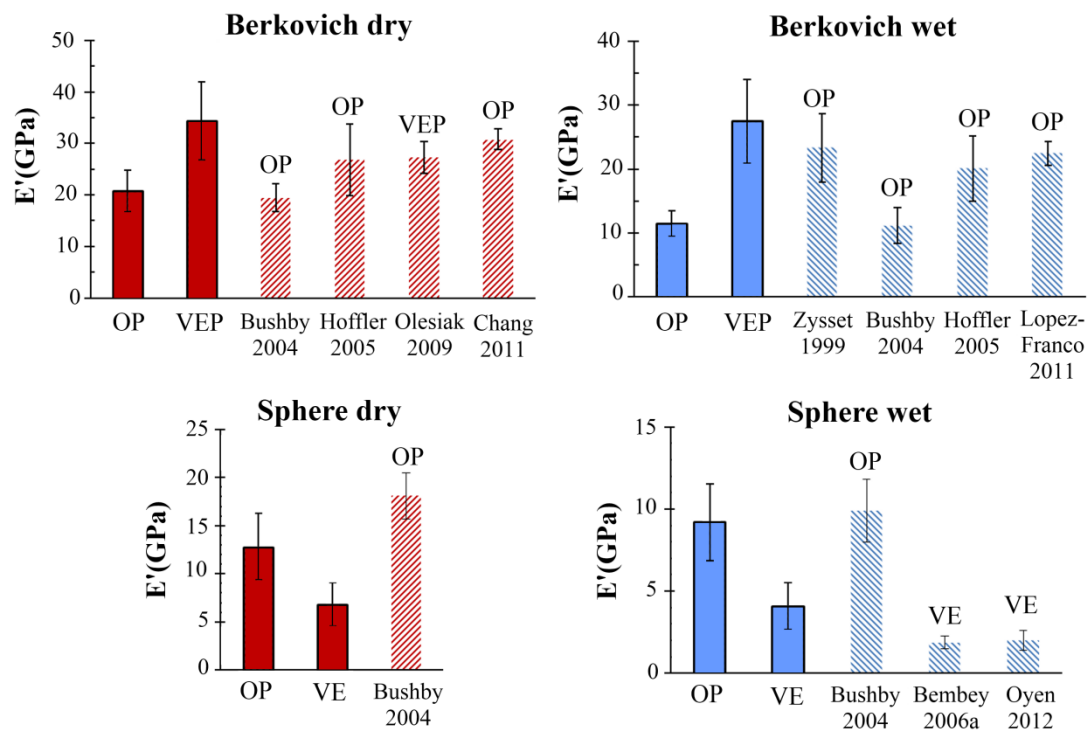


Figure 3.14: Comparison of mean/standard deviation of the plane strain moduli of the current study (solid colour) with literature. The analysis method is specified as OP, VE or VEP. The measured values are comparable with the range found in literature for different animal bones.

Bone hydration

Plane strain modulus was significantly higher in dry specimens than in rehydrated specimens in all the cases (Figure 3.12). This tendency is in accordance with literature (Bembey et al., 2006a, 2006b; Bushby et al., 2004; Hoffler et al., 2005). The deformation partitioning (Figure 3.13) showed that in all the cases the total deformation is larger in rehydrated bone than in dry bone. It must be noted that the wet samples considered here were not immersed in fluid while testing, and therefore the differences in values of fully rehydrated samples might be larger than the ones measured here. All

the methods trend in the same direction showing the capability of nanoindentation to capture differences in hydration states.

Probe geometry

Results demonstrate that probe geometry influences the indentation response. Berkovich indentations led to plastic deformations, indicated by the measured plastic deformation fraction 60-80% (Figure 3.13). In contrast, spherical tips allowed extended elastic to plastic transition, which can be detectable by plotting P - h curves in logarithmic scale (Oyen, 2010) (Figure 3.15). A curve parallel to $P \sim h^{3/2}$ is associated with an elastic regime, while a curve parallel to $P \sim h$ represents plastic behaviour. Figure 3.15 shows an initial rough phase (shaded in blue), where the tip has not made complete contact with the surface yet, followed by a curve parallel to the $P \sim h^{3/2}$ elastic law. Since there is not an elastic-to-plastic ($P \sim h^{3/2}$ to $P \sim h$) transition in the upper part of the curve, the assumption of no plasticity used in the viscoelastic model is valid.

On the other hand, the roughness observed in the first part of the curve in Figure 3.15 leads to an overestimation of the contact displacement, and thus the contact area. This might cause an underestimation in the value of plane strain moduli (Zhang et al., 2008). In this thesis, data was rejected if the initial roughness curve (shaded in blue) exceeded 5% of the maximum load. Nevertheless, roughness is the likely one cause of discrepancies between the Oliver-Pharr results for bone tested in the same condition—wet or dry—with the two different indenter tips.

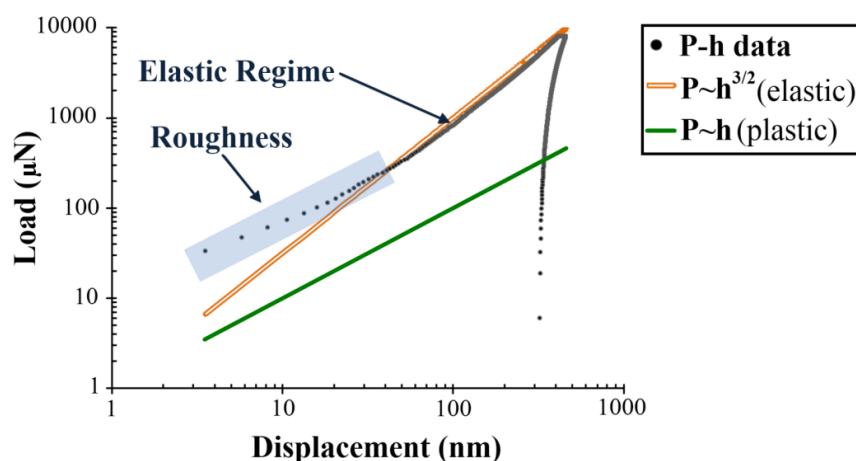


Figure 3.15: Logarithmic curve of P - h data for a spherical indent on epoxy-embedded dry bone. There is no elastic to plastic transition.

Viscous, plastic and elastic deformation fractions for both probe geometries are shown in Figure 3.13. In Berkovich indentations the viscous deformation fraction was less than 10%. Hence, even though Oliver-Pharr method did not capture time-dependent deformation, the deformation fractions for VEP and OP were similar. On the other hand, in spherical indentations the viscous deformation was about 25% of the total deformation. Although deformation partitioning in the OP case indicated that the plastic deformation was dominant even in spherical indentations, the P - h curves in logarithmic scale (Figure 3.15) showed that there was no plasticity induced in spherical indentations. This reflects that Oliver-Pharr method can incorrectly predict plasticity when it does not occur.

Embedding protocol

The embedding protocol did not result in significant differences between the plane strain moduli and viscosity values across the models. This demonstrates that nanoindentation measures local properties of bone. However, the VEP model showed that the hardness was higher for PMMA-infiltrated samples. Unlike in epoxy samples, in PMMA samples, a vacuum chamber was used to infiltrate the resin into the bone pores, which could contribute to an increase in hardness.

Analysis method: assumptions and limitations

Bone is a time-dependent material. However, OP analysis cannot capture the viscous behaviour of bone. The VEP is a single time constant model, where linear creep rate is assumed, and its prediction capability is limited when indenting a hierarchical material with different time scales such as bone (Wang and Lloyd, 2010). As such, the VEP model could only be fitted to the steady-state creep (Eq. 6.24). The VE model with two time constants gives a better approximation of the creep hold period than the VEP model, as the entire creep period can be fitted by the model. Time constants provide information about the time scales of deformation in the material relative to the time frame of the experiment.

All three models for data analysis here are based on the same fundamental elastic contact mechanics for indentation (Sneddon, 1965) (Figure 3.16). Hence, the elastic moduli should not differ from one approach to the next. The extension of elastic to VE (Lee and Radok, 1960) is the approach containing the most direct adaptation of elastic contact mechanics, and that containing the fewest simplifying assumptions. Once

plastic deformation is included, the picture gets more complicated. While Oliver-Pharr has been shown to be accurate for stiff materials, it overestimates modulus values for polymeric materials (Ngan et al., 2005; Tranchida et al., 2007), in part because of the time-dependent deformation in polymers (such as in bone). The VEP model has the most *a priori* assumptions. VEP assumes that the viscous, plastic and elastic deformations are in series and that the creep is linear, which is too simple to capture the more complex, multiple time constant behaviour observed in bone. This results in moduli that are larger than the ones obtained with the VE or OP approach. A tendency towards modulus overestimation was observed when this model was used for characterisation of polymers as well (Oyen and Cook, 2003). Therefore, VEP is useful for comparison of groups within studies, but further development of this model is required before quantitative material properties can be determined.

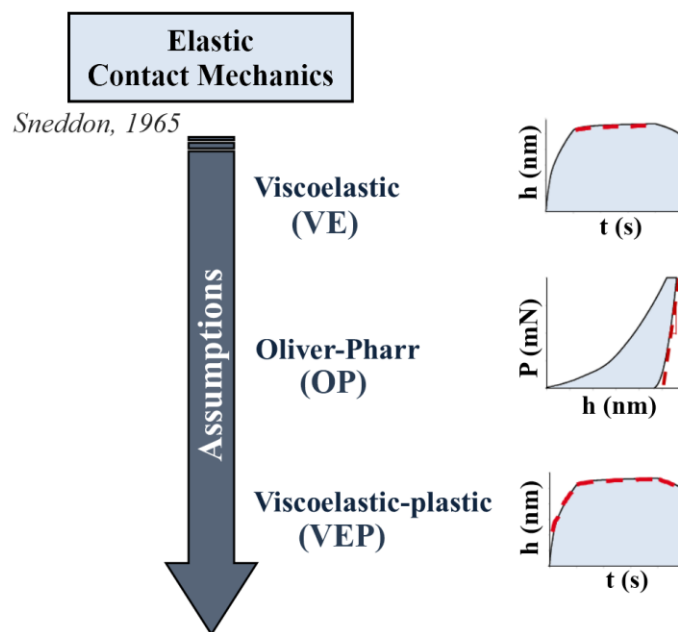


Figure 3.16: The three models (VE, OP, VEP) are based on the same elastic contact mechanics for indentation. The analysis method with the fewest assumptions is the VE. Each model is fit to different sections of the indentation response.

Each of the analytical models is fitted to different parts of the indentation P - h - t response (Figure 3.16). The most direct differences observed here were for spherical indentation using Oliver-Pharr, which is a fit only to the unloading data, and VE, which is a fit only to the load-hold data. The reasons for the large discrepancy between the obtained modulus values in these two cases certainly requires further detailed study in the future, but the most likely explanation is the failure of OP to account for

viscoelastic deformation during unloading. This study provides the most direct evidence yet of the extent of this effect in materials with time-dependent mechanical behaviour. While many studies have advocated for a hold period at peak load to “exhaust” viscoelastic deformation and minimise its effect during unloading, the results here demonstrate that with the load profile used in the current study, this approach does not provide reliable quantitative data on bone nanoindentation. Similar conclusions have also been previously suggested (Oyen and Ko, 2007). In the study by Oyen and Ko, two values of plane strain modulus that differed by a factor of 2 were used to generate two load-displacement curves derived from the VEP model. The combination of the chosen combination of E' and η_Q resulted in two curves with equivalent unloading slopes. Since in the OP approach the value of the stiffness S is calculated from the unloading slope, both curves yielded to similar values of stiffness. Using Eq. 3.3 to calculate E' for each curve with the inferred stiffness values led to two values of plane strain modulus that differed only by a factor of 1.2 (instead of 2). This again indicates the limitation of the OP method for analysing time-dependent materials.

Summarising, the OP method can be used for a fast identification of relative differences in the elastic moduli between samples. When OP is chosen, it is recommended to use a trapezoidal loading profile with a long holding period and rapid unloading, to minimise as much as possible the time-dependent behaviour of bone. The VEP model provides an estimation of the elastic, plastic and viscous contributions of the material behaviour in Berkovich indentations. When plastic deformation is not of interest, the VE approach using spherical indentation allows the analysis of the creep behaviour of bone. Overall, these methods can be used to make comparative analysis of the nanoindentation properties of bone: anisotropy of bone can be explored by comparing indents made in the longitudinal and transversal directions; and the effect of ageing or skeletal pathologies on tissue mechanics can be evaluated by comparing the nanoindentation properties of bones of different ages and pathologies.

3.4 Conclusions

This first systematic study of the same bones following different testing and analysis options demonstrated that **tissue level mechanical properties of bone measured by nanoindentation depend not only on the sample itself, but also on the hydration state, probe geometry and data analysis method.** The use of the viscoelastic approach is recommended for future experiments, as by avoiding plastic deformation, the VE method has the least simplifying assumptions when compared to the OP and VEP methods. At the same time, results indicated that **nanoindentation is capable of capturing relative trends in the mechanical properties of bone**, since all the approaches were able to distinguish between dry and rehydrated samples. Hence, nanoindentation provides the framework to compare tissue level mechanical properties of different type of bones, such as bones of different ages (Chapter 4) or pathologies (Chapter 6).

In this chapter, the time-dependent behaviour of bone was captured using a viscoelastic model. However, the used rheological model of springs and dashpots (Figure 3.5) does not reveal the actual structural and physiological reasons behind the time-dependent response. When bone is fully submerged in fluid, bone's time-dependence can be explored analysing the poroelastic behaviour induced by the fluid that flows through its porous network. Such poroelastic analysis can be used to directly obtain bone's elastic properties (E , ν) and the intrinsic permeability (k), thus characterising the biphasic nature of bone. In Chapter 4, nanoindentation is used to compare poroelastic properties of young and aged B6 mouse bone.

4 NANOINDENTATION TO MEASURE PERMEABILITY: APPLICATION IN AGEING

The research described in this chapter has been published in: N. Rodriguez-Florez, M.L. Oyen, S.J. Shefelbine, 2014, Age-related changes in mouse bone permeability, Journal of Biomechanics 47(5), 1110-1116. Copyright (2014) with permission of Elsevier.

In physiological conditions, bone exhibits not only the viscous-elastic-plastic response to load described in the previous chapter, but also poroelastic behaviour caused by the fluid movement. Evidence suggests that fluid flow through bone's lacunar-canalicular porous network may be the stimulus with which bone cells sense changes in the mechanical environment. Hence, the determination of lacunar-canalicular permeability, which measures the ease with which fluid passes through bone's porous network, could provide a better understanding of how bone adapts to its mechanical environments.

Experimentally measuring lacunar-canalicular permeability is challenging due to the heterogeneity of bone, small size of the pores and the interconnected dual porosity in bone (vascular and lacunar-canalicular). As a result, the estimates of lacunar-canalicular permeability found in literature vary by up to eight orders of magnitude (Cardoso et al., 2013). In this chapter, nanoindentation is proposed as a tool to determine permeability in cortical mouse bone. In particular, poroelastic properties of young and aged bone are compared to test the hypothesis that permeability deteriorates with age.

The objective of this chapter is to use a poroelastic approach based on nanoindentation data to characterise lacunar-canalicular permeability in mouse bone as a function of age.

4.1 Lacunar-Canalicular Permeability and its Role in Mechanotransduction

Bone is a dynamic tissue that continuously adapts its architecture and material properties in response to the mechanical demands. Previous studies have established that this functional adaptation is most likely coordinated by osteocytes, the mechanosensor cells in bone (Burger and Klein-Nulend, 1999; Cowin et al., 1995; Han et al., 2004). Osteocyte bodies lie in the lacunar pores in the hard mineralised bone matrix. Communication between neighbouring cells occurs through the mineralised matrix via the canaliculi. Together these structures constitute the lacunar-canalicular porosity (Figure 4.1), previously described in Chapter 2 (Figure 2.6).

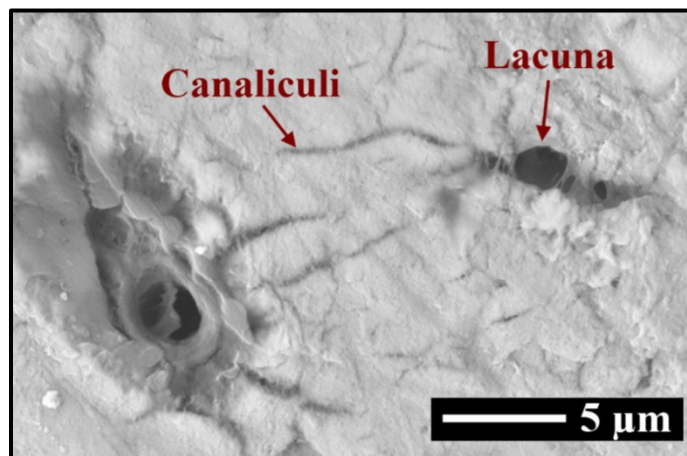


Figure 4.1: Lacunar pores and canaliculi channels of 2 month-old B6 tibia captured by SEM.

Due to the challenges associated with the *in vivo* observation of osteocytes, which lie deep within bone matrix, the precise mechanical stimulus that drives these cells to respond has yet to be elucidated. Evidence suggests that mechanical loads acting on bones at the organ level drive a flow of interstitial fluid through its porous network, which in turn activates osteocytes to regulate bone-adaptation. The primary loading-induced fluid motion appears to be through the lacunar-canalicular network, where osteocyte cells sense shear stress due to interstitial fluid movement (Anderson and Knothe Tate, 2008; Burger and Klein-Nulend, 1999; Fritton and Weinbaum, 2009; Gardinier et al., 2010; Jacobs et al., 2010; Klein-Nulend et al., 2013; Knothe Tate, 2003). Lacunar-canalicular permeability indicates the ease with which interstitial fluid passes through the lacunar pores and canaliculi of bone, and it can be described by Darcy's law.

Darcy's Law

Darcy's law states that fluid flows away from regions of high pressure to regions of low pressure according to:

$$q_i = -\kappa \frac{dp}{dx_i} \quad [4.1]$$

where q_i [m/s] is the fluid flux, κ [m²/Pa-s] is Darcy's hydraulic permeability and dp/dx_i [Pa/m] denotes the pore pressure gradient. Darcy's hydraulic permeability κ is a property of the structure of the matrix and the viscosity of the fluid, since it characterises the viscous drag that results as a fluid flows through a solid matrix. Hydraulic permeability is defined as the ratio of the intrinsic permeability k [m²] to the fluid dynamic viscosity μ (for water $\mu = 0.001$ Pa-s is assumed):

$$\kappa = k/\mu \quad [4.2]$$

The intrinsic permeability k is related to the porous bone structure solely (the connectedness of the porosity and the size and spatial arrangement of the pores), not the fluid in the pores. The values reported in the literature for lacunar-canalicular permeability correspond to the intrinsic permeability k [m²].

Estimates of Lacunar-Canalicular Permeability

Evidence suggests that characterising lacunar-canalicular permeability will help understand the role of fluid flow in the mechano-transduction mechanism of bone (Anderson et al., 2008; Burger and Klein-Nulend, 1999; Lemaire et al., 2011; Price et al., 2011). However, due to the challenges associated with the measurement of lacunar-canalicular permeability, the first estimates were purely theoretical. Lacunar-canalicular intrinsic permeability was traditionally estimated based on Biot's poroelasticity theory, giving values from 10^{-22} to 10^{-19} m² (Gururaja et al., 2005; Wang et al., 1999; Zhou et al., 2008). Finite element models also predicted permeability values in a similar order of magnitude, from 10^{-22} to 10^{-18} m² (Lemaire et al., 2011; Smit et al., 2002). Values from 10^{-23} to 10^{-19} m² were reported based on microstructural models of lacunar-canalicular porosity. However, recent experiments have measured smaller values of permeability than those predicted theoretically. Stress-relaxation of single osteons measured 10^{-25} - 10^{-24} m² (Gailani et al., 2009) and compaction of bone gave values of 10^{-23} m² (Gardinier et al., 2010). Nanoindentation studies measured permeability

between 10^{-24} to 10^{-21} m^2 for equine cortical bone (Galli and Oyen, 2009; Oyen, 2008; Oyen et al., 2012). Overall, these estimations of permeability range from 10^{-25} to 10^{-18} m^2 , as illustrated in Figure 4.2.

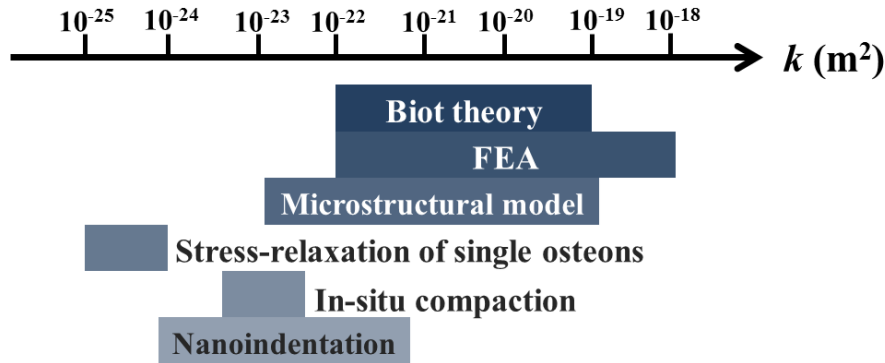


Figure 4.2: Reported intrinsic lacunar-canalicular permeability values (k) using different theoretical and experimental techniques.

This broad range of permeability values is mainly a result of the interconnected vascular and lacunar-canalicular porous network (Figure 4.3), which makes difficult to separate the contribution of each one to the permeability of bone (Benalla et al., 2012; Jast and Jasiuk, 2013; Knothe Tate et al., 2009).

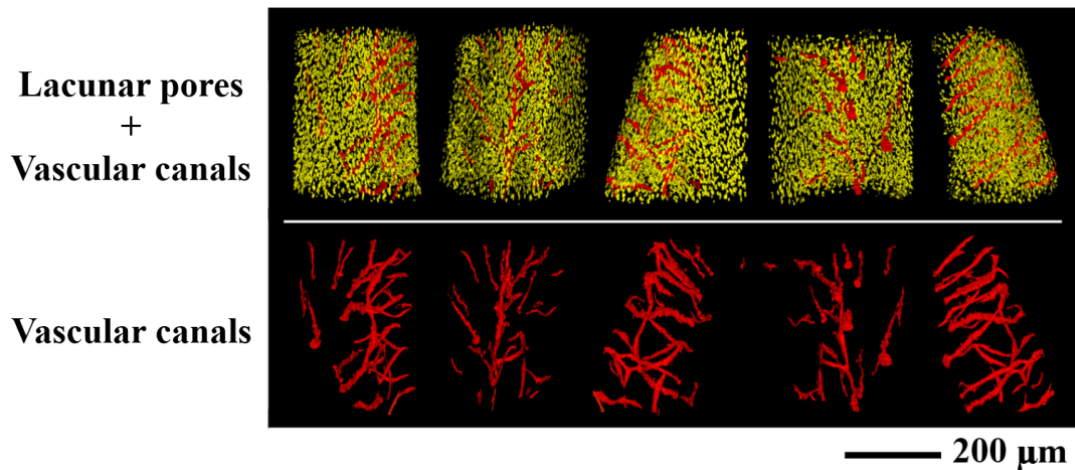


Figure 4.3: Nano-CT images of the porosity of 7 month-old B6 cortical tibia.

In this chapter, nanoindentation is chosen to isolate effects of lacunar-canalicular permeability independent of vascular permeability. To achieve this, bones are tested immersed in fluid and nanoindentation results are analysed following a poroelastic approach.

4.2 Nanoindentation of Bone Immersed in Fluid

A poroelastic analysis on nanoindentation data requires testing the samples fully submerged in fluid. However, due to the technical challenges that arise when samples are immersed in fluid, the vast majority of studies in bone nanoindentation have been performed on dry or partially rehydrated conditions, as described in Chapter 3. The protocol established in this thesis to indent hydrated bone is summarised in Appendix 9.1. This section encloses some experimental considerations that were taken into account in this thesis to perform nanoindentation on fully hydrated mouse bone.

Sample preparation

Due to the small size of the samples, mouse bones were embedded in epoxy resin following the protocol described in Chapter 3 (Figure 4.4). In this case, the final polishing was done using fine grid carbide paper instead of diamond slurry, in order to avoid diamond particles to penetrate into the micro-pores and affect the fluid flow. Since the indentations were automated, care was taken to keep the samples as flat as possible, as tilted samples could damage the transducer when moving to the next indent location.

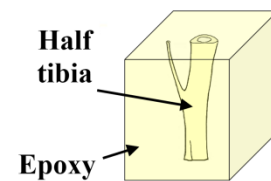


Figure 4.4: Schematic of half mouse tibia embedded in epoxy resin.

Fluid

Bones were submerged in distilled water instead of physiological saline to avoid any risk of damaging the nanoindenter.

Indenter probe

The poroelastic analysis required the use of spherical indenters to avoid inducing plasticity. Hence, spherical fluid cell probes were used. These are indenter tips that have an extended shaft to increase the distance between the liquid and the transducer (Figure 4.5). In the indentation of hydrated bone, it becomes increasingly complicated to keep the indenter tip clean, as collagen may act as glue. Sometimes, an air indent might aid

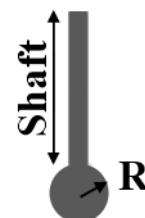


Figure 4.5: Schematic of a spherical fluid cell probe of radius R .

to remove the particles attached, but otherwise the tip should be carefully cleaned with acetone using cotton swaps, and checked under the microscope.

Container

A metallic container was used, where samples were glued. The container should be slightly bigger than the stage of the nanoindenter to allow the indenter to move within the whole range of movement and avoid the risk of hitting the edges of the container with the transducer. It should also be deep enough so that the samples are covered with ~2 mm of water above (Figure 4.6). The nanoindenter used in this thesis had a magnetic stage, which is why a metallic container was very useful. However, containers of other stiff materials, such as glass, could be used equally.



Figure 4.6: Schematic of a container filled with water. The sample should be covered with ~2 mm of fluid above.

4.3 Poroelastic Analysis via Nanoindentation

The poroelastic model used in this chapter was proposed by Oyen (2008) and further developed by Galli and Oyen (2009). This approach examines spherical indentation creep responses of fully saturated bone incorporating fluid motion through a porous elastic network based on a previously built master-curve library. Bone is considered an isotropic linear poroelastic material with incompressible constituents. Such assumption simplifies considerably the equations governing the poroelastic constitutive behaviour (Detournay and Cheng, 1993).

Poroelastic indentation theory

During the indentation ($t > 0$), the rapid local deformation from the spherical indenter causes fluid to be forced out of the material, resulting in pore pressure, which supports part of the applied load. As the fluid leaves the material, pore pressure decreases, resulting in a time-dependent deformation response, which is measured by the nanoindenter. As time passes ($t = \infty$) pore pressure reaches equilibrium and the load is supported by the solid skeleton. A schematic of a step load is shown in Figure 4.7.

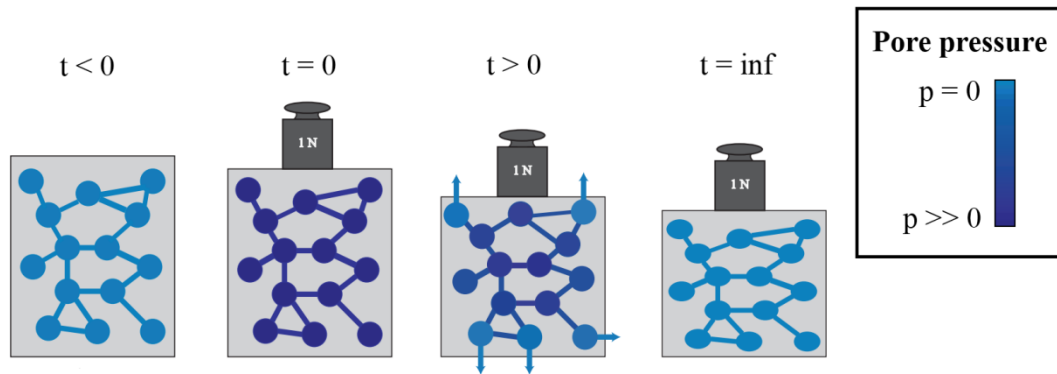


Figure 4.7: Schematic of the response of a poroelastic material to a step load. Pressure in the pores of the material range from $p = 0$ (light blue) to $p \gg 0$ (dark blue). Adapted from (Strange, 2013).

During the indentation process, there is a fluid flux from high pressure pores to regions of low pressure. This fluid motion is described by Darcy's law (Eq. 4.1). The material's poroelastic response can be fully characterised with five parameters (four elastic constants and the permeability): the shear modulus G ; the drained Poisson's ratio ν ; the undrained Poisson's ratio ν_u , ranging between $\nu - 0.5$; the Biot effective stress coefficient α , ranging between 0 and 1; and the Darcy hydraulic permeability κ .

The elastic properties (G and ν) correspond to the porous medium considered as a homogeneous linear elastic material. The undrained and drained cases of a fluid-infiltrated porous material represent its limiting behaviours (Figure 4.7). The undrained response characterises the condition where the fluid is trapped in the porous solid ($t = 0$); while the drained response describes the state of the material after the internal pore pressure has reached equilibrium with the applied load (pore pressure zero or the pressure corresponding to empty pores) ($t = \infty$).

The Biot effective stress coefficient α represents the variation of the fluid volume in a material unit volume due to the volumetric change of the element when loaded under the drained condition. For an ideal isotropic poroelastic material, the dimensionless α is defined as:

$$\alpha = 1 - \frac{K}{K_s} \quad [4.3]$$

where K [N/m^2] is the bulk modulus of the drained material (bone with pores); and K_s [N/m^2] refers to the bulk modulus of the material of which the solid skeleton is made (bone material). The bulk modulus is described as a function of the elastic properties (G , ν) of the bone with pores in the case of K or the bone material for K_s :

$$K = \frac{2G(1 + \nu)}{3(1 - 2\nu)} \quad [4.4]$$

Identification of poroelastic properties: The master-curve approach

There is no closed-form analytical solution to the problem of spherical indentation of a poroelastic material; hence, numerical solutions are required to determine the poroelastic parameters (G , ν , ν_u , α , κ). This was first addressed by Agbezuge and Deresiewicz (1974) and further elaborated by Deresiewicz (1976), who proposed a numerical framework to solve the problem of a spherical indenter applying a step load on a saturated poroelastic material (Agbezuge and Deresiewicz, 1974; Deresiewicz, 1976). However, step-loading conditions do not take into account the actual rise-time that is inevitable in experimental setups. Galli and Oyen (2009) extended this solution to ramp-hold test profiles and proposed a method to rapidly identify poroelastic constitutive parameters from the creep period of load-controlled spherical indentation tests. This is the method followed in this chapter.

Galli and Oyen (2009) solved the spherical indentation problem with varying ramp times using finite element modelling (Abaqus v 6.7) for several materials. When the time-displacement data during the creep period is normalised (Eq. 4.5-4.9), the time-dependent behaviour of poroelastic materials collapses onto a single curve. Therefore, by normalising the time-displacement indentation response (h^*-t^*) of the modelled materials Galli and Oyen (2009) created a master-curve library.

The non-dimensional creep displacement h^* relates the transition between the undrained and drained states and it is defined as:

$$h^* = \frac{h(t) - h_0(t)}{h_\infty(t) - h_0(t)} \quad [4.5]$$

where $h(t)$ is the time-dependent displacement of the indenter, $h_0(t)$ is the indentation depth that corresponds to step-loading conditions, and $h_\infty(t)$ is the indentation displacement at $t = \infty$ when the pore pressure field vanishes. These two values can be computed from the elastic solutions (Oyen et al., 2012) as follows:

$$h_0(t) = \left(\frac{3P(t)(1 - \nu_u)}{8GR^{1/2}} \right)^{2/3} \quad [4.6]$$

$$h_\infty(t) = \left(\frac{3P(t)(1 - \nu)}{8GR^{1/2}} \right)^{2/3} \quad [4.7]$$

where $P(t)$ represents the indentation load, R is the indenter tip radius, and G stands for the shear modulus. The normalised time t^* is given by:

$$t^* = \sqrt{\frac{ct}{Rh(t)}} \quad [4.8]$$

$\sqrt{Rh(t)}$ represents the contact radius of the indentation. The diffusivity coefficient c is a function of the five constitutive parameters:

$$c = \frac{2\kappa G(1 - \nu)(\nu_u - \nu)}{\alpha^2(1 - 2\nu)^2(1 - \nu_u)} \quad [4.9]$$

Using the master-curve library built by Galli and Oyen (2009), three constitutive parameters can be identified from the spherical indentation data. From Eq. 4.5-4.9, it can be observed that the permeability κ and the effective stress coefficient α only appear in the definition of the diffusivity coefficient c . Thus, even when G , ν and ν_u are

known, only κ/α^2 can be determined using this routine. Hence, in this approach, G , ν , and κ are considered unknown, while the value for α is set to 1 (Oyen, 2008) and the value of ν_u is set to 0.5 (Galli and Oyen, 2009; Oyen et al., 2012). Therefore, the constituents are considered incompressible. The indenter tip is considered to be impermeable.

The poroelastic analysis framework proposed and validated by Galli and Oyen (2009) is based on the above described normalisation of the time-displacement data and it consists of two optimisation steps (Figure 4.8).

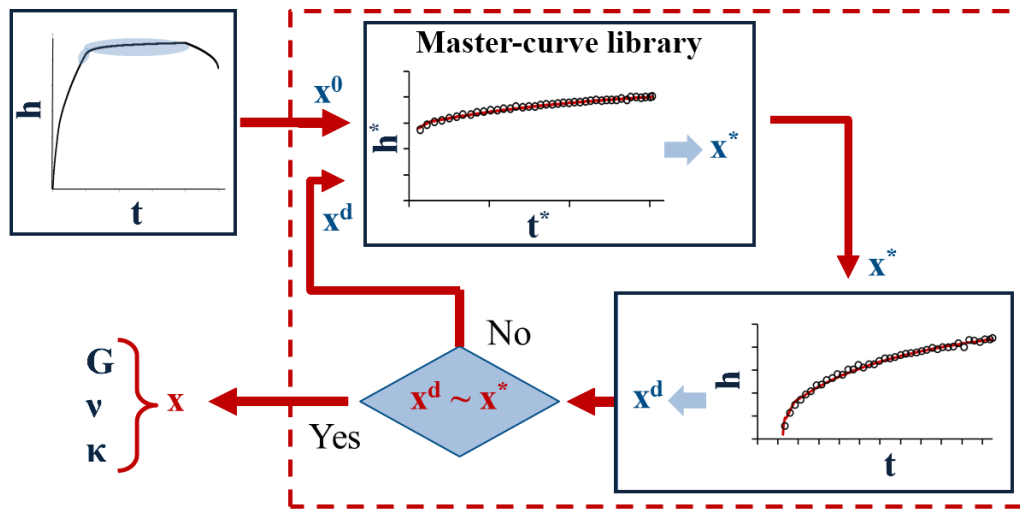


Figure 4.8: Spherical indentation poroelastic analysis framework proposed by Galli and Oyen (2009). The fitting in the normalised domain (h^*-t^*) based on the master-curve library results in x^* ; the result in the dimensional domain ($h-t$) is x^d . The final result is $x = [G, \nu, \kappa]$.

The first step occurs in the normalised domain (h^*-t^*), where based on initial guesses x^0 , the normalised curves of the indented material are fitted to the master curves in the database. In theory, the optimisation in the non-dimensional domain should already give the best fit for the dimensional domain. However, by normalising the indentation response, the noise of the experimental data can have a greater influence on the magnified holding region. Hence, the second optimisation routine is used to verify that the non-dimensional solution (x^*) gives also the best fit in the dimensional domain. The second routine uses the solution from the normalised domain (x^*) as initial guess to fit the curves in the time-displacement domain ($h-t$) and compute a new dimensional solution (x^d). Convergence is achieved when the difference between the parameters identified in each domain is negligible ($x^* \sim x^d$). The final solution (x) contains the values of G , ν , and κ .

4.4 Age-related Changes in Mouse Bone Permeability

It is known that bone's material properties in a single individual change with age: old bone is stiffer but less tough than young bone (Cowin, 2001; Ferguson et al., 2003; McCalden et al., 1993; Raghavan et al., 2012; Zimmermann et al., 2011). Despite the differences between murine and human bone, mice are often utilised to explore aspects of age-related bone loss in humans (Halloran et al., 2002; Jilka, 2013). As explained in Chapter 2 (Figure 2.4), unlike human bone, mouse bone does not have osteons or Haversian systems (Figure 4.9). This is why values of porosity and permeability of human or other animal bone cannot be directly transferred to mouse bone. Recent poroelastic FE models of mouse cortical bone mechanotransduction have shown that bone's response to load-repetition is highly influenced by the assumed value for lacunar-canalicular permeability (Pereira and Shefelbine, 2013). Therefore, it is essential to include accurate experimental lacunar-canalicular permeability values into computational models to explore the influence of fluid flow in bone remodeling.

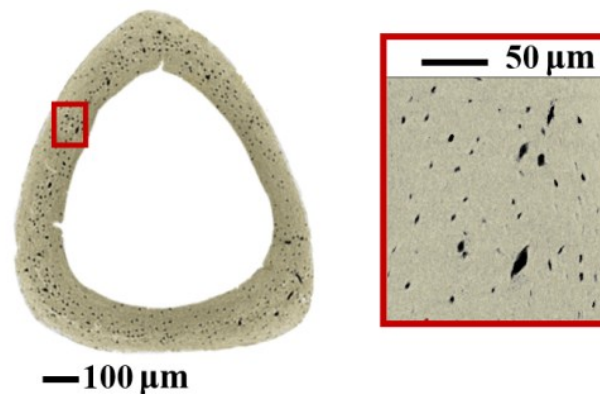


Figure 4.9: Cross-section of B6 mouse tibia (no osteons) from SEM images.

Li et al. (1987) showed that age has significant influence on permeability and found that cortical bone permeability of canine tibiae was bigger in young bone. These measurements were made using an experimental setup consisting of cylindrical containers to measure the volume of fluid flow; hence, the values obtained included both vascular and lacunar-canalicular permeability. However, it is unclear whether the permeability values also decrease with age at the lacunar-canalicular level.

Nanoindentation was used in this study to measure the lacunar-canalicular permeability in young and aged B6 mouse bone. Previous nanoindentation tests of hydrated

biological tissues have established that the values of bone permeability are dependent on the indentation contact size and reported nanoindentation-measured permeability values approximately three orders of magnitude smaller than those given by microindentation (Oyen et al., 2012). Results from porosity studies also suggest that the probability of measuring specific porosity-ranges in a sample is affected by the inherent structure or density of the interconnected porous network (Jast and Jasiuk, 2013; Knothe Tate et al., 2009). Overall, these findings suggest that different indentation contact sizes might measure different hierarchies of bone permeability. In order to investigate this further, spherical indenters of two different radii were used to indent bones of different ages.

Analysing age-related changes in mouse bone permeability will provide a better insight into the complex nature of bone permeability and its influence on mechanotransduction.

4.4.1 Materials and Methods

The outline of the experimental setup is shown in Figure 4.10.

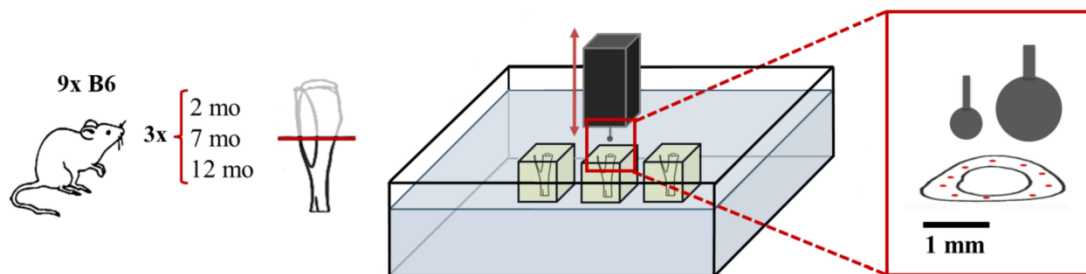


Figure 4.10: Schematic of materials and methods. The distal halves of the tibiae of three B6 mice of 2, 7 and 12 months were embedded in epoxy resin and tested submerged in distilled water. Indents were made along the circumference of the bone using spherical tips of two different radii.

Sample preparation

Nine C57BL/6 (B6) female mice of 2, 7 and 12 months, which correspond to young, skeletally mature and old mice respectively, were used for this study. Three right tibiae from each age-group were cleaned of surrounding soft tissue, dried in air for an hour and embedded in epoxy resin, as explained in Chapter 3. The resin and hardener were mixed and let cool for 15 minutes to increase the viscosity and avoid the infiltration of the resin into the pores. Tibiae were cut at the mid-diaphysis using a low speed

diamond saw. The distal sections were cut in cubes and polished using increasing grades of carbide papers (from P800 to P4000). SEM images verified that the pores of the bones were not filled with resin (Figure 4.11).

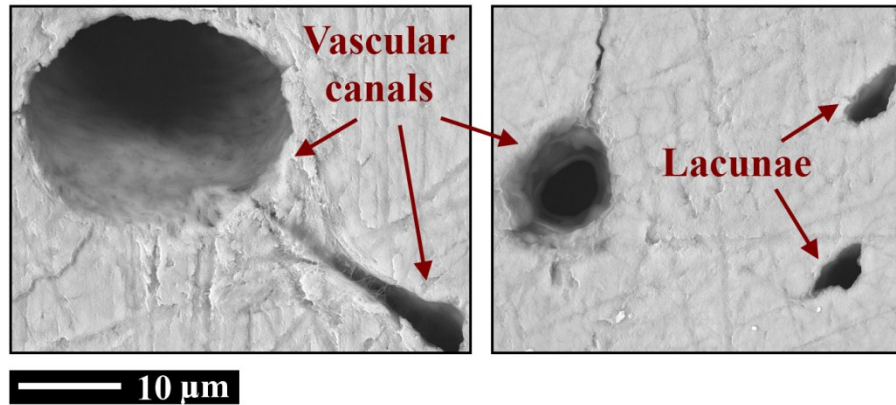


Figure 4.11: SEM images of 12 month-old B6 tibia embedded in epoxy. Pores are not filled with resin.

Nanoindentation

Tests were carried out using a TI700 U nanoindenter in load control, following the testing protocol defined in the Appendix (9.1). Distal halves of the tibiae were glued to a metallic container that covered the stage. Indents were done in the longitudinal direction on the tibia mid-diaphyseal cross-sections (Figure 4.10). Tests were conducted after fully submerging the specimens in distilled water for at least 15 minutes to fill the pores with fluid. Spherical fluid cell indenter tips of two sizes were used: radius of 238 μm and 500 μm . A trapezoidal loading profile was applied with a rising time of 10 s to a maximum load of 6 mN and a holding time of 30 s. Different peak loads were tested ($P_{\text{max}} = 3, 6, 8, 9 \text{ mN}$) and $P_{\text{max}} = 6 \text{ mN}$ was chosen because this value gave the most consistent set of displacements. A minimum of ten indents were made on each sample in the mid-cortex around the circumference of the bone and the indents that fell in pores were excluded –as the indentation depth was exceeded. Experimental data from all tests was exported as load-displacement-time (P - h - t) for poroelastic analysis.

4.4.2 Analysis

Poroelastic Analysis

The poroelastic analysis was performed as detailed in Section 4.3. The percentage of loading ramp analysed ranged between 1-4%. The parameters A and B were used to calculate the initial guesses of h_0 and h_∞ based on the displacement at the end of the ramp $h(t_R)$ and at the end of the creep holding period $h(t_R+t_c)$:

$$h_{0guess} = (1 - A) \times h(t_R) \quad [4.10]$$

$$h_{\infty guess} = (1 + B) \times h(t_R + t_c) \quad [4.11]$$

with A and B ranging between 0.05-0.15 until the best fit was achieved. These guesses were used for calculating the guess for the non-dimensional displacement h^* according to Eq. 4.5. Hence, in order to avoid h^* to tend to infinite, h_{0guess} and $h_{\infty guess}$ had to have different values. In addition, these guesses were also used to obtain the drained Poisson's ratio ν from Eq. 4.6-4.7 following:

$$\frac{h_\infty}{h_0} = \left(\frac{1 - \nu}{1 - \nu_u} \right)^{2/3} \quad [4.12]$$

Both curve fittings, in the non-dimensional and dimensional domain, were carried out following the non-linear least-squares optimisation routine from MATLAB Optimisation ToolboxTM, as described by Galli and Oyen (2009).

Statistical Analysis

The number of indents per age group ranged between 30 and 55 and thus, unequal sample sizes had to be considered for statistical analysis. Hochberger's GT2 test (post-hoc test) was utilised to compare the means between the three age-groups when Levene's test proved homogeneity of variances. If the variances were inhomogeneous, Games-Howell test was used. In order to compare the means of the same bones when indenting with different tips, the Wilcoxon signed-rank test was used. The significant level assumed was $p = 0.05$. Statistical analysis was performed using SPSS (v 21, SPSS Inc., Chicago, IL).

4.4.3 Results

Elastic properties

The measured elastic properties are summarised in Figure 4.12. Shear modulus increased significantly with age from 2 to 7 months for both indenter tips ($p < 0.001$) and then decreased again ($p < 0.001$) in the oldest bones. The 238 μm radius tip measured larger shear modulus for the three ages, but this was significant only for the 7 month-old bones ($p = 0.002$). No statistical difference was found in Poisson's ratios between the age-groups when indenting the bone with the largest tip. The 238 μm measured smaller Poisson's ratio values for the youngest bones compared to the 7 and 12 months ($p < 0.001$). There were no significant differences in the Poisson's ratio for the two tips.

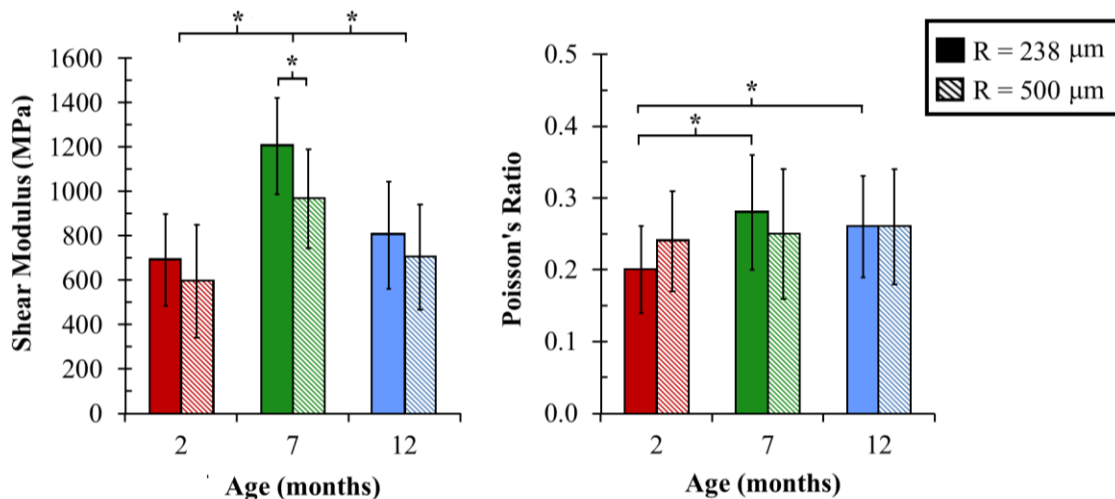


Figure 4.12: Average/standard deviations of shear modulus (left) and Poisson's ratio (right) of B6 tibiae of 2, 7 and 12 months ($p < 0.001$).

Permeability

Measurements with the small tip revealed that the youngest bones had a larger permeability value ($p < 0.01$) than the older ones but no statistical difference was found between the 7 and 12 month-old bones (Figure 4.13).

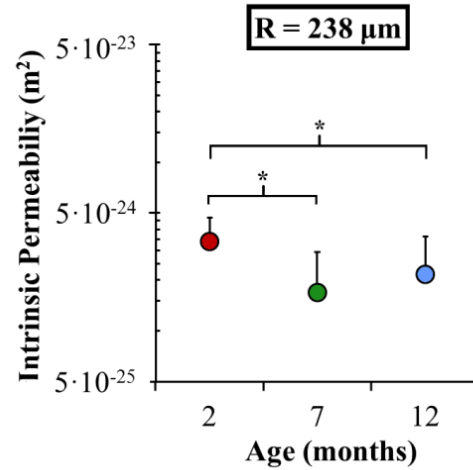


Figure 4.13: Intrinsic permeability of B6 tibiae decreased with age when using a 238 μm radius tip ($p < 0.01$).

The permeability distribution captured with the two tips is shown in Figure 4.14. For the larger radius indents young bones had a narrower range of permeability values (10^{-24} to $5 \times 10^{-23} \text{ m}^2$) than the old bones (5×10^{-25} to 10^{-21} m^2).

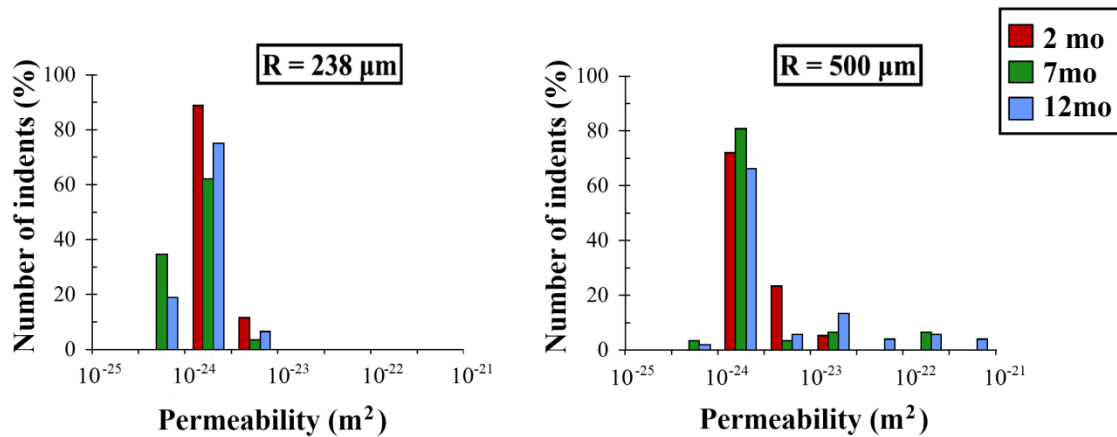


Figure 4.14: Intrinsic permeability distribution as a function of age when indenting bone with a 238 μm (left) and 500 μm (right) radius tip ($p < 0.05$). A wide distribution of permeability is captured with the bigger tip.

An example of the wide range of permeability values captured with the larger tip ($R = 500 \mu\text{m}$) can be observed in Figure 4.15, where displacement-time (h - t) curves of two indents on the same 12 month-old bone result in permeability values that vary three orders of magnitude. Hence, small indentation depths ($\sim 300 \text{ nm}$) were related to low permeability values ($\sim 10^{-24} \text{ m}^2$), while bigger indentation depths ($\sim 2500 \text{ nm}$) were related to high permeability values ($\sim 10^{-21} \text{ m}^2$).

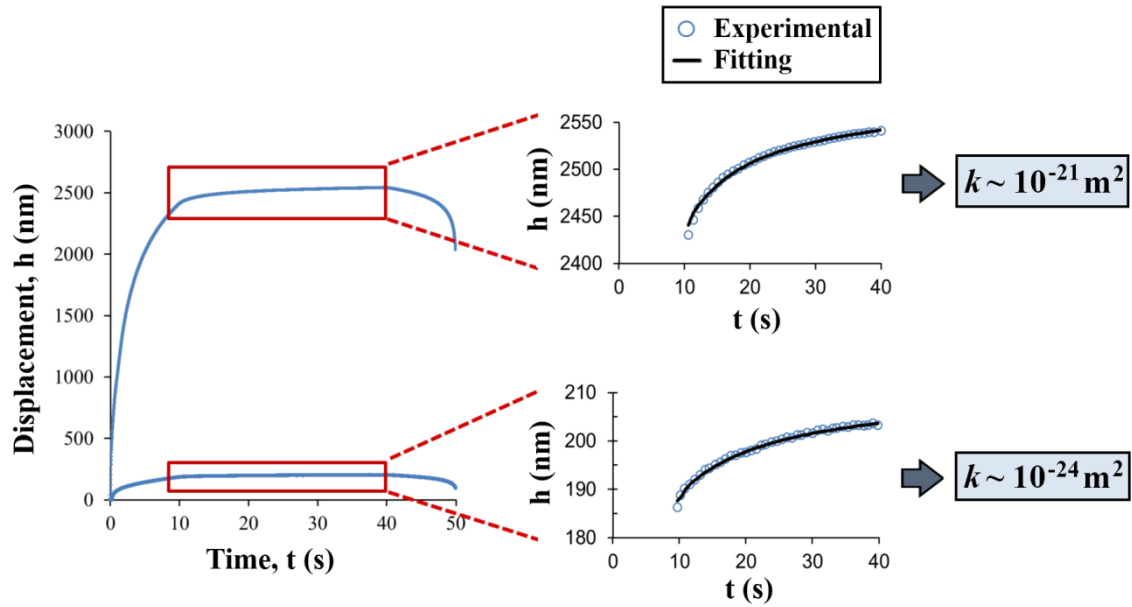


Figure 4.15: Displacement-time (h - t) curves corresponding to two indents on the same 12 month-old bone using the 500 μm sphere tip. Fitting the high-displacement curves result in permeability values $\sim 10^{-21} \text{ m}^2$, while low-displacement curves give values $\sim 10^{-24} \text{ m}^2$.

These differences in indentation depths can be translated to differences in contact sizes, as the indentation contact radius is defined as $a = \sqrt{Rh(t)}$. As shown in Figure 4.16, the 238 μm radius tip induced contact radii between 5-12 μm , whereas the bigger tip induced a wider range of contact radii of up to 40 μm . Big contact radii were related to high permeability values (Figure 4.16).

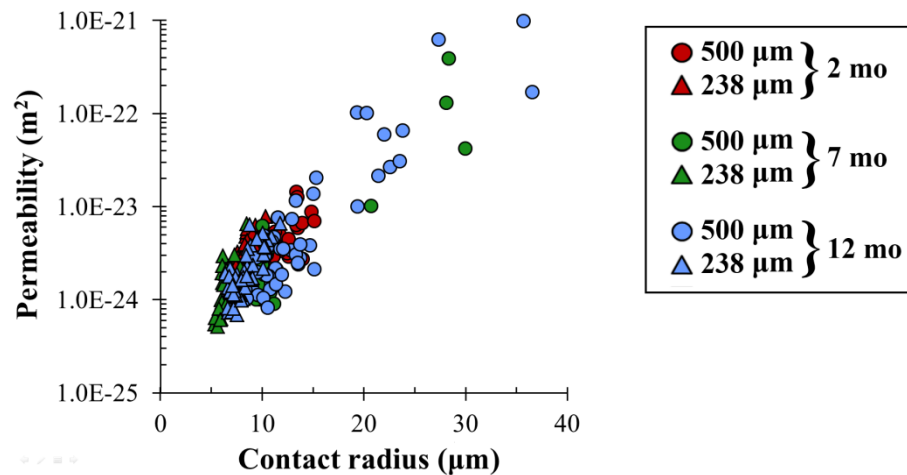


Figure 4.16: Relationship between indentation contact radius a and intrinsic permeability k for indents made with the 500 μm (circle) and 238 μm (triangle) radius sphere tips. The bigger tip induced indents with bigger contact radii, which led to higher values of permeability.

4.4.4 Discussion

In this chapter, nanoindentation was used to determine the poroelastic properties of B6 wild-type mouse tibia as a function of age. The indentations with the small tip revealed a decrease in the lacunar-canalicular permeability from young to skeletally mature cortical bone. The 500 μm tip imposed larger contact sizes, which captured both lacunar-canalicular and vascular permeability of bone.

The elastic properties derived from the poroelastic analysis did not show such dependence on the indentation contact size and their values were in agreement with published data. Shear modulus increased from young to skeletally mature (2 to 7 months) bone and then decreased in aged mice (12 months). Previous nanoindentation tests on hydrated bone have reported shear moduli of 430-500 MPa (Bembey et al., 2006a; Oyen, 2008; Oyen et al., 2012). Some studies have measured larger values of shear modulus in dry bone (Bushby et al., 2004; Chang et al., 2011; Lopez Franco et al., 2011); however, as shown in Chapter 3, shear modulus measured by nanoindentation decreases almost an order of magnitude when spherical indentation is used on hydrated bone, in contrast to sharp indentation in dry bone (Rodriguez-Florez et al., 2013). Previous studies have also shown similar age-related changes in elastic properties of B6 murine bone with different measurement techniques at the whole-bone (Brodt et al., 1999; Ferguson et al., 2003; Somerville et al., 2004) and tissue (Raghavan et al., 2012) level. The drained Poisson's ratios measured in this study are similar to the values obtained by Oyen et al. (2012) and close to the $\nu = 0.3$ often assumed for bone.

The 238 μm tip showed that lacunar-canalicular permeability decreased from 2 to 7 months with no significant changes from 7 to 12 months (Figure 4.13). In vivo tracer transport experiments through the lacunar-canalicular network of rat bone showed that transport becomes more restricted in aged bone due to a more compact mineralised tissue (Knothe Tate et al., 1998). In human cortical bone, porosity of lacunae decreases slightly with age (Wang and Ni, 2003). Their findings on osteonal bone seem to follow the same trend of a decrease of lacunar-canalicular permeability with age as the one found here for mouse bone.

The measured permeability values (5×10^{-25} to 10^{-21} m^2) are within the range of previous measurements using other experimental methods (Figure 4.2). Curve fitting of stress-

relaxation of single osteons gave lacunar-canalicular permeability values of 10^{-25} - 10^{-24} m² for bovine femoral bone (Gailani et al., 2009). This technique cannot be directly employed in murine bone, which does not have osteons. Compaction of intact bone by Gardinier et al. (2010) measured values of 10^{-23} m² for lacunar-canalicular permeability of canine metacarpal in situ. Oyen (2008) measured permeability of equine bone using spherical nanoindentation assuming incompressible constituents ($\alpha = 1$ and $\nu_u = 0.5$) and found values of 10^{-24} m². In further analysis on different equine bone specimens and using the master curve library together with the poroelastic framework, nanoindentation tests measured permeability values of 10^{-21} - 10^{-23} m² (Galli and Oyen, 2009; Oyen et al., 2012). In the current study, the majority of indents (60-80%) indicated permeability values between 10^{-24} and 5×10^{-24} m² for both tips for the three ages evaluated.

Though permeability values measured in this chapter are within the range of previously reported values, quantitative values measured by nanoindentation depend on the testing and analysis methods, as concluded in Chapter 3. The epoxy-embedded bones were dry and then rehydrated before testing, which may have caused changes to the cellular structures inside the lacunae. Anderson et al. (2008) found that permeability decreased two orders of magnitude when the cellular structure was taken into account in a computational model. This model did not include the effects of lipids and collagen matrix, which decreases permeability a further three orders of magnitude (Wen et al., 2010). Nevertheless, all the samples in the current study underwent the same preparation and testing protocol, and therefore, the effects of freshness are assumed to have affected all the bones in the same manner. The assumptions made in the analysis also influence the measured values. In the poroelastic approach, the values of ν_u and α were assigned a priori. The undrained bone was considered incompressible ($\nu_u = 0.5$), which does not influence the permeability value significantly when compared to $\nu_u < 0.5$ (Oyen et al., 2012). The value of $\alpha = 1$ does not influence the elastic properties, but since the algorithm identifies k/α^2 from the diffusivity coefficient (Eq. 4.9), the assumed value affects the quantitative value of permeability. Theoretical studies have estimated values of 0.14 (Cowin, 1999) and 0.15 (Smit et al., 2002) for α in osteonal bone and it has been shown that its value increases with increasing micro-porosity or decreasing mineral content (Hellmich and Ulm, 2005). The value $\alpha = 1$ was chosen in this study because there are no estimates of what it should be for non-osteonal bone,

nor how it changes with age. If $\alpha \neq 1$ was used, all the reported permeability values would differ by a constant value α^2 , and thus the age-related trends would not be altered. Overall, the measured values give a first experimental estimate of the lacunar-canalicular permeability of non-osteonal bone.

In order to investigate the influence of the contact size in the measured permeability level, indents made with the 238 μm and 500 μm radius tip were compared. The larger tip imposed larger contact sizes and revealed a wider distribution of permeability for the 7 and 12 month-old bones, reaching permeability values as large as $9 \times 10^{-22} \text{ m}^2$ (Figure 4.14 and Figure 4.16). These large values were not captured when indenting old bone with the small tip. This difference can be explained by looking at the lacunar and vascular pores of cortical bone (Figure 4.17). For an average displacement of $\sim 250 \text{ nm}$ the imposed contact diameter was $\sim 15 \mu\text{m}$ for the small tip and $\sim 22 \mu\text{m}$ for the larger tip. The long radius of elliptical lacunae in murine bone measures 1-10 μm with a spacing of $\sim 30 \mu\text{m}$ between lacunae; while the diameter of vascular canals is $>10 \mu\text{m}$ with a vascular spacing of $\sim 100 \mu\text{m}$ (Carriero et al., 2014a; Schneider et al., 2007, 2010; Voide et al., 2011; Wang et al., 2005). Figure 4.17 shows a nano-CT image of a 12 month-old tibia where the long radius of a lacuna is 7.0 μm and the diameter of a vascular canal is 14.2 μm . This suggests that with a larger contact area there is a higher likelihood of indenting a hole or part of a hole. When the indent fell into a pore, the time-displacement curve was either distorted or the displacement limit was exceeded before making contact with the surface. In both cases, this data was excluded from the analysis.

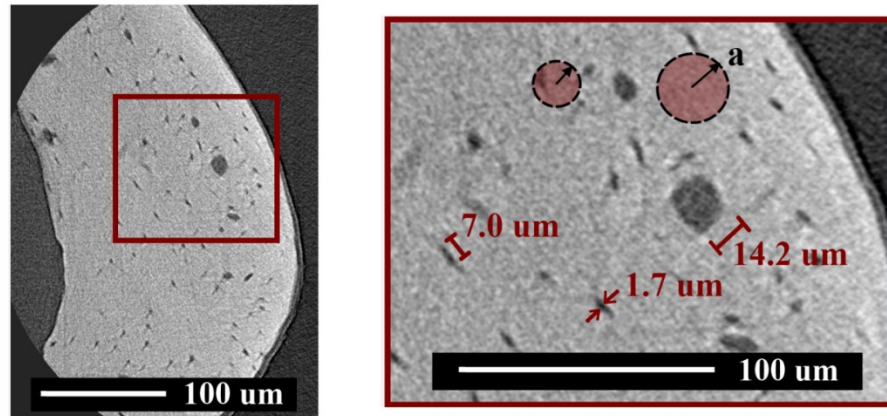


Figure 4.17: Nano-CT of a 12 month-old tibia at a resolution of $0.6 \mu\text{m}/\text{pixel}$. Pores of varying sizes are shown. The shaded areas correspond to the contact areas imposed by the indenter. The $238 \mu\text{m}$ radius tip imposed contact radii $a \sim 7 \mu\text{m}$, whereas the $500 \mu\text{m}$ tip imposed $a \sim 11 \mu\text{m}$.

Results indicate that the $500 \mu\text{m}$ tip was able to identify both lacunar-canalicular and vascular permeability, showing a continuum distribution of permeability over three orders of magnitude (Figure 4.14). These differences in permeability are in accordance with the multi-scale permeability response of equine bone obtained with varying contact sizes: when the contact diameter increased from $6\text{--}14 \mu\text{m}$ to $18 \mu\text{m}$, permeability increased almost two orders of magnitude; and it increased three orders of magnitude further when the contact radius reached $200\text{--}300 \mu\text{m}$ (Oyen et al., 2012). In these studies, nanoindentation measured different levels of permeability in a discrete manner; the transition across length scales was not explored further. In the current study, the indents with the $500 \mu\text{m}$ tip captured this transition and revealed that the permeability distribution was narrower in the youngest bones. This may be a result of the increasing intra-cortical and cortical porosity with advancing age found in B6 mice femora (Courtland et al., 2013; Ferguson et al., 2003), similar to the increased size of vascular Haversian canals seen in human bone (Wang and Ni, 2003).

4.5 Conclusions

This chapter provided a first experimental approximation based on mechanics from nanoindentation of lacunar-canalicular permeability of mouse bone, and determined that **lacunar-canalicular permeability of bone decreases from young to skeletally mature bone**. Results suggest that **nanoindentation with varying contact sizes might provide the tool to understand the dual-porosity nature of bone**. Thus, characterising permeability via nanoindentation may help indicate alterations in bone mechano-adaptation.

As explained in the current chapter, fluid flow through bone's porous network is likely involved in mechanotransduction of the bone's mechanics environment into a cellular mechano-adaptive response. This porous network has an effect on bone's mechanical behaviour. In particular, evidence suggests that pores affect the way cracks propagate at the tissue level, hence affecting bone's ability to resist fracture. Chapter 5 explores the influence of intra-cortical porosity on crack propagation using novel computational techniques.

5 EFFECTS OF MICRO-POROSITY ON BONE MECHANICS

Biologically, fluid flow through bone's porous network is expected to play a major role in the mechanotransduction mechanism of bone, as discussed in the previous chapter. From a mechanics point of view, bone's porosity is expected to influence its ability to resist fracture. Bone fracture is a serious problem in our societies, as the fracture risk is rising due to the increase in the average age of the population. In the elderly and in severe conditions of brittle bone diseases, this increase in fracture risk can be the cause of mortality. A detailed description of the fracture mechanism of bone is vital for the implementation of appropriate therapies. However, this mechanism is yet not fully understood, mainly because bone has a complex fracture behaviour comprising various toughening mechanisms at different length-scales. At the micro-scale, intra-cortical porosity might affect crack propagation through bone tissue.

In osteogenesis imperfecta murine (*oim*) bones, which represent the moderate to severe condition of osteogenesis imperfecta in humans, a genetic mutation results in high bone fragility. *Oim* bones have altered porosity, and thus, they provide an excellent platform to analyse the influence of intra-cortical porosity on crack propagation. As explored in the next chapter, these bones also exhibit deteriorated material properties. Computational techniques become very useful to isolate the effect of intra-cortical porosity only. Crack initiation and propagation can be analysed following the Extended Finite Element Method (XFEM). This approach allows to model crack propagation along an arbitrary, solution-dependent path. However, there are difficulties associated with the propagation of cracks through holes when using XFEM in the commercial software Abaqus.

This study aims to 1) establish a technique to propagate cracks through holes using XFEM, and to 2) understand the influence of intra-cortical bone porosity on crack propagation using a two-dimensional model of the porosities of osteogenesis imperfecta murine (*oim*) cortical bone.

5.1 Exploring Crack Initiation and Propagation at the Micro-scale: the Role of Intra-cortical Porosity

Intra-cortical porosity has been shown to be related with the mechanical behaviour of cortical bone (Albert et al., 2014; Cowin et al., 2009; McCalden et al., 1993; Schneider et al., 2013). At the tissue level, bone porosity is expected to have a significant influence on bone toughness and crack propagation (Carriero et al., 2014b; Turnbull et al., 2014; Ural and Vashishth, 2014; Voide et al., 2011; Yeni et al., 1997). Pores might affect the crack propagation path through crack deflection, serve as stress concentration for the creation of new micro-cracks or stop the crack from propagating further by blunting the crack front (Figure 5.1). As a consequence, pores might act as defects or as toughening mechanisms which slow down crack growth (Christen et al., 2012; Donaldson et al., 2014; O'Brien et al., 2005; Taylor et al., 2007).

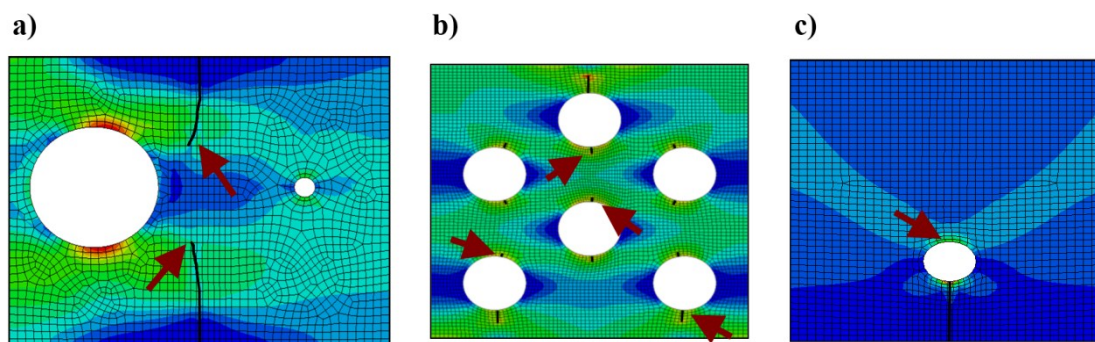


Figure 5.1: Qualitative representation of the influence of pores on crack propagation by a) deflecting the crack, b) starting new micro-cracks, c) blunting the crack tip (indicated by arrows). The three plates are loaded in tension. Colour maps refer to maximum principal stresses with increasing stress from blue to red.

Evidence suggests that the increased intra-cortical porosity found in ageing or osteoporotic bone (Besdo and Vashishth, 2012; Budyn et al., 2008; Ural and Vashishth, 2007a), as well as in severe conditions of brittle bone diseases such as osteogenesis imperfecta (Carriero et al., 2014a; Vardakastani et al., 2014), might adversely affect the micro-scale fracture mechanism of bone. However, due to the complex and interconnected toughening mechanisms of bone, the effect of intra-cortical porosity on crack initiation and propagation remains to be elucidated.

Mouse bones provide an excellent platform to investigate the sole role of porosity on bone toughness, without taking into account the effect of other micro-structural features found in osteonal bone (osteon, cement line). Recent experimental studies on two wild-

type mouse strains of different porosities (B6 and C3.B6) have indicated that their micro-crack behaviour is strongly influenced by vascular canals (Schneider et al., 2013; Voide et al., 2011), while lacunae are thought to provide guidance for crack propagation at the micro-scale (Voide et al., 2011). Although the lack of osteons in mouse bone facilitates to measure the implications of porosity in fracture toughness, the experimental values are also affected by possible differences in material properties or lamellar structure. Computational studies have the ability to isolate the individual influence of each of these features. As such, modelling techniques can provide additional insight into the role of intra-cortical porosity on crack initiation and propagation.

Computational Techniques to Model Crack Growth in Cortical Bone

The finite element method (FEM) is a well-known numerical technique for finding approximate solutions to partial differential equations. However, simulating the propagation of cracks using traditional FEM is computationally expensive and cumbersome (Abdelaziz and Hamouine, 2008; Yazid et al., 2009). The FEM mesh needs to conform to the geometry of the crack. Since the topology of the domain changes as the crack propagates, continuous re-meshing is required to align the element edges with the crack.

Cohesive finite element modelling is an alternative technique which does not require re-meshing as the crack grows. Cohesive elements are placed on the interfaces of regular finite elements and a damage model is implemented in these cohesive elements, as shown in Figure 5.2. Cohesive zone models (CZM) have captured the toughening behaviour of human cortical bone with age (Ural and Vashishth, 2006, 2007a). This technique has been used to demonstrate the contribution of increased age-related intra-cortical porosity on fracture toughness in human bone (Ural and Vashishth, 2007b). However, CZM is not capable of capturing the influence of the micro-structure on the crack path, as crack extension has to follow a predefined path around elements of the mesh (Figure 5.2).

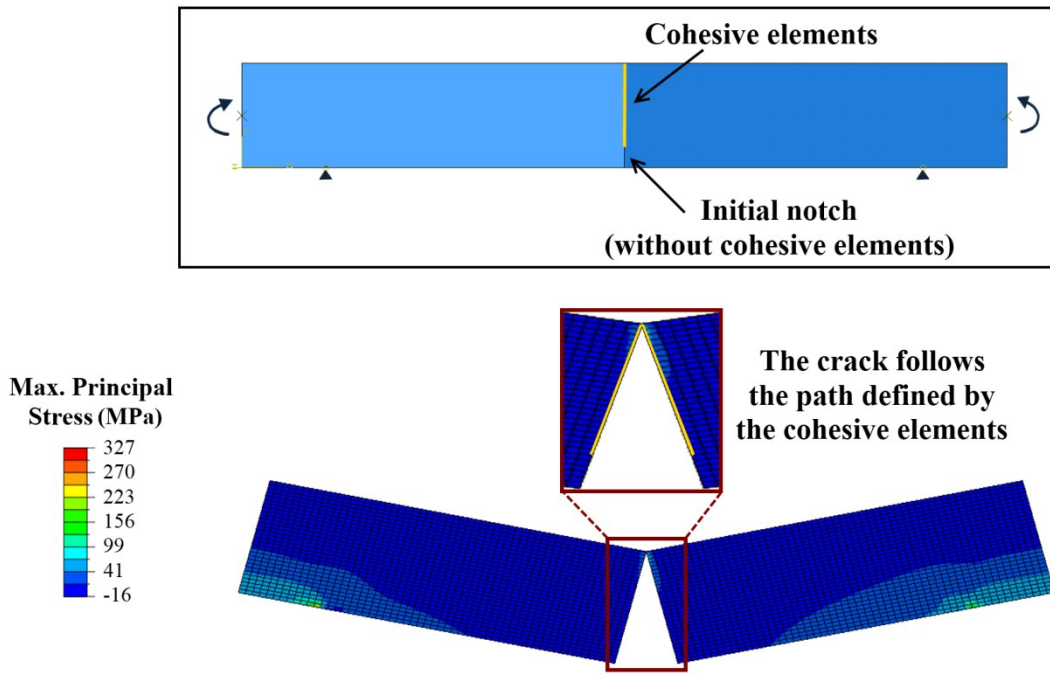


Figure 5.2: 3-point bending setup of a steel beam with an initial notch. Cohesive elements are placed along the boundary of standard elements and a damage criterion is assigned to them. When the damage criterion is met, crack will propagate along the pre-defined path.

The extended finite element method (XFEM) can predict crack initiation and propagation along an arbitrary, solution dependent path; hence overcoming the limitation of the cohesive zone model. In XFEM, enrichment functions are applied to standard FEM elements in order to model the discontinuity caused by the crack. Using this approach, the crack does not have to be confined to the boundaries of the elements. XFEM has been used to explore crack propagation on osteonal bone at the macro-scale (Feerick et al., 2013), micro-scale (Abdel-Wahab et al., 2012; Gao et al., 2013; Li et al., 2013a; Vergani et al., 2014) and from a multi-scale perspective (Budyn and Hoc, 2007; Budyn et al., 2008). The studies including bone micro-structure in XFEM have investigated the combined effect of osteons, cement lines and porosity on simplified geometries of osteonal bone (Abdel-Wahab et al., 2012; Budyn and Hoc, 2007; Li et al., 2013b). However, the effect of the real geometry of the pores has not been evaluated and hence, it is still unclear how intra-cortical bone porosity affects crack growth. Taking into account that XFEM is available in commercial software, such as Abaqus, the XFEM technique was chosen in this chapter as a promising approach to investigate the role of intra-cortical porosity in crack initiation and propagation. A more detail background on XFEM will be presented in the next Section.

5.2 Introduction to Extended Finite Element Method (XFEM) for Crack Initiation and Growth

Extended Finite Element Method (XFEM) was introduced by Belytschko and Black (1999) based on the notion of partition of unity (Melenk and Babuska, 1996) to computationally model crack initiation and propagation along an arbitrary, solution dependent path (Belytschko and Black, 1999; Moës et al., 1999; Sukumar et al., 2000). Crack location in XFEM is not confined to the boundaries of the mesh, and hence, there is no need of re-meshing (like in traditional FEM) or specifying a pre-defined path for crack growth (as in the cohesive zone model) (Figure 5.3). Since this seems to be a promising technique to model crack growth in bone, the XFEM capability of the commercial software Abaqus (v 6.12) (Giner et al., 2009) is employed in this chapter. Although XFEM can be used to model stationary and moving cracks, the interest of this chapter resides on the initiation and propagation of cracks based on the cohesive damage approach.

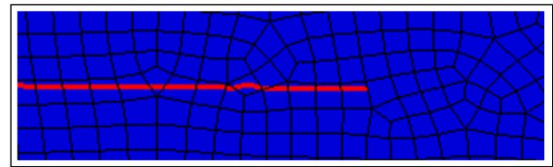


Figure 5.3: The crack (in red) does not have to follow a predefined element boundary.

XFEM models crack growth based on the concepts below:

- First, since cracks *introduce discontinuities* in the model, a method is required to incorporate these discontinuities into the conventional FEM.
- In order to quantify the magnitude of the introduced discontinuity, such as the opening of the crack, the *phantom node approach* is used in conjunction with the *cohesive segment method*.
- The crack is located and tracked following the *level-set method*.
- *Damage initiation and propagation criteria* are defined to specify when the crack initiates and where it propagates.

These concepts are further discussed in the following sections. More complete reviews of XFEM can be found in (Fries and Belytschko, 2010; Karihaloo and Xiao, 2003; Yazid et al., 2009).

Extended Finite Element Method to Incorporate Discontinuities

In XFEM, discontinuities are incorporated by adding extra “enrichment functions” to the classical finite element nodal displacement functions in the domain k (Figure 5.4). The nodes where the enrichment term has been added are referred to as enrichment regions, contained in the enriched subdomain k^* .

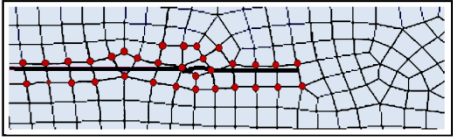
$$u(x) \approx \underbrace{\sum_{i \in k} N_i(x) u_i}_{\text{Classical}} + \underbrace{\sum_{i \in k^*} N_i(x) H(x) a_i}_{\text{Enriched}}$$


Figure 5.4: Conventional FEM nodal functions $N(x)$ enriched by the Heaviside distribution $H(x)$ in the nodes belonging to elements cut by the crack (in red).

In the case of propagating cracks, the conventional nodal shape function $N(x)$ is enriched by a Heaviside step function $H(x)$, which represents the displacement jump across the crack face in the nodes belonging to elements cut by the crack (Moës et al., 1999). $H(x)$ takes the value 1 above the crack face and -1 below the crack. The standard nodal displacement vector associated with the nodes without XFEM is denoted u_i , while the nodal degrees of freedom enriched by a jump discontinuity are named a_i .

Phantom Nodes and Cohesive Segment Method

When describing the displacement of enriched nodes with the formulation shown in Figure 5.4, it is assumed that a crack grows across an entire element at a time. The discontinuity of the cracked elements is reproduced by phantom nodes (Song et al., 2006), which are superposed to the original real nodes, as represented in Figure 5.5. It must be noted that phantom nodes are not assigned any displacement, as they serve just as additional degrees of freedom for the real nodes.

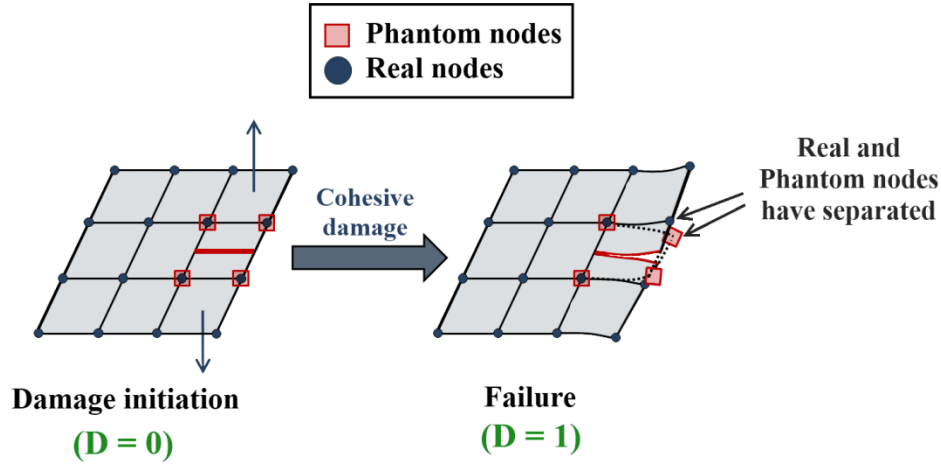


Figure 5.5: When damage initiates ($D = 0$), phantom nodes are superposed to real nodes. At the point of failure ($D = 1$), real and phantom nodes move apart opening the crack.

Damage initiation and evolution are implemented in the enrichment regions according to the cohesive segment method (Moës and Belytschko, 2002; Remmers et al., 2003), as represented in Figure 5.6. When the adopted failure criterion is met in an enriched element, damage is initiated (damage, $D = 0$) and phantom nodes are superposed to the original real nodes. At this point, the two surfaces of the cracked element are held together by equal and opposite forces referred to as cohesive traction strength. Degradation of traction strength occurs until failure (damage, $D = 1$), when the phantom and real nodes can move apart resulting in the splitting of the element and opening of the crack. The area under the cohesive traction and crack opening curve G_c (Figure 5.6) represents the critical value of fracture energy required to make the crack grow.

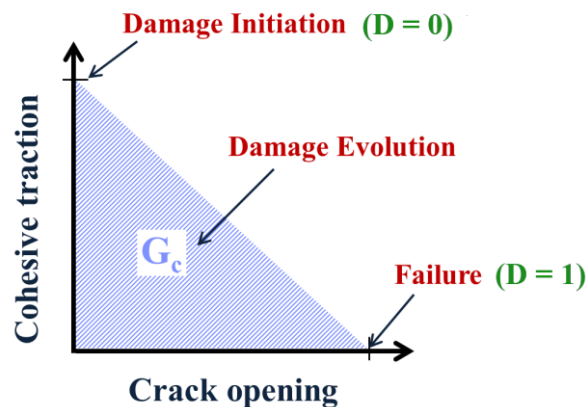


Figure 5.6: Traction-separation law. Linear degradation of traction strength between crack surfaces from damage initiation ($D = 0$) to failure ($D = 1$). The area under the curve is the fracture energy required to propagate the crack, G_c .

Level-set method

In order to describe the topology of the crack and track its propagation, XFEM relies on the level-set method. The functions $\Phi(x)$ and $\Psi(x)$ are used to fully describe the location of the crack (Figure 5.7). The nodal value of $\Phi(x)$ is the signed distance of the node from the crack face. Hence, $\Phi(x) = 0$ represents the crack face. The second function required to define the crack is $\Psi(x)$, which is the signed distance of the node from an almost-orthogonal surface passing through the crack front. The intersection of level-sets $\Phi(x) = 0$ and $\Psi(x) = 0$ denotes the crack front.

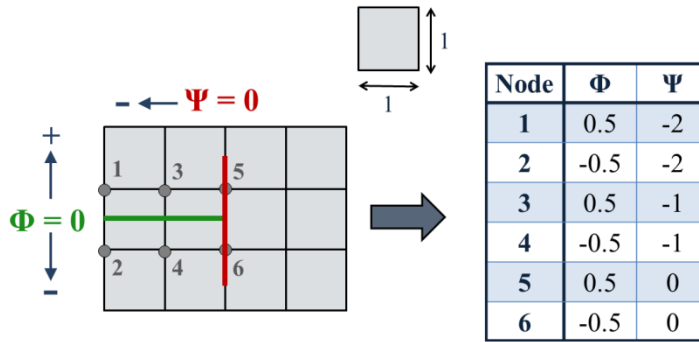


Figure 5.7: Schematic representation of the level-set functions of a crack. $\Phi(x) = 0$ represents the crack path while $\Psi(x)$ is zero on the orthogonal surface passing through the crack front.

Damage initiation and evolution criteria

Abaqus XFEM provides stress or strain based criteria for damage initiation (Abaqus 6.12 documentation). The failure criterion used in this thesis for bone is based on the maximum principal stress σ or strain ε (Doblaré et al., 2004; Nalla et al., 2003; Schileo et al., 2008):

$$f = \frac{\langle \sigma \rangle}{\sigma_{max}} \quad \text{or} \quad f = \frac{\langle \varepsilon \rangle}{\varepsilon_{max}} \quad [5.1]$$

where σ_{max} and ε_{max} correspond to the maximum allowable principal stress and strain respectively. Damage initiation occurs when the maximum principal stress/strain ratio reaches a value of unity ($f = 1$).

Damage evolution is described using an energy-based approach. Once damage initiates, linear degradation of the traction strength between the two faces of the element will occur governed by the user-defined critical fracture energy value G_c .

5.3 XFEM to Model Crack Propagation through Holes

In this thesis, the XFEM capability in Abaqus (v 6.12) is used to model crack growth through bone's micro-structure with the objective of exploring how intra-cortical porosity affects crack propagation. However, there are some limitations associated with introducing holes in the model:

“Within an enrichment region – which is the domain where the damage criteria is implemented-, a new crack initiation check is performed only after all existing cracks have completely separated. This may result in the abrupt appearance of multiple cracks.” (Abaqus 6.12 documentation).

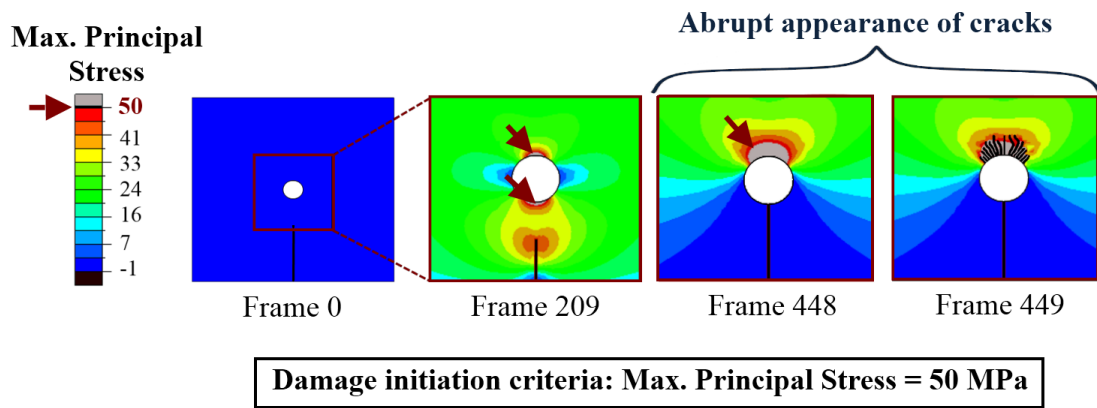


Figure 5.8: A plate with an initial notch subjected to horizontal tension. The damage initiation criterion is set to a maximum principal stress of 50 MPa. As the crack propagates (Frame 209), there are grey-shaded areas (pointed by the arrow) in which the damage initiation criterion is met. However, a damage initiation check is not performed until the existing crack reaches the hole. There is an abrupt appearance of multiple cracks from Frame 448 to 449.

This is represented in Figure 5.8, where a square plate with an initial notch and one hole is subjected to tension in the horizontal direction. The whole plate corresponds to one enrichment region, where damage initiation is set to a maximum principal stress of 50 MPa. Hence, as tension is applied to the model and the crack propagates, new cracks should appear in the elements where the maximum principal stress reaches the critical value of 50 MPa (the areas shaded in grey in Figure 5.8). However, this is not the case. In Frame 209, the maximum principal stresses around the hole already exceed the 50 MPa but there are no new cracks starting at these grey regions. Instead, the existing crack keeps growing until it reaches the hole (Frame 448). The hole is considered as a boundary of the enrichment region; thus, after the existing crack reaches the hole, a

new crack initiation check is performed. When this happens, there is a big area on the other side of the hole where the damage initiation criterion is met, which results in the abrupt appearance of multiple cracks from Frame 448 to Frame 449.

In this chapter, two approaches are considered in order to overcome this limitation (Figure 5.9). The first method consists of assigning void material properties to the holes (with Young's modulus, $E_{\text{void}} \ll E_{\text{plate}}$) so that the crack is free to propagate through the holes without reaching the boundary of the enrichment region, hence avoiding the abrupt appearance of multiple cracks (Besdo and Vashishth, 2012; Li et al., 2013b). The second method is a novel approach where multiple partitions are employed and multiple enrichment regions are assigned with independent crack growth possibilities.

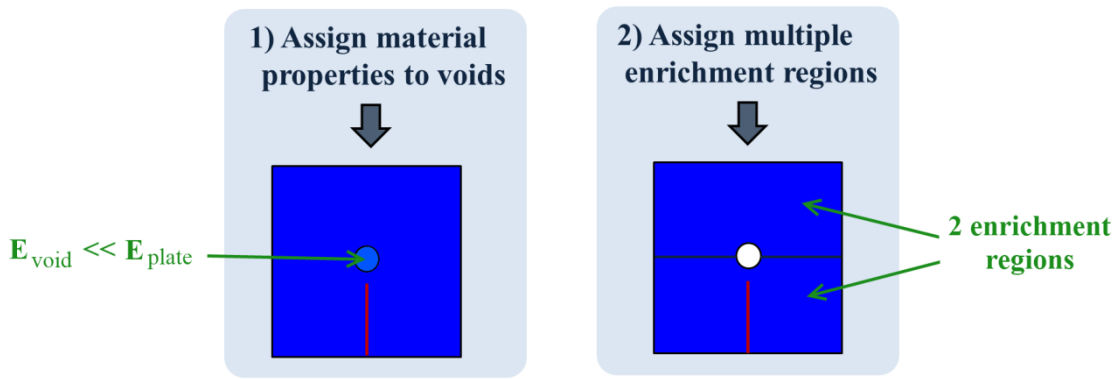


Figure 5.9: Two techniques to model crack growth through a hole. 1) Assigning material properties with reduced Young's modulus, E , to the void. 2) Employing partitions and assigning two enrichment regions, so that the damage initiation check is performed in each of the regions independently.

This section explores the proposed two techniques to propagate cracks through holes using XFEM within Abaqus by considering a simple case of a 2D plate in tension with holes located along the crack path.

5.3.1 Materials and Methods

Model setup

The commercial finite element software Abaqus (v 6.12, Dassault Systemes 2012) was used. The model setup is shown in Figure 5.10. A square plate (5 mm x 5 mm) with an initial notch ($a_0 = 2.1$ mm) was subjected to a displacement ($\delta = 0.018$ mm) in the right edge. One hole and three holes ($R = 0.25$ mm) were placed in the plate along the crack path. In order to explore the two techniques presented in Figure 5.9, different models

were created from each of the setups with one and three holes. Two models were created with material properties assigned to holes, “1 hole filled” and “3 holes filled”. And other two models were created with partitions through the empty holes, “1 hole” and “3 holes”. In the first approach, the whole model was considered as one enrichment region. In the second approach, each partitioned region was considered an independent enrichment region: the one-hole model had two enrichment regions ($e1$, $e2$) and the model with three holes had four enrichment regions ($e1$ - $e4$).

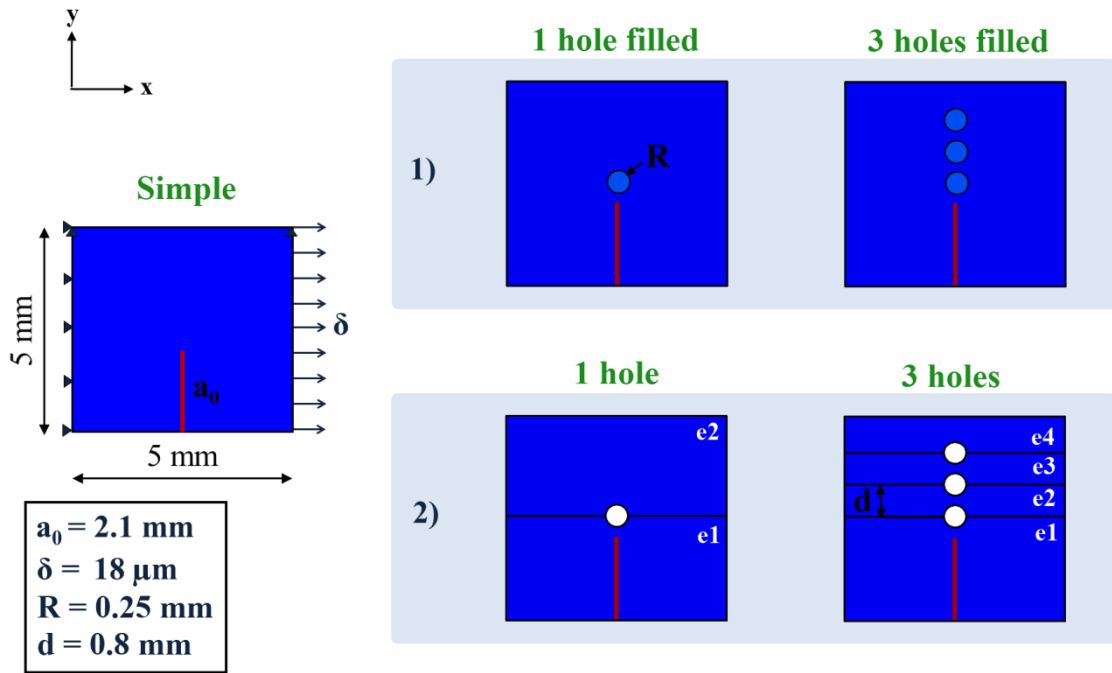


Figure 5.10: A plate with an initial notch a_0 has the left edge constraint in x , while a displacement δ is applied to the right edge. Two modelling techniques are compared: 1) assigning material properties to holes, and 2) creating partitions that correspond to different enrichment regions $e1$ - $e4$. Two setups are considered for each of the technique: a plate with a hole of radius R in the middle, and a plate with three holes of radius R with distance d between them.

The configuration of the used element-types and meshes is represented in Figure 5.11. Four-node bilinear plane-strain quadrilateral elements with reduced integration (CPE4R) were used in enrichment regions, while three-node linear plane-strain triangle elements (CPE3) were employed in the edges. This was done in order to reduce the computational time. An approximate element size of 0.05 mm was used in the enrichment regions, with smaller elements in the filled holes (~ 0.03 mm) and bigger elements in the non-enriched regions (~ 0.08 mm). Structured meshes were implemented in regions where crack initiation occurs, as this has been shown to aid in

the convergence (Feerick et al., 2013). The notch was placed in the middle of the elements and the crack tip coincided with the edge of the element, as shown in Figure 5.11.

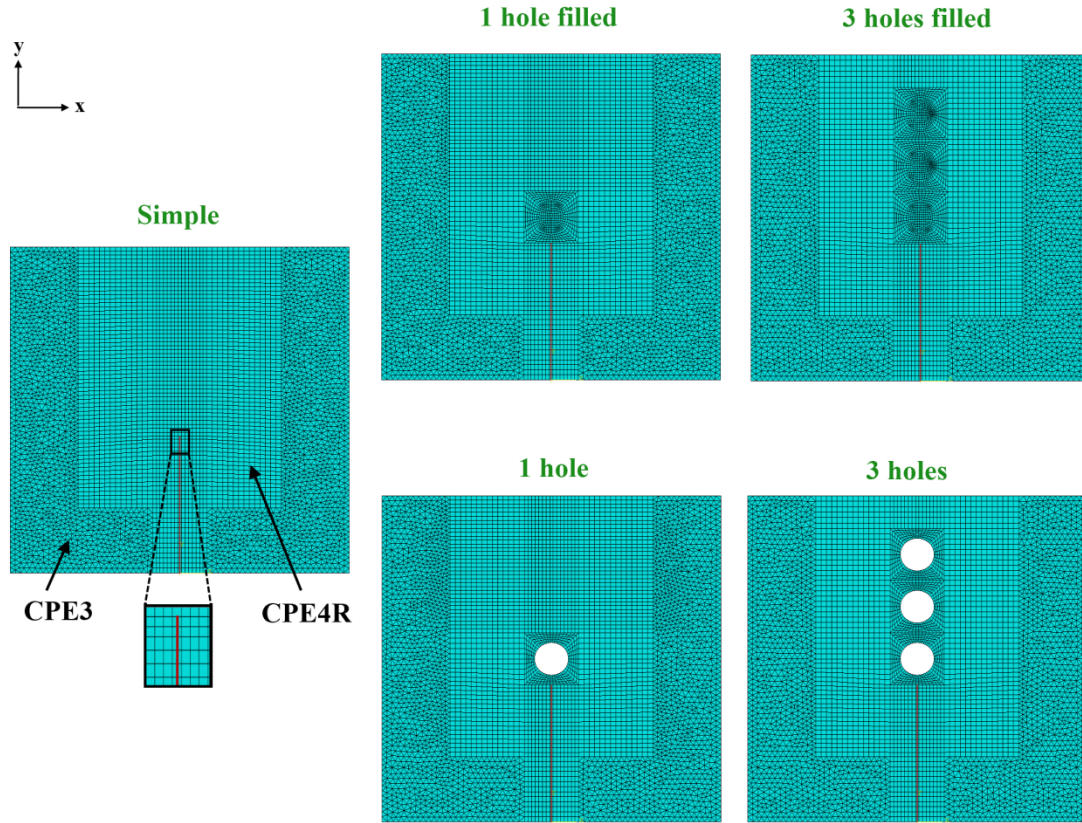


Figure 5.11: The mesh configuration of the five XFEM models. 3-node linear plane-strain triangles were used in non-enriched areas, while 4-node bilinear plane-strain quadrilateral elements were employed in the structured enriched areas.

Material properties

Damage initiation and evolution were modelled following the cohesive segment method (Figure 5.6) with maximum principal strain ε_{max} failure criterion for damage initiation and a fracture energy G_c based damage evolution law for crack propagation. Material properties are summarised in Table 5.1. The plate was considered linear elastic and homogeneous and the material properties were chosen based on previously reported properties of transverse cortical bone (Abdel-Wahab and Silberschmidt, 2011; Budyn et al., 2008; Li et al., 2013a). The maximum principal strain ε_{max} was chosen to be higher than the usually reported values for bone (0.4-0.6%) (Budyn and Hoc, 2007; Li et al., 2013b), in order to have only one crack propagating from the initial notch and avoid having sequential nucleation of cracks below and above the hole. Given the value of G_c

and according to the damage law in Figure 5.6, higher values of ε_{max} lead to a later initiation but a faster propagation of damage. Therefore, once the crack initiates from the notch, this crack propagates across the plate before the strain values reach the initiation criterion on other sites. If sequential propagation occurred, it would not be possible to track the crack front. In the case of voids, the assigned elastic modulus E was 1000 times smaller than the one for the plate, while the rest of the parameters were kept constant.

Table 5.1: Elastic and damage properties of the plate and void. *The void properties were only applied to holes in “1 hole filled” and “3 holes filled”.

		Plate	Void*
<i>Elastic properties</i>	Young’s modulus, E (MPa)	10000	10
	Poisson’s ratio, ν	0.15	0.15
<i>Damage properties</i>	Max. principal strain, ε_{max} (%)	0.8	0.8
	Fracture energy, G_c (N/mm)	0.2	0.2

5.3.2 Analysis

Tracking of the crack front

As explained previously, Abaqus uses the level-set method to capture the location of the crack face $\Phi(x)$ and crack tip $\Psi(x)$. The values of the level-set functions are stored only at the nodes of the elements where the crack passes through. Although the visualisation module of Abaqus allows tracking the location of the crack based on the nodal values of the level-set functions (Figure 5.12), the crack location is not stored in any variable and thus, it is not possible to directly evaluate the evolution of crack extension.

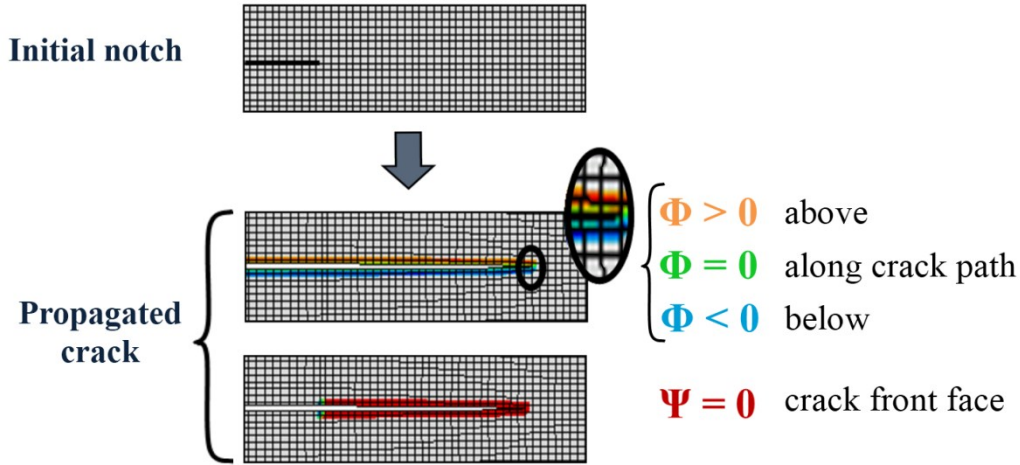


Figure 5.12: Initial notch and the level-set functions of the propagated crack. $\Phi(x) = 0$ represents the crack path, while $\Psi(x)$ is zero on the orthogonal surface passing through the crack front.

A MATLAB code (The MathWorks, Natick, MA, USA) was developed in order to track the location of the crack front while the crack propagated from the initial notch. The nodal values of $\Psi(x)$ in all frames and coordinates of these nodes were extracted as text files from Abaqus visualisation module. A representation of the nodal values of $\Psi(x)$ as the crack propagates is shown in Figure 5.13.

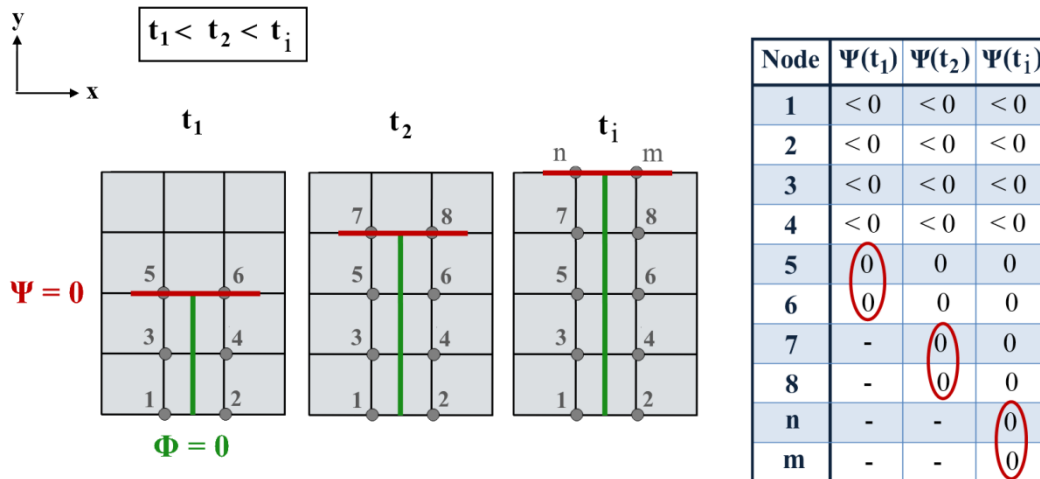


Figure 5.13: Representation of the nodal values of the level-set function $\Psi(x)$ at different time steps ($t_1 < t_2 < t_3$) as the crack propagates. The location of the crack front is defined by identifying the location of the nodes in which $\Psi(x)$ turns zero at each time-step (circled in red).

The extension of the crack along the y axis at time t_i was calculated from the y coordinates of the crack front nodes at time t_i (y_n and y_m) and the y coordinates of the initial crack front nodes (y_5 and y_6).

$$Crack_extension(t_i) = Average(y_n, y_m) - Average(y_5, y_6) \quad [5.2]$$

The total crack length at a given time t_i was computed as the sum of the extension of the crack at t_i and the length of the initial notch, in this case 2.1 mm:

$$Crack_length(t_i) = Crack_extension(t_i) + Notch_length \quad [5.3]$$

5.3.3 Results

The results of the simulations after applying the displacement of 0.018 mm are presented in Figure 5.14. As expected, the crack started from the notch and propagated straight up through the plate in all five cases, splitting the plate in two parts.

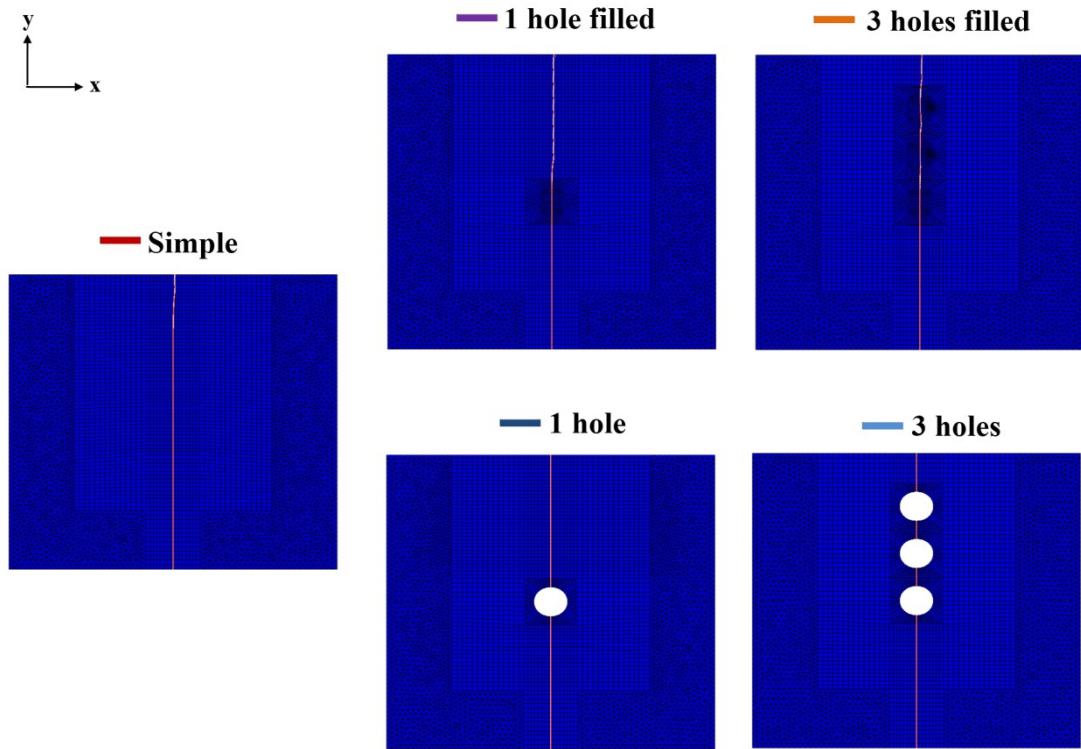


Figure 5.14: The crack propagated straight up through the plate in all the models.

The crack length was computed throughout all the frames starting from the 2.1 mm initial notch, as shown in Figure 5.15. Similar behaviour was observed when comparing the models where the holes had been assigned material properties (“1 hole filled”, “3 holes filled”), and the models where multiple enrichment regions were employed (“1 hole”, “3 holes”). Overall, the crack started propagating earlier in the plates with holes using both approaches, and the 3-hole plate showed least resistant to crack propagation.

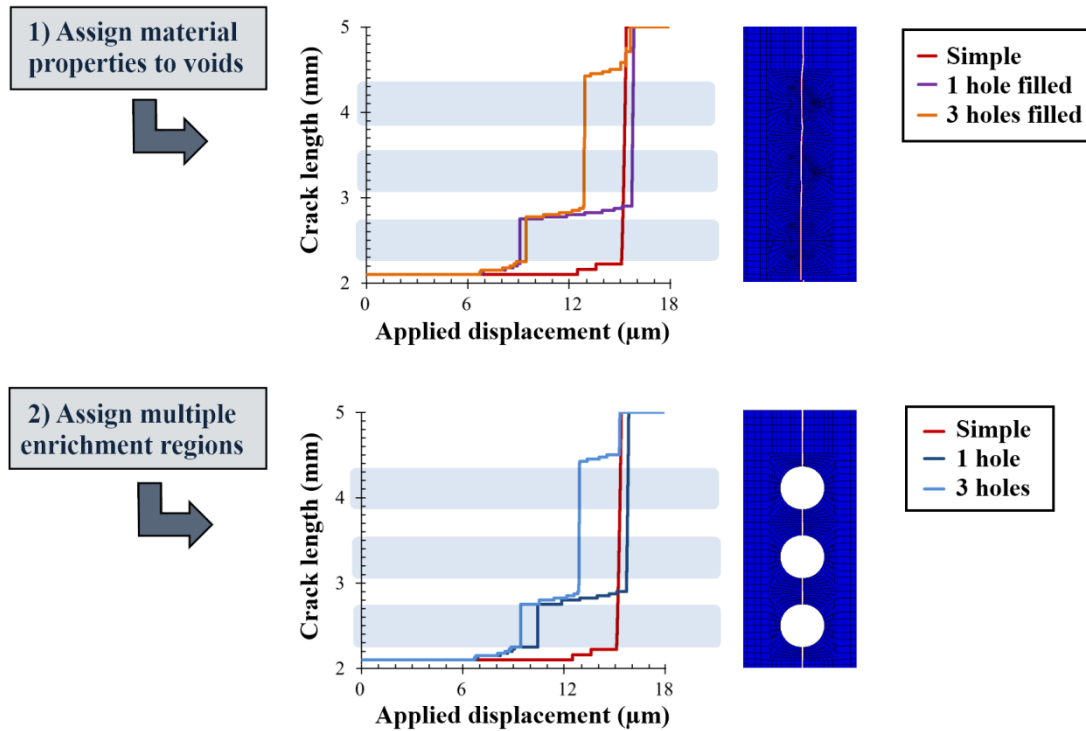


Figure 5.15: Crack length against the applied displacement for the models where the holes are filled with material properties (1) and the models where multiple enrichment regions are assigned (2). The blue-shaded areas represent the location of the holes along the crack path. Similar behaviour is observed in both techniques.

The reaction force on the left edge of the plate was then computed against the applied displacement and the crack length for all the models (Figure 5.16). Again, similar behaviour was observed for both modelling techniques. The measured reaction force was biggest for the simple plate and decreased as the number of holes increased.

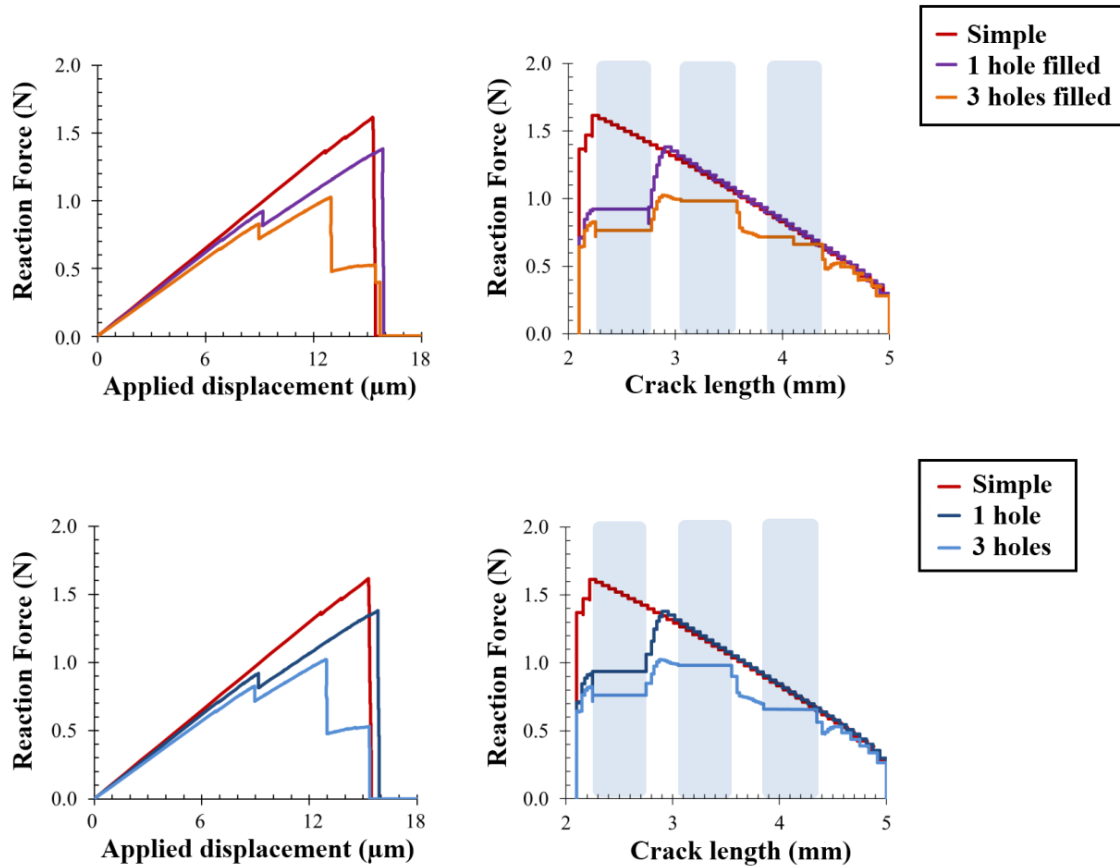


Figure 5.16: Reaction force at the left edge of the plate against the applied displacement and crack length. The blue-shaded areas represent the location of the holes along the crack path. Similar behaviour is observed in both techniques. The reaction force is biggest for the simple plate and smallest for the plate with three holes.

5.3.4 Discussion

In this study, two techniques were compared to propagate cracks through holes using the XFEM capability of Abaqus. In the first technique, material properties were assigned to the holes. The second technique consisted of a new approach of assigning multiple enrichment regions within the model. In the simple configurations studied here (Figure 5.10), where there was only one crack propagating from the notch, similar results were obtained with both approaches. Although effort was made to keep the mesh as similar as possible for all the models, the slight differences between the two techniques are thought to be attributed to the differences in the mesh, since the crack propagates one element at a time.

The increased porosity resulted in an earlier initiation of the crack for models with one and three holes, compared to the simple plate without holes (Figure 5.15). In the simple model, the crack propagated almost instantly soon after crack initiation occurred. In

contrast, for the models with holes, crack propagation was slowed down after propagating through the first hole. However, the second hole did not have the same effect, as the crack shot across the second and third holes. The evolution of the crack as it propagated through the three-hole plate is shown in Figure 5.17.

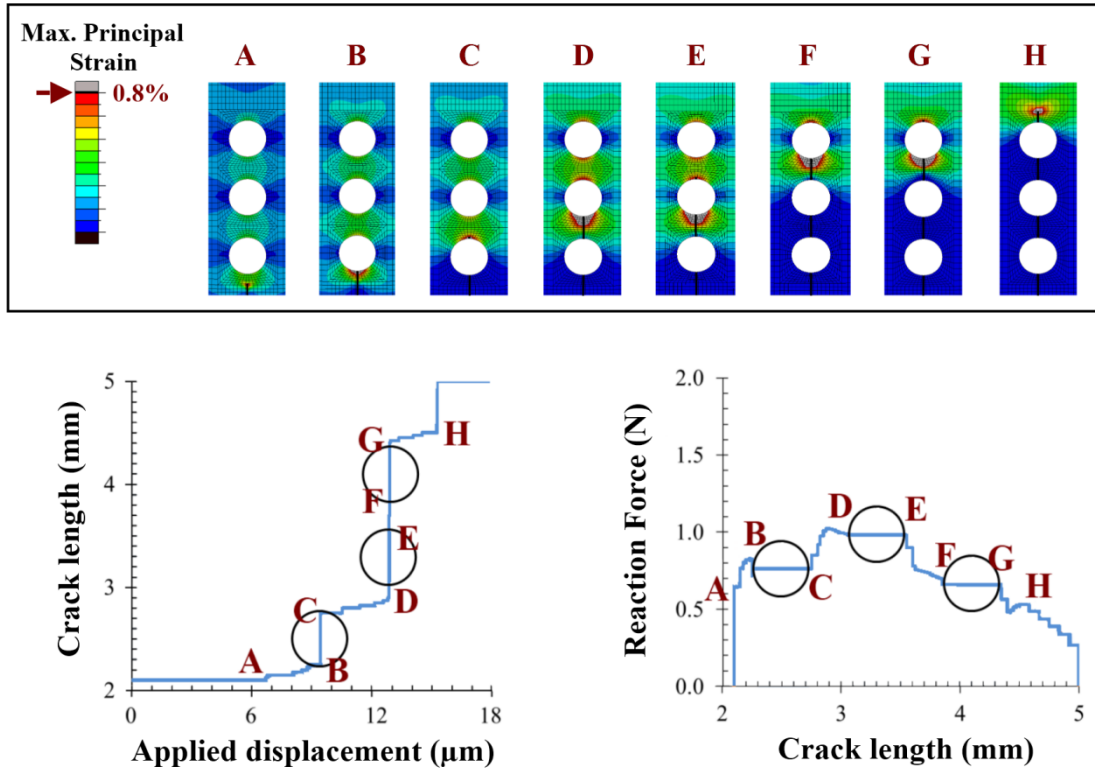


Figure 5.17: Propagation of the crack through the “3 holes” model. Maximum principal strain for damage initiation is set to 0.8%. The location of the holes and points of interest is identified in the curves of crack length vs. applied displacement and reaction force vs. crack length.

This preliminary study illustrates the capability of XFEM to propagate cracks through holes following the proposed two techniques. Previous studies have used XFEM in Abaqus to explore the effect of voids on the stress intensity factor (Jiang et al., 2014; Singh et al., 2011) or on neighbouring cracks (Haboussa et al., 2011). However, these studies have not considered the propagation of cracks through the voids. Recently, material properties have been assigned to voids in order to understand the influence of porosity on the crack path of osteonal cortical bone (Besdo and Vashishth, 2012; Li et al., 2013b). In this chapter, a novel enrichment region-based technique was proposed to propagate cracks through pores. The advantages of this technique will become clear in Section 5.5 of this chapter. In conclusion, XFEM within Abaqus can provide insight into the interaction between cracks and holes. As such, XFEM can be used to study how pore shape, size and location affect the propagation of the crack.

5.4 Influence of Pore Shape, Size and Location on Crack Propagation using XFEM

The proposed multiple enrichment region technique in XFEM-Abaqus can be applied to various configurations of bone porosity. This section uses the setup described in Figure 5.10 and the material properties defined in Table 5.1 to provide a set of examples that can be tested to evaluate the influence of pore shape (elliptical vs. circular pore), size (small vs. big pore) and location (closer vs. further from the notch) on the propagation of the crack through bone's micro-structure.

Pore shape

In bone's transverse direction, vascular pores can be approximated to circles, while lacunar pores have a more elliptical shape (Figure 4.17). It has been shown that in certain skeletal pathologies which affect whole bone toughness the shape of intra-cortical porosity is altered (Carriero et al., 2014a). This is why it is important to evaluate the influence of pore shape in crack propagation. Here three pore shapes are considered: circle ($R = 0.25$ mm) and vertical and horizontal ellipses (*long-axis* = 0.5 mm, *short-axis* = 0.2 mm). Figure 5.18 shows the models in the beginning of the simulation and when the crack has fully propagated. Due to the symmetry in all models, the crack propagated straight through the pores. However, differences can be observed in the force-displacement response. The force required for the crack to propagate through the horizontal ellipse is the highest, when compared to the circle and vertical ellipse, indicating that the horizontal ellipse provides most resistance to crack growth in this simple configuration.

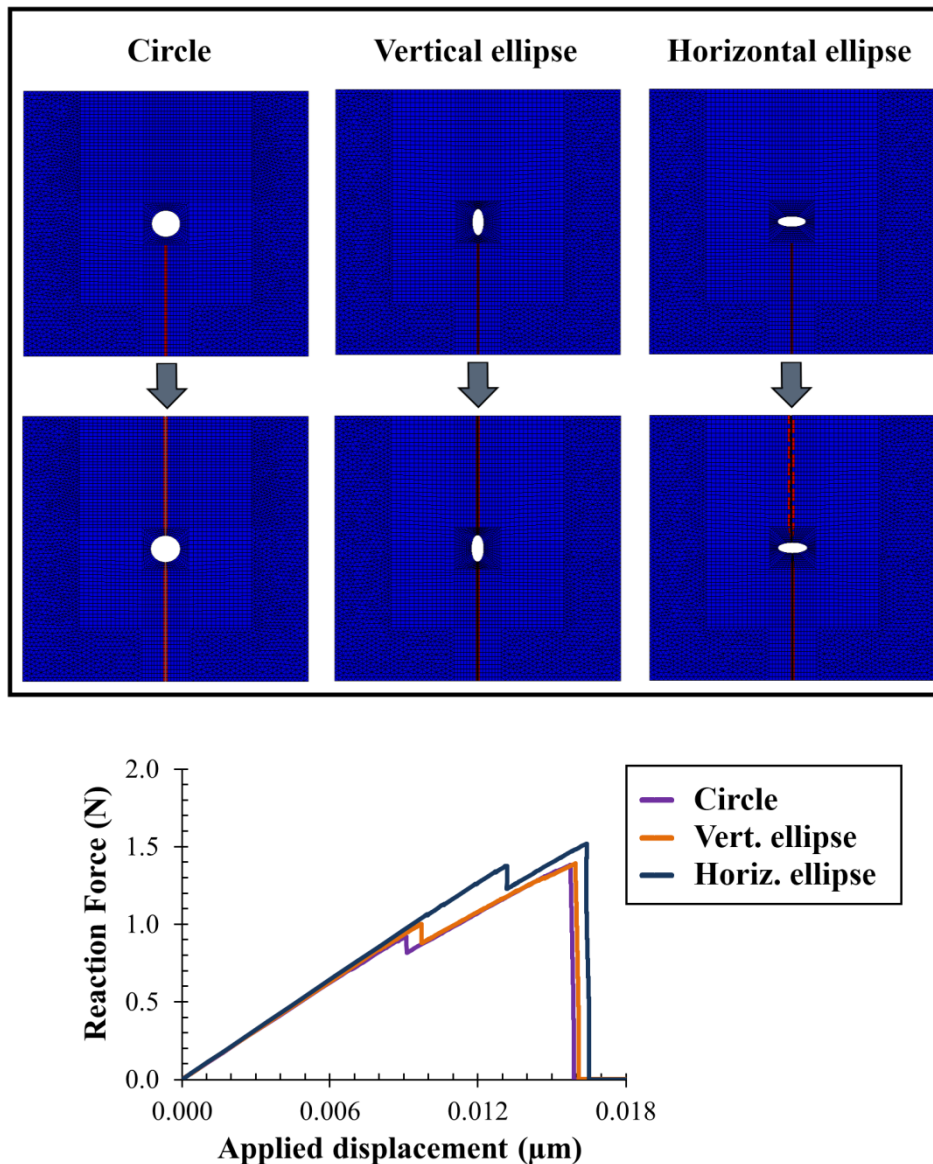


Figure 5.18: Effect of pore shape on crack propagation. The horizontal ellipse provides most resistance to crack growth when compared to a circle pore or a vertical ellipse.

Pore size

A distribution of different vascular and lacunar pore sizes can be found within a single bone (Figure 7.2). In addition, the size of the pores might be affected by age, as suggested by the differences in permeability values found in Chapter 4 (Rodriguez-Florez et al., 2014a). Here, a small ($R = 0.1 \text{ mm}$), medium ($R = 0.25 \text{ mm}$) and big ($R = 0.35 \text{ mm}$) pore sizes are considered (Figure 5.19). Comparing these three configurations, the small hole provides most resistance to crack growth.

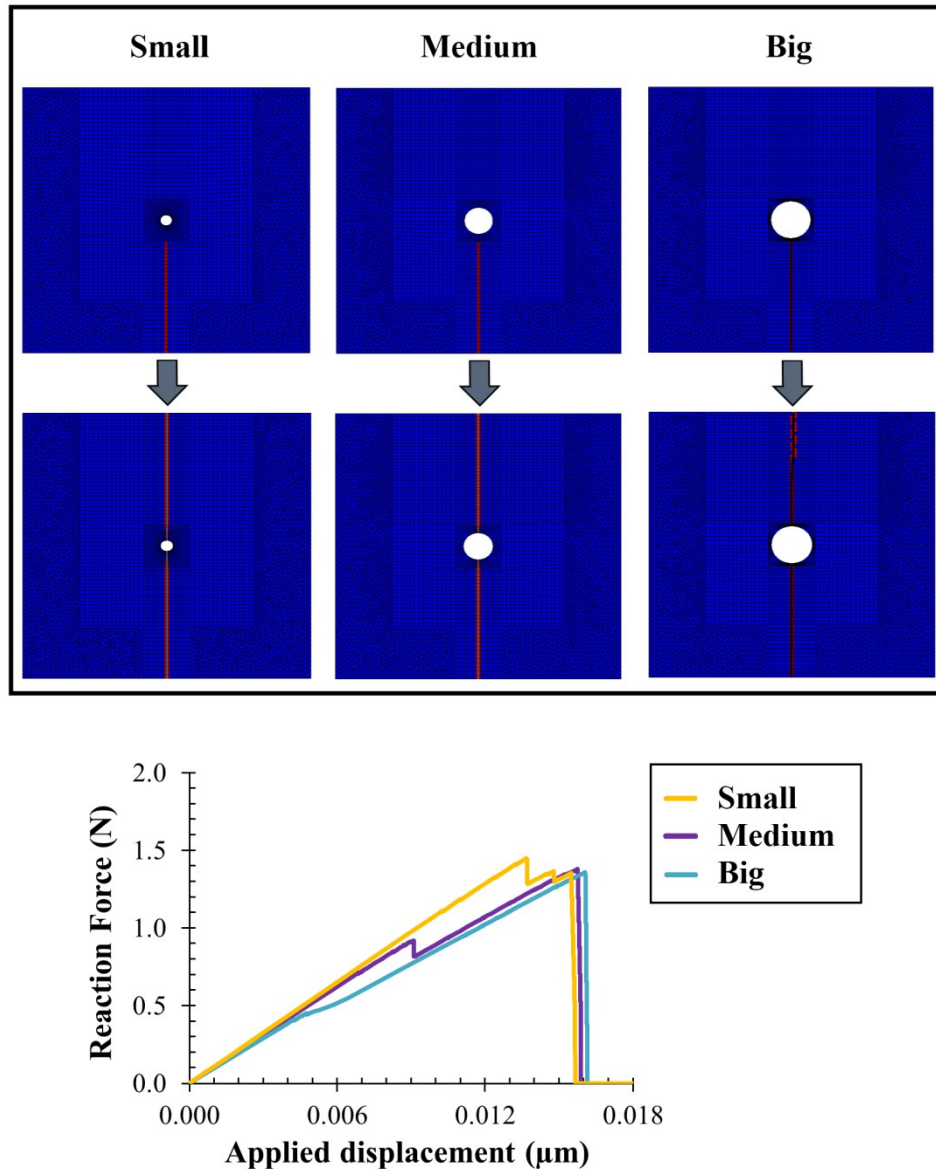


Figure 5.19: Effect of pore size on crack propagation. The small hole provides most resistance to crack growth.

Pore location

In the examples above the pores and notch were located in the middle. In bone, certain distributions of the pores might make the crack deflect, while other distributions might make the crack propagate faster. In order to investigate this, a hole in the middle, on the left of the notch (*horizontal distance from notch to hole centre* = 0.5 mm) and further left (*horizontal distance from notch to hole centre* = 0.75 mm) are considered (Figure 5.20). Moving the hole to the left makes the crack deflect; however, moving the hole further to the left does not alter the crack path. Among these three configurations, placing the hole in the middle provides least resistance to crack growth as the reaction

force is the smallest and a smaller displacement is required to make the crack propagate through the plate.

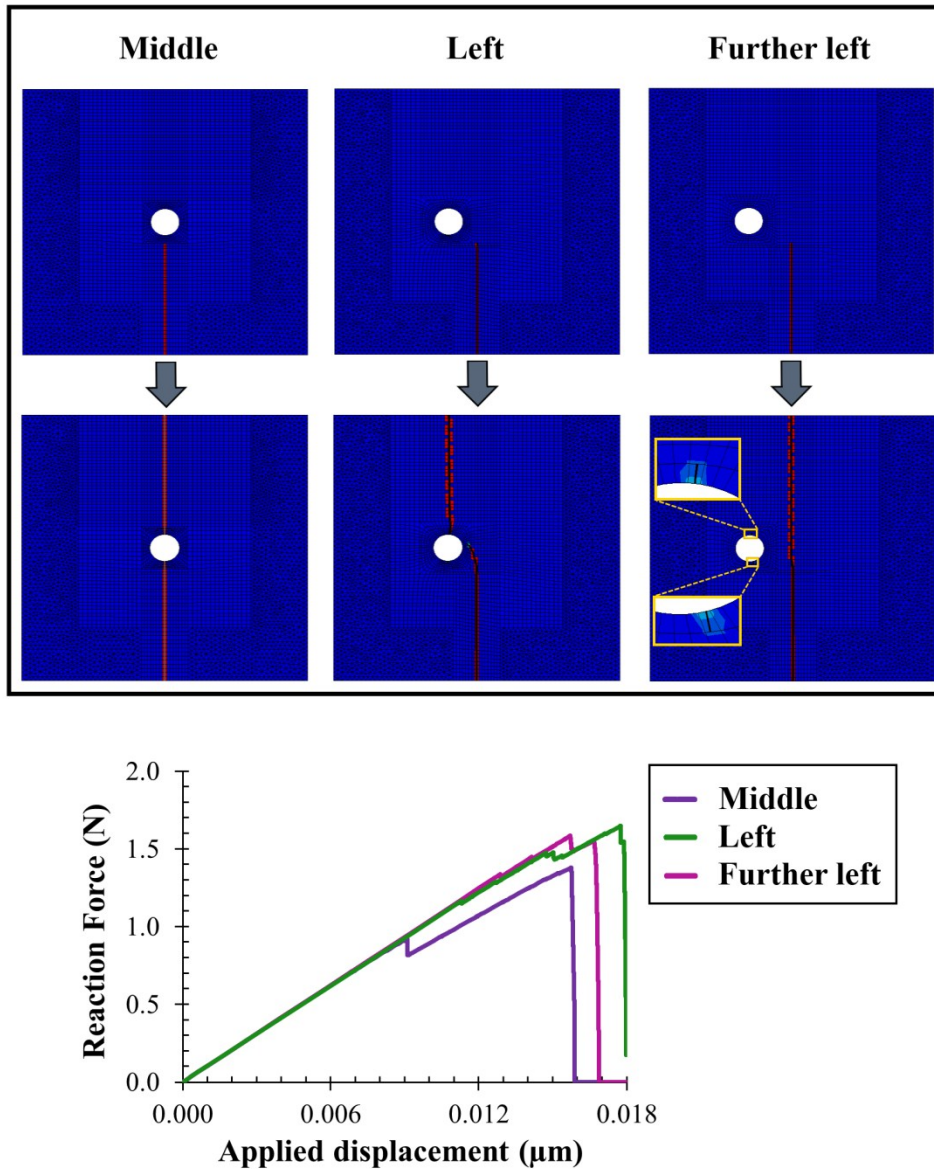


Figure 5.20: Effect of crack location on crack propagation. The crack growth path is altered by the hole located on the left, while by locating the hole further left the path is straight. The hole in the middle provides least resistance to crack growth.

In summary, the novel multiple partition technique in XFEM-Abaqus provides the framework to study several pore configurations in 2D. This will be applied to brittle bone disease (osteogenesis imperfecta) in the following section.

5.5 The Use of XFEM to Assess the Influence of Intra-cortical Porosity on Crack Propagation: Application to Brittle Bone Disease

Healthy bone is a tough material, thanks to the multiple interconnected toughening mechanisms that act from the molecular level up to the macro-scale (Buehler, 2007; Gupta et al., 2013; Launey et al., 2010; Nalla et al., 2005; Ritchie et al., 2009; Ural and Vashishth, 2014; Wang and Gupta, 2011; Zimmermann et al., 2014). Although it is known that ageing or certain skeletal diseases increase bone's fracture risk, it is still not fully understood how specific diseases affect those toughening mechanisms. This understanding is essential for the future design of effective treatments (Ural and Vashishth, 2014).

Osteogenesis imperfecta is a disease that is caused by mutations in the collagen genes and leads to skeletal fragility, as explained in the Introduction. The mouse model of the severe case of osteogenesis imperfecta (*oim*^{-/-}) has smaller and denser vascular canals with a branched architecture compared to their wild-type controls (*oim*^{+/+}) (Carriero et al., 2014a). A recent study has hypothesised that the altered intra-cortical porosity might contribute to the increased fracture risk in *oim*^{-/-} bone (Carriero et al., 2014b).

In order to test this hypothesis and assess the sole contribution of intra-cortical porosity on crack growth, computational techniques can be used. Previous computational studies have developed simplified geometric representations of the micro-structure of osteonal (human and bovine) cortical bone to model crack growth at the tissue level (Abdel-Wahab et al., 2012; Budyn et al., 2008; Gao et al., 2013; Li et al., 2013b; Mischinski and Ural, 2013; Ural et al., 2011). These studies have focused on the role of osteons and cement lines as the main toughening mechanisms at the micro-scale. However, mouse bone does not have osteons. Very recently, Donaldson et al. (2014) developed an elaborate multi-level voxel-based FE model to investigate the contribution of porosity on wild-type mouse bone toughness (Donaldson et al., 2014). This approach represented a localised brittle behaviour of bone, as elements were removed according to a stress-based damage initiation criterion. In contrast, the cohesive segment method in XFEM has the capability of capturing damage evolution –representing the toughening behaviour of bone– following the user-defined traction-separation curve (Figure 5.6).

In the current chapter, the XFEM capability of Abaqus was chosen to assess the influence of micro-porosity on crack propagation in *oim* bone. The two modelling techniques presented in Section 5.3 (assigning material properties to voids and employing multiple enrichment regions) were tested using 2D models of the vascular porosity of healthy *oim*^{+/+} and brittle *oim*^{-/-} cortical bone.

5.5.1 Materials and Methods

Model setup

Abaqus (v 6.12, Dassault Systemes 2012) was used for the XFEM studies. The model setup is represented in Figure 5.21. The topology of transverse cross sections of healthy *oim*^{+/+} and brittle *oim*^{-/-} tibial mid-diaphysis was captured with synchrotron radiation-based computed tomography (CT) in the Swiss Light Source by Carriero et al. (Carriero et al., 2014a) and used to create 2D models of vascular and lacunar porosity. In this study, only the vascular porosity was considered. Hence, regions containing vascular canals were chosen. A frame of non-porous material was introduced around the model, in order to minimise the influence of the boundary conditions on the failure analysis.

A notch ($a_0 = 30 \mu\text{m}$) was placed in the centre of the lower edge and a displacement ($\delta = 2 \mu\text{m}$) was applied perpendicular to the notch in the x-direction. The left edge was constraint in the x-direction. The two nodes corresponding to the corners of the bottom edge were also constraint in the y-direction to aid in the convergence.

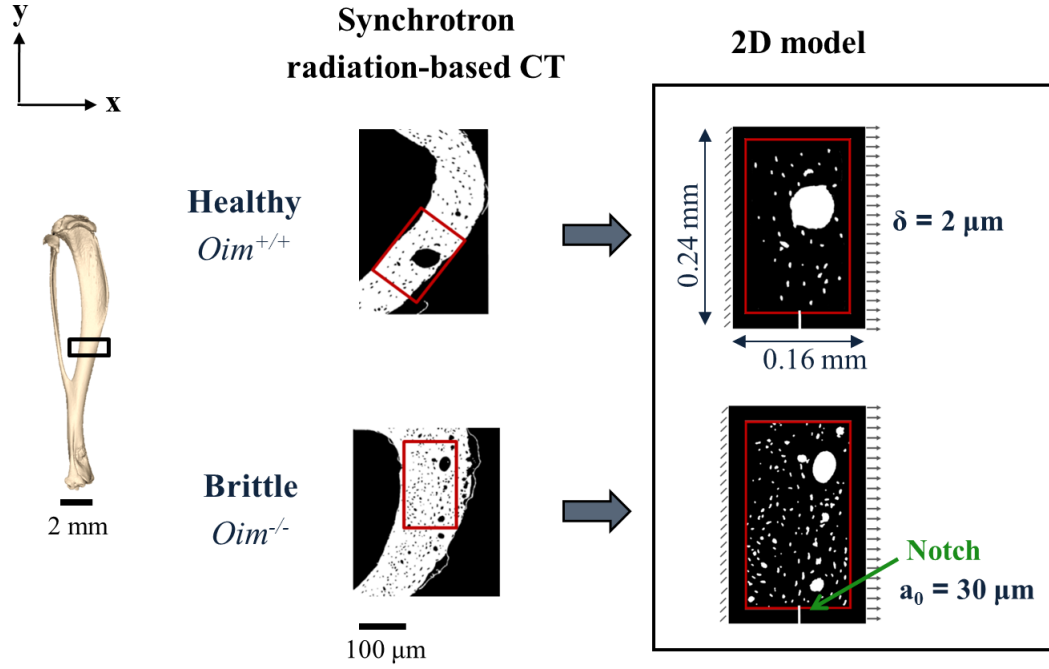


Figure 5.21: Model geometry setup. The topologies of transverse cross sections of healthy $oim^{+/+}$ and brittle $oim^{-/-}$ tibial mid-diaphysis were captured with synchrotron radiation-based CT (Carriero et al., 2014a) and used to create 2D models of vascular and lacunar porosity. A notch a_0 was placed and a displacement δ was applied on the right edge, while the left edge was constraint in the x-direction.

Only vascular canals were considered to test the two modelling approaches, as shown in Figure 5.22. For the first approach, void material properties were assigned to vascular canals and only one enrichment region was considered. In the second approach, several partitions were made and multiple enrichment regions were assigned. Enrichment regions were chosen progressively looking at maximum principal stress maps so that a crack would initiate in all the elements where the damage initiation criterion was met.

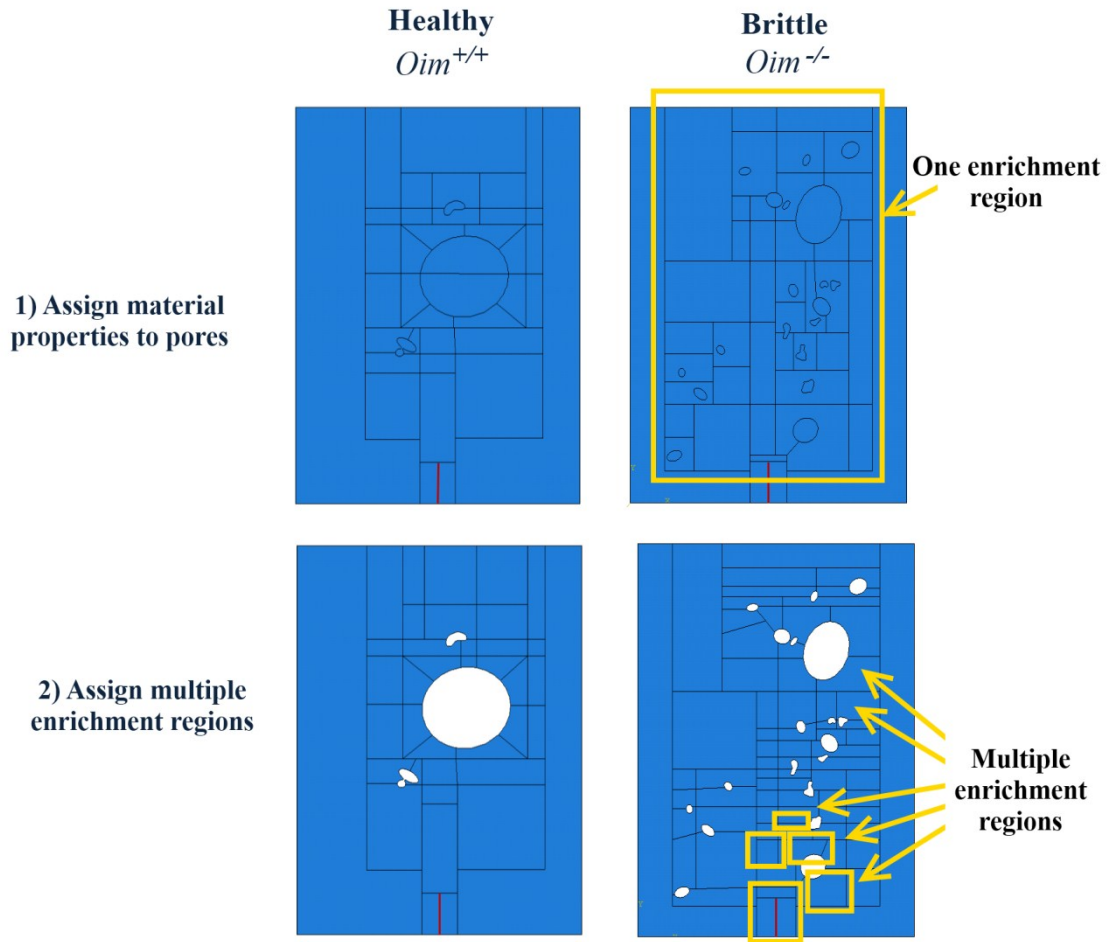


Figure 5.22: $Oim^{+/+}$ and $oim^{-/-}$ models of vascular canals with several partitions. For the first technique, partitions are made only to aid the meshing process but there is one enrichment region assigned to the model. In contrast, for the second technique, different enrichment regions are assigned to each partition.

The configuration of the used element-types and meshes is shown in Figure 5.23. Four-node bilinear plane-strain quadrilateral elements with reduced integration (CPE4R) were used in the majority of the model and three-node linear plane-strain triangle elements (CPE3) were employed in the edges. The average element size ranged from $0.5\ \mu\text{m}$ around the pores to $2\ \mu\text{m}$ in the edges. Effort was made to keep the meshes as structured as possible.

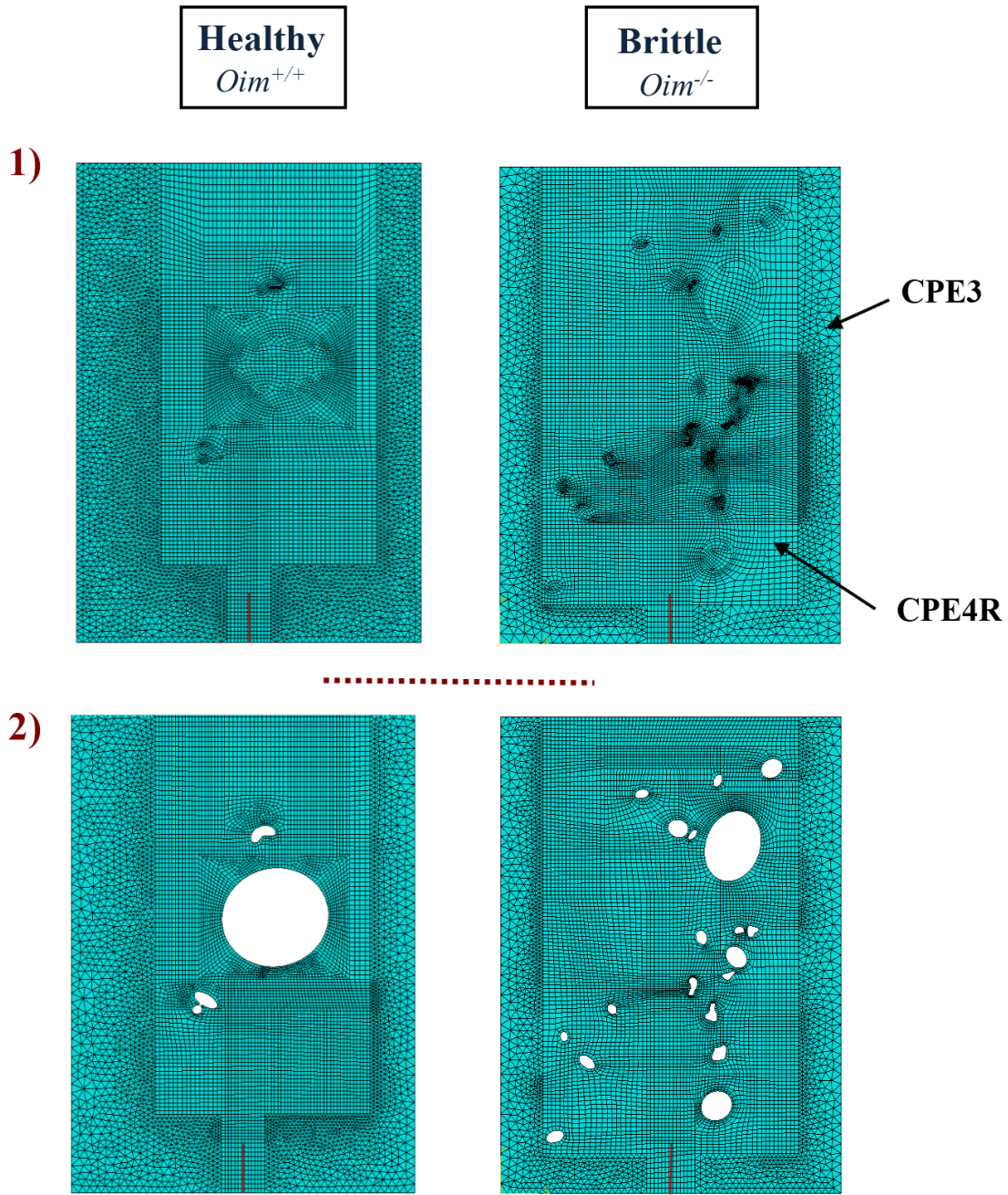


Figure 5.23: Configuration of the meshes for $oim^{+/+}$ and $oim^{-/-}$ 2D models of vascular canals in the case of 1) material properties assigned to voids and 2) multiple enrichment regions.

Material properties

The cohesive segment method was used with maximum principal stress failure criterion for damage initiation and an energy based damage evolution law for crack propagation (Abdel-Wahab et al., 2012; Feerick et al., 2013). Bone was considered isotropic linear elastic and homogeneous and bone material properties were maintained constant for $oim^{-/-}$ and $oim^{+/+}$ (Table 5.2), to explore the influence of porosity only. It must be noted

that cortical bone damage properties used in the literature vary between 40-150 MPa for maximum principal stress (in tensile) and 0.2-3 N/mm for fracture energy (Abdel-Wahab et al., 2012; Feerick et al., 2013; Gao et al., 2013; Mischinski and Ural, 2011). Since $oim^{-/-}$ bones are known to have low fracture toughness (Carriero et al., 2014b), low values of damage properties were chosen. Hard contact was implemented on the faces of the elements split by the crack, to prevent non-physical over closure, with a coefficient of friction of 0.3 (Feerick et al., 2013).

Table 5.2: Material properties assigned to $oim^{+/+}$ and $oim^{-/-}$ models.

		Bone	Pore
<i>Elastic properties</i>	Young's modulus, E (MPa)	10000	10
	Poisson's ratio, ν	0.15	0.15
<i>Damage properties</i>	Max. principal stress, σ_{max} (MPa)	50	50
	Fracture energy, G_c (N/mm)	0.238	0.238

5.5.2 Results

Prior to the implementation of the failure criterion, a simple FEM model of the vascular and lacunar porosities of $oim^{+/+}$ and $oim^{-/-}$ was used in order to compare the levels of stress achieved and roughly identify the regions of maximum stress, which should correspond to regions of crack initiation. Figure 5.24 indicated that brittle bone reached higher maximum principal stresses than healthy bone. The areas of maximum principal stress were located around the vascular pores.

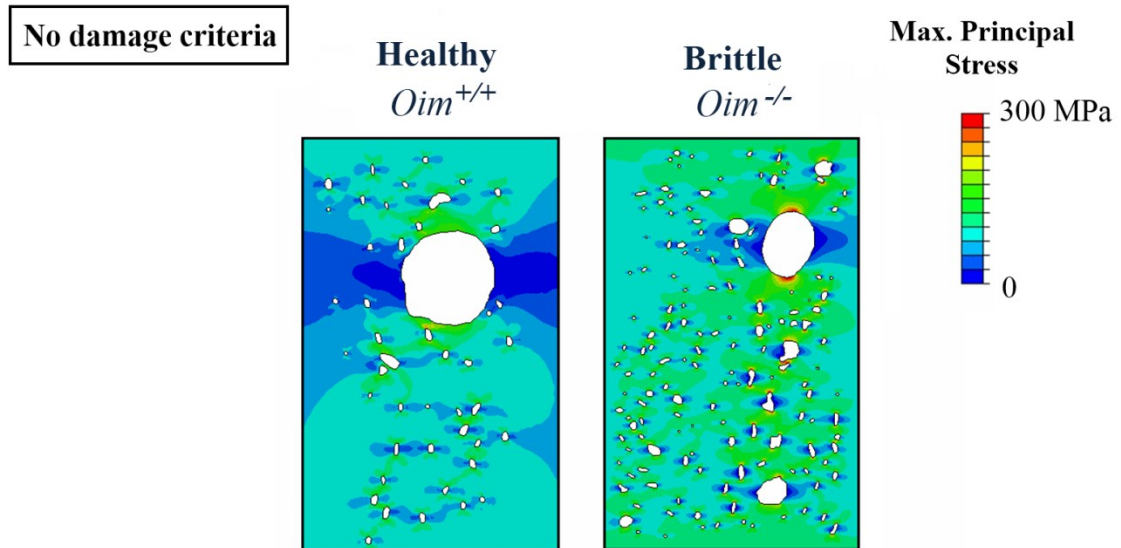


Figure 5.24: FE models of *oim^{+/+}* and *oim^{-/-}* after applying a displacement of 2 μm to the right edge. Brittle bone reached higher stresses than healthy bone. Regions of maximum stresses correspond to areas around the vascular pores.

The crack propagation path for the four models is shown in Figure 5.25. When material properties were assigned to the vascular pores, only one crack propagated through the model. In contrast, when multiple enrichment regions were assigned, several cracks started progressively following the vascular pores.

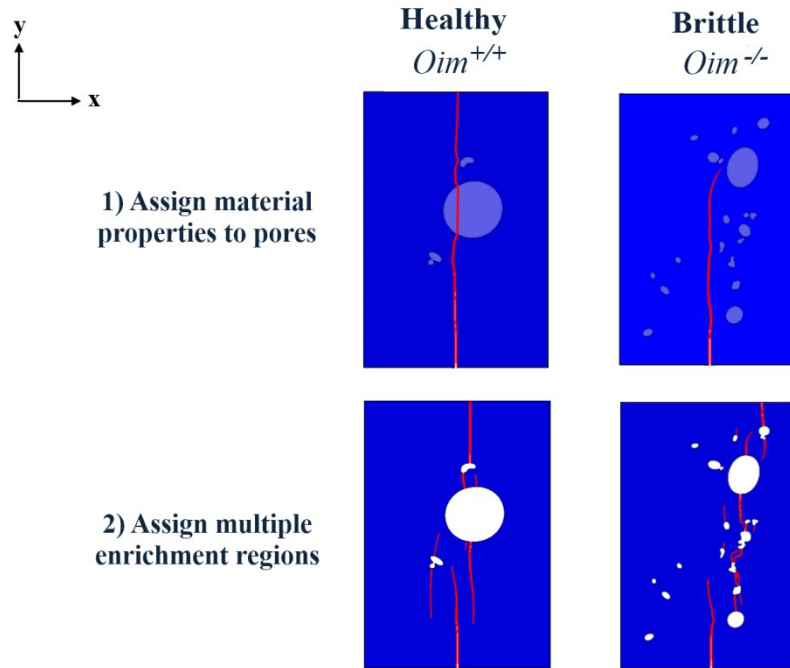


Figure 5.25: Crack propagation (in red) in $oim^{+/+}$ and $oim^{-/-}$ models of vascular pores. Only one crack propagates when material properties are assigned to voids, while multiple cracks propagate following the vascular pores when using the multiple enrichment region technique.

The reaction force on the left edge was computed against the applied displacement (Figure 5.26). The force-displacement curves showed that when the material properties were assigned to the voids, the reaction force was bigger in the $oim^{-/-}$ bones. In contrast, when multiple partitions were employed, $oim^{-/-}$ bones showed smaller reaction forces than healthy $oim^{+/+}$ bones.

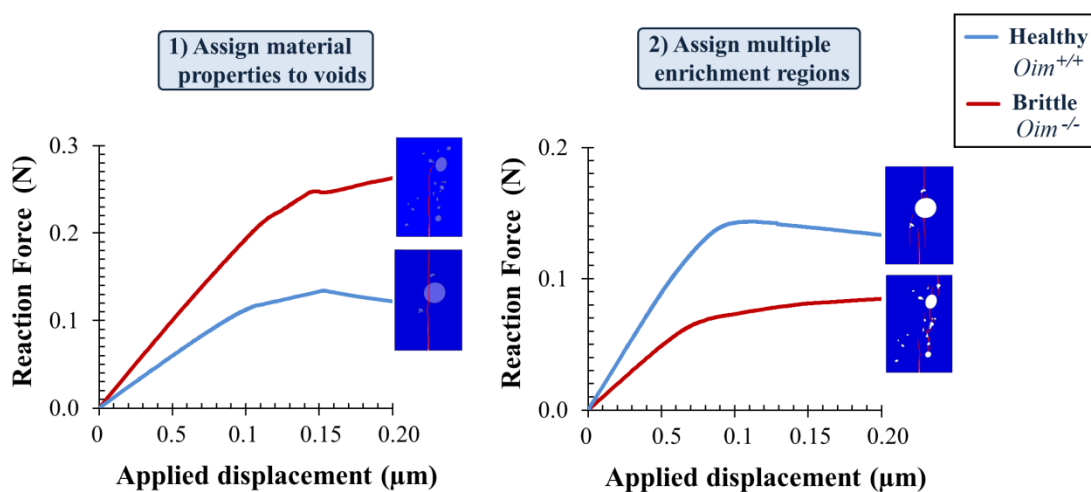


Figure 5.26: Reaction force on the left edge against the applied displacement on the right edge of the model. With the first technique (left), $oim^{-/-}$ model exhibits a bigger reaction force, while with the second technique (right) $oim^{-/-}$ has lower reaction forces than healthy $oim^{+/+}$.

5.5.3 Discussion

The influence of the transversal topology of vascular canals on crack propagation of healthy $oim^{+/+}$ and brittle $oim^{-/-}$ tibial bone was studied using XFEM techniques in Abaqus. The first modelling approach (assigning material properties to voids) did not capture the sequential crack nucleation, revealed by the second method (assigning multiple enrichment regions). Results suggest that crack propagation is strongly affected by the micro-porosity, as the main crack follows the vascular canals.

The proposed modelling techniques yielded to different crack propagation trajectories. In Abaqus-XFEM, additional cracks cannot nucleate until all pre-existing cracks in an enriched feature have propagated (Abaqus 6.12 documentation). Thus, when assigning material properties to the voids and considering the whole model as one enrichment region, new cracks cannot initiate until the existing crack from the notch has fully propagated reaching the boundary. Hence, although some regions might have met the damage initiation criteria, sequential crack nucleation cannot be captured, as shown below in Figure 5.27.

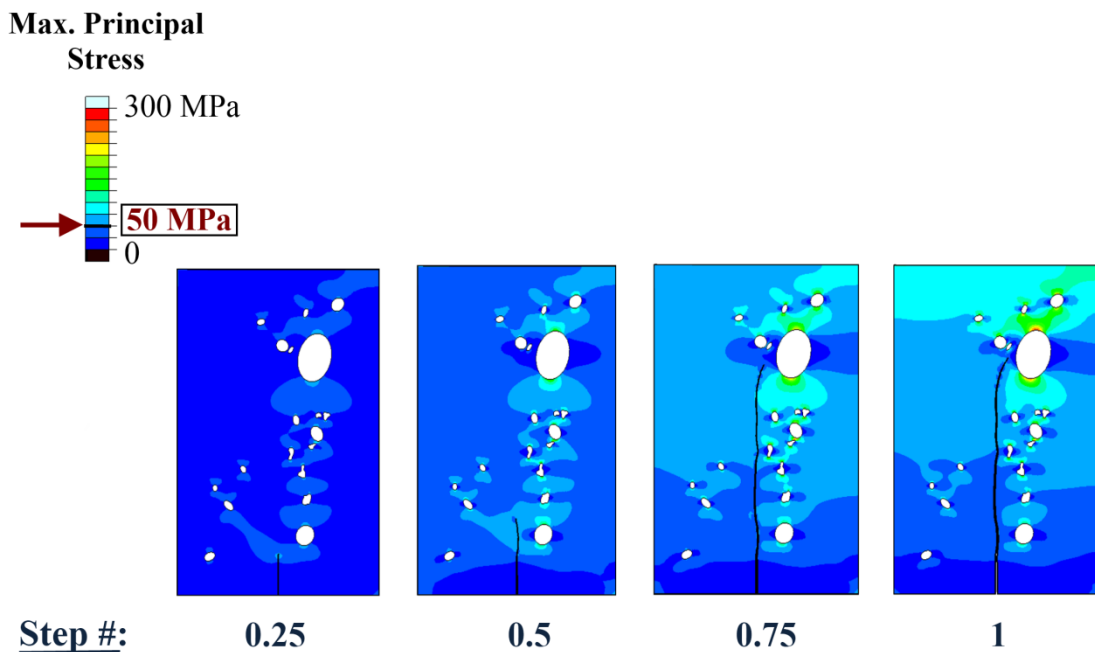


Figure 5.27: Crack growth in the $oim^{-/-}$ model where material properties were assigned to voids (the pores are shown white to aid in the visualisation). Although the critical maximum principal stress of 50 MPa is reached around the pores by step 0.5, new cracks do not initiate.

The sequential crack nucleation was captured when using the second technique and specifying multiple enriched features in the model. However, there are some limitations

that must be taken into account when employing this technique. The most obvious one is that it is time consuming to create partitions in the model. This will become increasingly difficult when including lacunar porosity or modelling bone micro-porosity in 3D under mixed loads. Other limitations are associated with the XFEM capabilities in Abaqus, as cracks cannot branch nor interact with each other (Abaqus 6.12 documentation). Therefore, as shown in Figure 5.28, two cracks which are directed towards the same element might lead to high stress concentrations.

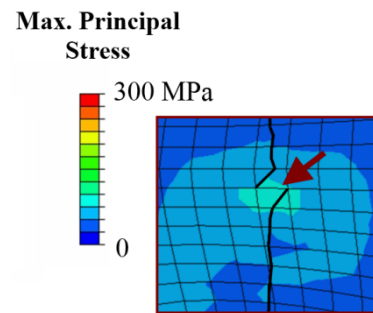


Figure 5.28: Since cracks cannot interact, cracks directed towards the same element lead to stress concentrations.

Despite these limitations, the multiple enrichment region technique indicated that the main crack follows the vascular canals as it propagates. This is in accordance with previous experimental studies in healthy mouse cortical bone which have demonstrated that the number of micro-cracks correlate significantly to the amount of vascular canals (Voide et al., 2011). The current study further indicates that the increased density of vascular canals found in *oim*^{-/-} cortical bone (Carriero et al., 2014a) contributes to its brittleness (Carriero et al., 2014b), even when the same material properties were assigned to *oim*^{-/-} and *oim*^{+/+} models.

As a conclusion, the novel technique of assigning multiple enrichment regions is suggested for future Abaqus-XFEM modelling of bone micro-architecture.

5.6 Conclusions

This chapter explored the use of extended finite element models in Abaqus to investigate the influence of bone micro-porosity on crack formation and growth at the micro-scale. A new modelling technique based on multiple enrichment regions was suggested and it was compared to assigning material properties to voids. These two approaches yielded to similar results in the simple case of a plate with one and three holes located along the crack trajectory with no sequential crack nucleation. However, when the complex topology of *oim* vascular porosity was modelled, assigning material properties to the pores resulted in non-physical propagation of the crack. Hence, **the novel method of assigning multiple enrichment regions is recommended for future XFEM models that include intra-cortical porosity.** To conclude, **the preliminary results shown in this chapter indicate that altered intra-cortical porosity contributes to the brittleness of *oim*^{-/-} bone at the tissue level.** A detailed study of the individual influence of different bone features in bone toughness is essential to develop strategies to inhibit fracture risk.

In the XFEM model of *oim* bone used in this chapter, the sole influence of micro-scale structure was explored. Changes in the composition and organisation of bone's building blocks –collagen, mineral and water- also affect the ability of bone to resist fracture. Chapter 6 analyses the implications of altered bone toughness in the mineral properties of cortical bone by comparing mouse models that exhibit brittle and ductile behaviour at the macro-scale.

6 ALTERED MECHANICS IN PATHOLOGIC BONE: IMPLICATIONS IN THE MINERAL

The research described in this chapter has been published in: N. Rodriguez-Florez, E. Garcia-Tunon, Q. Mukadam, E. Saiz, K.J Oldknow, C. Farquharson, J.L. Millan, A. Boyde, S.J. Shefelbine, 2014, An investigation of the mineral in ductile and brittle cortical mouse bone, Journal of Bone and Mineral Research, doi: 10.1002/jbmr.2414.

Skeletal pathologies often affect bone's basic building blocks. When bone's mineral phase, organic matter and/or water (or their assembly) is altered, the mechanical integrity of the whole bone can be compromised. Here, the composition and structure of two different pathologic bone types that have very different whole bone mechanical properties, *oim* (mouse model of osteogenesis imperfecta) and *PhosphoI* (mouse model of rickets), are investigated.

Osteogenesis imperfecta (OI, or brittle bone disease) is characterised by a mutation in the collagen. As a result of an abnormal collagen template, the mineral composition and structure of *oim*^{-/-} bones is altered. Other skeletal diseases do not directly alter bone's constituents, but instead interfere with the process of formation of these constituents, as it occurs in rickets. *PhosphoI*^{-/-} bones are missing an enzyme required for the mineralisation process, which leads to altered amount and composition of the mineral. The consequences of these two pathologies in whole bone mechanics are very different: *oim*^{-/-} bones are brittle, while *PhosphoI*^{-/-} are ductile. Despite representing the two extremes of toughness, brittle and ductile bones are expected to have similar alterations in the mineral.

The aim of this chapter is to explore the mineral of brittle *oim*^{-/-} and ductile *PhosphoI*^{-/-} cortical bone at the mineral (nm) and tissue (µm) levels.

6.1 Pathologic Bone: Extremes of Toughness

Bones considered in this chapter belong to two mouse strains that exhibit the two extremes of toughness. At the whole bone level, bones of B6C3Fe-a/aCol1a2oim/oim (*oim*^{-/-}) are brittle, while Phospho1-R74X null mutant (*Phospho1*^{-/-}) mice have ductile bones (Carriero et al., 2014b, 2014d) (Figure 6.1). A brief review of the studies that have characterised the mechanics, structure and composition of these two mouse strains is presented here.

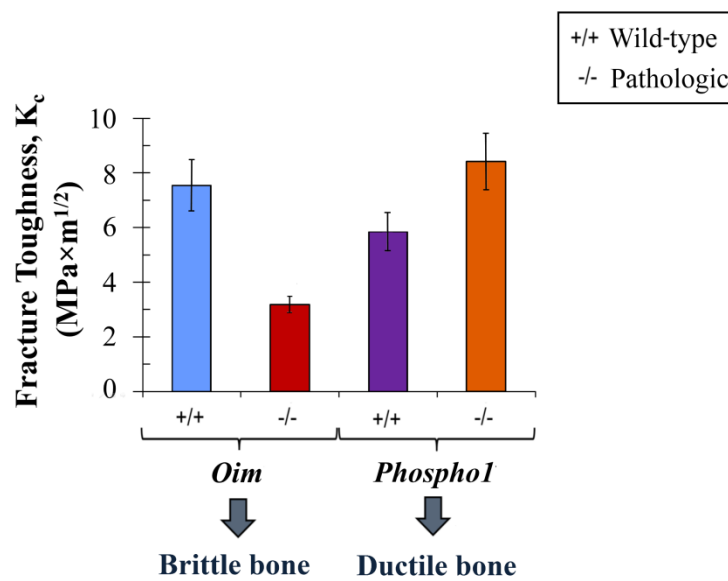


Figure 6.1: Values of fracture toughness adapted from literature (Carriero et al., 2014b, 2014d). K_c from all groups is significantly different ($p < 0.05$).

Osteogenesis imperfecta murine (Oim^{-/-})

Osteogenesis imperfecta murine (*oim*^{-/-}) is a mouse model of the disease similar to moderate-severe OI in humans (Chipman et al., 1993). Osteogenesis imperfecta is caused by a defect in the genes coding for collagen (Rauch and Glorieux, 2004). It is an excellent model of the implications of molecular defects on whole bone integrity. This is why numerous studies have explored the structure, mechanics and composition of *oim*^{-/-} bones.

At the whole bone level, *oim*^{-/-} bones exhibit decreased ultimate stress (Vanleene et al., 2012), toughness (Carriero et al., 2014b, 2014d) and torsional stiffness (McBride et al., 1998) and are smaller in size and body mass (Chipman et al., 1993) than their wild-type controls (*oim*^{+/+}). At the tissue level, *oim*^{-/-} bones have decreased elastic modulus

(Mehta et al., 2009; Vanleene et al., 2012), increased resistance to plastic deformation (Grabner et al., 2001) and a higher degree of mineralisation of the bone matrix (Grabner et al., 2001; Vanleene et al., 2012). Depending on the technique used, the number of animals, and the age of the animals, studies have found the mineral to matrix ratio of *oim*^{-/-} bone to be smaller (Bart et al., 2014), the same (Camacho et al., 1999) or larger (Carriero et al., 2014b; Vanleene et al., 2010) than wild-type *oim*^{+/+} bone. At the nano-scale, the *oim*^{-/-} apatite thickness is smaller, the mineral crystals are more tightly packed and not as well aligned as in wild-type bone (Camacho et al., 1996; Fratzl et al., 1996; Grabner et al., 2001; Vanleene et al., 2012). The collagen structure is disorganised (Nadiarnykh et al., 2007) and there is increased water content (Miles et al., 2002). Tensile tests on demineralised *oim*^{-/-} and wild-type bone revealed no difference in material properties, suggesting that abnormalities in the mineral and in the interaction between the mineral and collagen contribute to the poor material properties in *oim*^{-/-} bone (Miller et al., 2007a).

Phospho1-R74X null mutant (Phospho1^{-/-})

Phospho1-R74X null mutant (*Phospho1*^{-/-}) is a mouse model that represents defective mineralisation process caused by the ablation of the phosphatase PHOSPHO1 (MacRae et al., 2010). PHOSPHO1 is essential for the generation of inorganic phosphate for bone mineralisation (Huesa et al., 2011; Millán, 2013; Yadav et al., 2011). As such, it can be used to study skeletal diseases associated with altered mineralisation such as rickets in children and osteomalacia in adults (Yadav et al., 2011, 2014).

Phospho1^{-/-} mice have more ductile (Huesa et al., 2011) and tougher (Carriero et al., 2014d) bones at the macro-scale when compared to their wild-type controls (*Phospho1*^{+/+}). They exhibit reduced elastic modulus and hardness at the tissue level and reduced local mineral to matrix ratio (Huesa et al., 2011). The collagen-mineral level of *Phospho1*^{-/-} mice has not yet been explored.

6.2 Mineral Characterisation Techniques

The mineral properties of brittle and ductile bones were determined combining some of the techniques presented in the Introduction (Section 2.2.2). Thermogravimetric analysis, nanoindentation, BSE SEM and XRD were combined to explore the mechanics, structure and composition of *oim* and *Phospho1* mouse cortical bone at different length-scales (Figure 6.2).

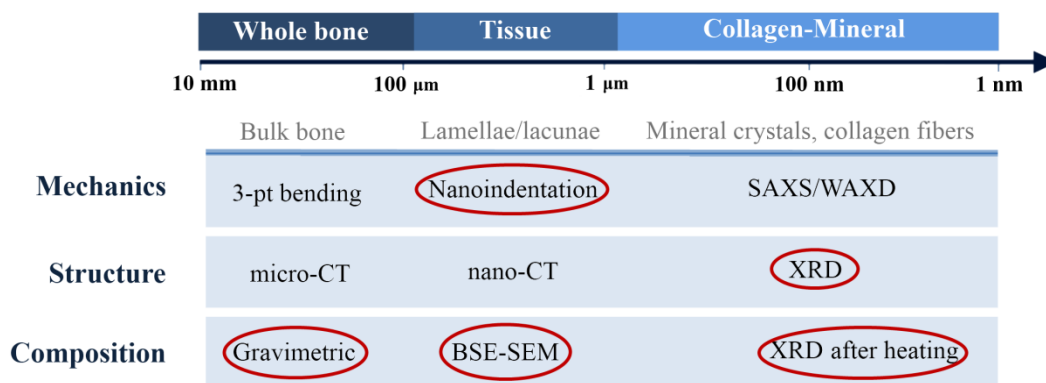


Figure 6.2: Multi-scale techniques used in this chapter indicated in comparison with the bone characterisation techniques described in the Introduction (Figure 2.11).

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) measures the loss of mass with temperature and monitors the changes associated with dehydration and organic decomposition. Hence, it provides a quantification of the bulk mineral to matrix ratio of the sample. In bone, the change in mass monitored by TGA is considered to be due to loss of water for temperatures up to 200°C, organic content from 200°C to 600°C, and carbonate content above 600°C (Legros et al., 1987; Peters et al., 2000; Wang et al., 2003) (Figure 6.3).

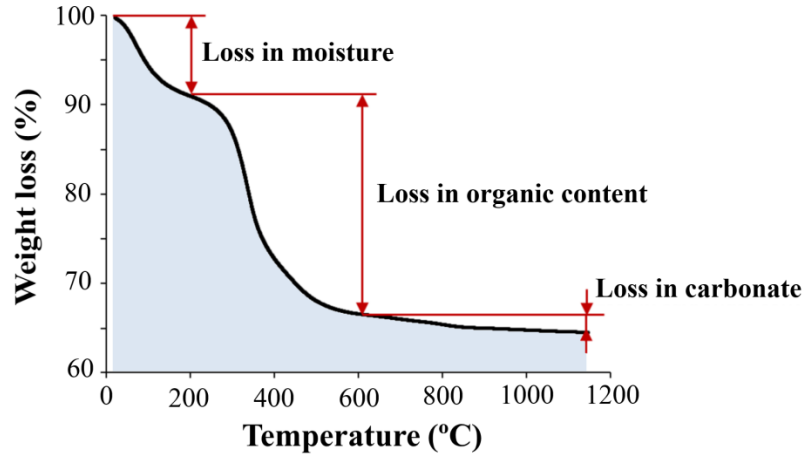


Figure 6.3: Weight loss (%) with temperature in *oim*^{+/+} femur powder measured by TGA. Moisture is lost until 200°C, organic content until 600°C and carbonate afterwards.

Bulk mineral to matrix ratio can be calculated as the ratio between the percentages of mass remaining after heating to 600°C and the organic mass loss between 200°C and 600°C (Peters et al., 2000):

$$\frac{\text{Mineral}}{\text{Matrix}} = \frac{m_{600^{\circ}\text{C}}(\%)}{m_{200^{\circ}\text{C}}(\%) - m_{600^{\circ}\text{C}}(\%)} \quad [6.1]$$

The percentage of mass at 600°C represents the weight percentage of mineral content and it depends on the amount of mass lost due to moisture, which might be altered due to skeletal pathologies or due to sample preparation. In order to avoid this influence, the mass percentage at 600°C is translated to dry weight percentage for the calculation of the mineral to matrix ratio:

$$m_{600^{\circ}\text{C}}(\% \text{ dry weight}) = \frac{m_{600^{\circ}\text{C}}(\% \text{ initial weight})}{m_{200^{\circ}\text{C}}(\% \text{ initial weight})} \times 100 \quad [6.2]$$

Nanoindentation

Nanoindentation is used to characterise tissue elastic properties of bone. This technique was described in detail in Chapter 3.

Backscattered electron microscopy (BSE SEM)

Backscattered electron scanning electron microscopy (BSE SEM) provides information about the degree of mineralisation of the mineralised bone matrix at the micro-scale (Roschger et al., 1998). As the primary electron beam is focused and scanned across the

specimen surface, the electrons interact with the atoms in the sample and generate different types of signals, one of them being backscattered electrons. BSE signal is dependent on the atomic number of the material and it reflects the local electron density providing a grey-level image of the surface layer of the specimen (penetration depth $\sim 1\text{-}2\text{ }\mu\text{m}$) (Bloebaum et al., 1997). To better capture the contrast, it is often useful to normalise the grey-values of the pixels according to the lower (A) and upper (B) bounds of the histogram of the pixel distribution:

$$p_n = \frac{(p - A)}{(B - A)} \times r \quad [6.3]$$

where p_n is the normalised pixel value, p is the current pixel, and r is the bin range, in this case 255.

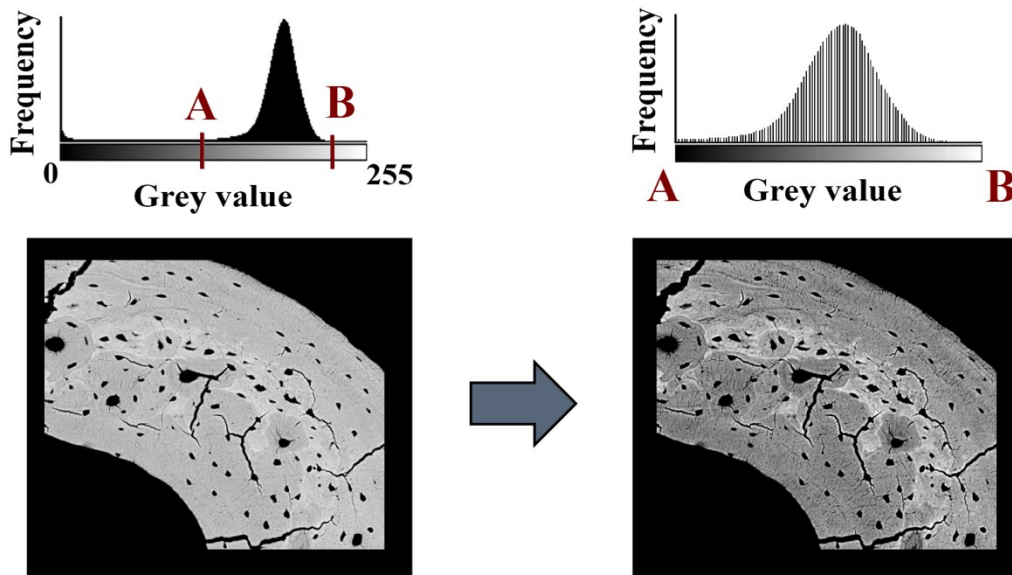


Figure 6.4: BSE SEM image of *Phospho1*^{-/-} bone with the original pixel distribution (left) and after normalising the grey-values between A and B (right). A better contrast is achieved when the pixel values are normalised (Eq. 6.3). The brighter the BSE intensity value, the bigger the degree of mineralisation of the matrix.

When the grey levels are calibrated against reference materials such as monobromo and monoiodo dimethacrylate, the BSE signal provides quantitative approximation of the mean mineral content (in wt% Ca) in bone tissue (Boyde et al., 1995; Roschger et al., 1998).

X-ray Diffraction of bone powder (XRD)

X-ray diffraction enables probing the crystalline structure of a wide range of materials. When an X-ray beam interacts with the planes of atoms that form the crystal lattice (Figure 6.5), Bragg's Law can be used to determine the distance between such planes:

$$n\lambda = 2d\sin(\theta) \quad [6.4]$$

where λ is the wavelength of the X-ray, θ is the angle of incidence of the X-ray beam, and d is the distance between adjacent planes of atoms (Figure 6.5). When n is an integer, the reflected waves from different layers are perfectly in phase, and thus, constructive interference occurs and a diffraction peak will be produced. Since the X-ray wavelength λ is fixed in most diffractometers, a family of planes produces a diffraction peak only at a specific angle 2θ .

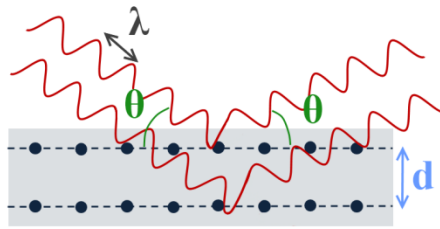


Figure 6.5: Schematic representation of Bragg's law. λ is the wavelength of the X-ray, θ is the incidence angle, and d is the distance between planes of atoms.

Based on a diffraction peak, the Scherrer equation (Scherrer, 1918) is used together with the FWHM method (full width at half maximum) to calculate the mean crystal size B along the family of planes that produced such diffraction peak (Suryanarayana and Norton, 1998):

$$B = \frac{k\lambda}{L\cos(\theta)} \quad [6.5]$$

where k is the shape factor, λ is the wavelength of the X-ray, L is the peak width at half maximum and θ is the Bragg angle where the peak is located (Figure 6.6).

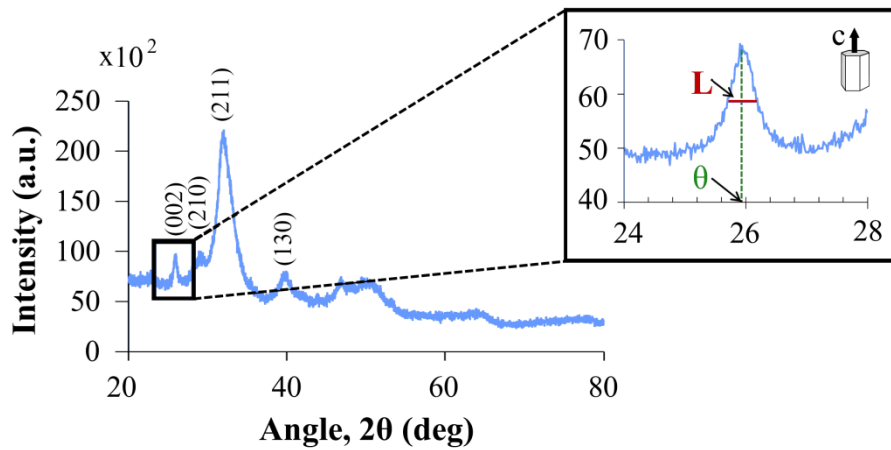


Figure 6.6: XRD pattern of *oim*^{+/+}. The peak at 26° is the is used to calculate the mean crystal size in the c-direction (Eq. 6.5). *L* is the peak width at half maximum; θ is the Bragg angle.

Bone exhibits low crystallinity, which results in broad and overlapping peaks. The diffraction peak at $2\theta = 26^\circ$ corresponds to the (0 0 2) c-axis and it is the only peak that does not exhibit overlapping, which is why this peak is used to measure the average crystal size along the c-axis (Figure 6.6) (Boskey et al., 2003).

The positions of the peaks in the XRD spectra (2θ) provide information about the phases present in the bone powder. Although unheated bone exhibits low crystallinity and peak overlapping, heating the bone results in the recrystallization of the mineral to different phases depending on crystallite size and composition (Figure 6.7) (Rogers and Daniels, 2002). These phases can be identified by comparing the peak positions against the International Centre for Diffraction Data (ICDD) powder diffraction reference patterns.

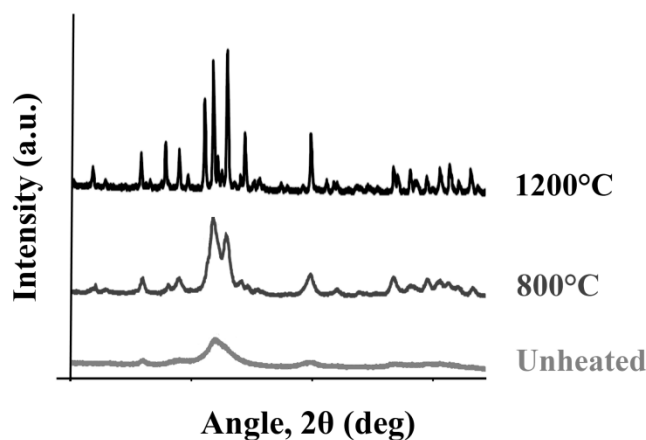


Figure 6.7: XRD spectra of *oim*^{+/+} bone unheated, previously heated to 800°C and 1200°C. The mineral becomes more crystalline with temperature.

6.3 Comparison of the Mineral in Brittle and Ductile Bone

Variations in composition and organisation of the apatite mineral, collagen protein and water often lead to the deterioration of bone's overall mechanical behaviour (Dong et al., 2010; Miller et al., 2007a; Sroga and Vashishth, 2012; Wilson et al., 2006; Yeni and Norman, 2000). Although collagen is expected to play a major role in bone toughness (Fratzl et al., 2004; Nyman and Makowski, 2012; Zimmermann et al., 2011), it is also known that the mineral phase is altered in pathologic bones which exhibit decreased or increased bone toughness (Camacho et al., 1996; Donnelly et al., 2010; Grynepas, 1993; Huesa et al., 2011; Phillips et al., 2000; Ural and Vashishth, 2014; Vanleene et al., 2012).

This study characterised mineral properties of brittle and ductile cortical bone. The two mouse phenotypes described in Section 6.1 were chosen based on known fracture toughness values (Figure 6.1) to represent brittle and ductile bone (Carriero et al., 2014b, 2014d). In brittle *oim*^{-/-} bones the mutation affecting collagen structure has a profound effect on mineralisation (Camacho et al., 1996; Sarathchandra et al., 1999; Vanleene et al., 2012), whereas in ductile *Phospho1*^{-/-} bones the mineral is directly affected by the lack of PHOSPHO1 (MacRae et al., 2010), which in turn might also affect collagen structure (Huesa et al., 2011).

Multi-scale techniques were combined to explore mineral structure and composition, tissue mechanics and mineralisation and the bulk mineral content of brittle *oim*^{-/-} and ductile *Phospho1*^{-/-} bones (Figure 6.2). Nano-structure was analysed by X-ray diffraction (XRD) of bone powder, which determined the average mineral crystal size. Thermogravimetric analysis (TGA) was then carried out to compare the bulk mineral to matrix ratios. This method complements Raman or FTIR (Fourier transform infrared spectroscopy) analyses (Bart et al., 2014; Carriero et al., 2014b; Huesa et al., 2011; Vanleene et al., 2012), which only measure certain components of the mineral (Phosphate) and collagen (Amide I). TGA measures total mineral and organic contents, including carbonated mineral and non-collagenous proteins, providing additional insight into bulk composition. Changes in composition at the nano-scale were investigated with a second XRD analysis of the bone powder after the TGA. Heating the mineral increases its crystallinity, and thus differences in composition and structure become more apparent. The ramifications of the changes in mineral structure and

composition were evaluated at the tissue level by analysing the mineralised matrix. The extent of mineralisation of the bone matrix was compared using backscattered electron scanning electron microscopy (BSE SEM). Though density is often used as a correlate of elastic modulus, this is not always true in pathologic bone, where altered mineral organisation and structure at the nano-scale might influence micro-scale mechanical properties (Oyen et al., 2008; Vanleene et al., 2012). Nanoindentation was performed on embedded bones to identify changes in tissue elastic properties.

It was hypothesised that these two pathologies, which map the extremes of whole bone mechanics (brittle and ductile), will have altered mineral crystal size, composition, mineral fraction, degree of mineralisation of the bone matrix and tissue elastic properties compared to healthy bone.

6.3.1 Materials and Methods

6.3.1.1 Specimens

Bones from brittle *oim*^{-/-} and ductile *Phospho1*^{-/-} mice were compared to their corresponding wild-type controls (*oim*^{+/+} and *Phospho1*^{+/+}). In total 24 male mice of 7 weeks-of-age were used (six per group). Femora were utilised to analyse the mineral, while tibiae were used to explore bone tissue (Figure 6.8).

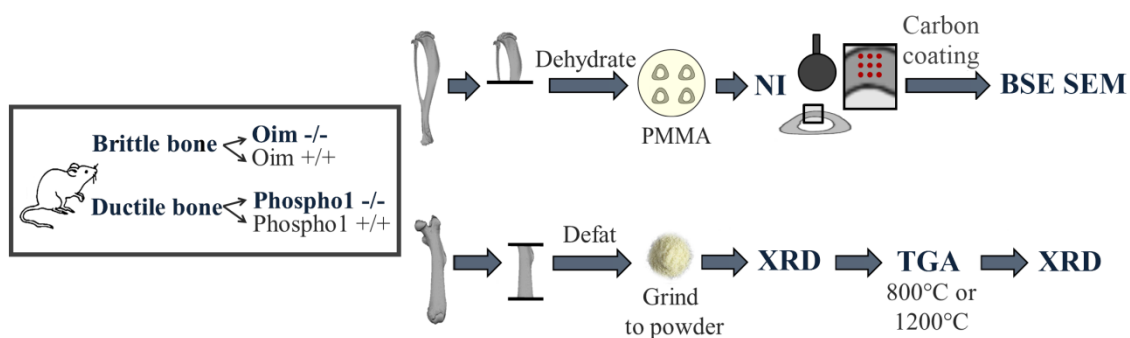


Figure 6.8: Schematic of methods. Tibiae were used for tissue analysis and femora were utilised to determine properties of the mineral.

6.3.1.2 Mineral characterisation

The protocol for mineral characterisation is summarised in Figure 6.9. Right femora of six mice per group (total 24 femora) were used for XRD and TGA analysis. Bones were cleaned of soft tissue and bone marrow, and both epiphyses were cut off with a water cooled low speed diamond saw. Femora were then defatted using first a 2:1 and then a

1:2 chloroform/ethanol solution for 48 hours each. The specimens were dehydrated in increasing concentrations of ethanol (70% to 100%) for 10 minutes each. Each bone was wet ground in acetone until a uniform and homogeneous powder was obtained and the vials were left under the fume hood until the evaporation of the acetone.

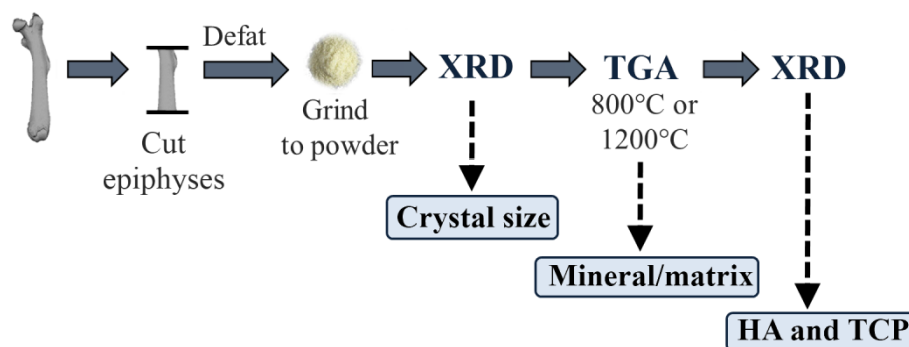


Figure 6.9: Mineral characterisation protocol. Femora were defatted and ground to powder to measure average mineral crystal size using XRD, and to monitor the bulk mineral to matrix ratio via TGA. XRD scans were run after TGA to assess the fractions of HA and TCP.

X-ray Diffraction of bone powder (XRD)

XRD patterns were obtained using a PANalytical® XRD X'Pert Pro diffractometer operated at 40 kV and 40 mA with no spinning. The initial data collection was in the 2θ range of 20° to 80° , with a step size of $0.0334^\circ/2\theta$ (kept constant) and a count time at each step of 3 s. A set of slower scans was carried out from 24° to 28° with 250 sec/step in order to better capture the diffraction peak at $2\theta = 26^\circ$ (Figure 6.6). The mean crystal size was calculated following the Scherrer equation (Eq. 6.5) with the FWHM method, as explained in Section 6.2. The standard $B_{std}(2\theta) = 0.14^\circ$ was obtained measuring a Si substrate under the same conditions as the samples. An in-built tool provided by PANalytical® X'Pert software was used for the calculations.

Thermogravimetric Analysis (TGA)

TGA was carried out on the Netzsch STA 449 C Jupiter ® simultaneous thermal analyser at a constant heating rate of $10^\circ\text{C}/\text{min}$ in controlled air atmosphere. Femur powder, which weighted 8-12 mg depending on the sample, was heated to the required temperature in a platinum crucible. Three of the samples per group were heated from room temperature to 800°C , and the other three to 1200°C . The mineral to matrix ratios were calculated according to Eq. 6.1-6.2.

XRD after heat treatment

Compositional differences become more apparent after thermal annealing of the mineral, which evolves mainly to hydroxyapatite (HA) and tricalcium phosphate (TCP). After TGA, XRD scans were collected with a time of 52 sec/step from $2\theta = 20^\circ$ to 80° . PANalytical® Xpert Highscore software was employed to identify the phases present comparing peak positions against the International Centre for Diffraction Data (ICDD) powder diffraction reference patterns. Peaks corresponding to hydroxyapatite (HA) were traced using hydroxylapatite (reference code: 01-074-0566), while calcium phosphate (ref. code: 01-070-2065) was used to identify β -tricalcium phosphate (TCP) peaks. The mass fractions of the identified phases were estimated based on their Reference Intensity Ratios (RIR). The percentages of HA and TCP were used to estimate differences in calcium to phosphate (Ca/P) ratios, taking into account that Ca/P of stoichiometric HA is 1.67, whereas TCP has a ratio of 1.5. A higher conversion to TCP is associated to a calcium-deficient apatite, which exhibits a lower Ca/P ratio (Holden et al., 1995; Kannan et al., 2005; Raynaud et al., 2002).

Tissue characterisation

The protocol for tissue characterisation is summarised in Figure 6.10. Right tibiae of four mice per group (total 16 tibiae) were used for nanoindentation and BSE SEM. Tibiae were cleaned of surrounding soft tissue and fixed in 70% ethanol for 48 hours. They were dehydrated in a series of increasing concentrations of ethanol (80, 90 and 100% for 24, 24 and 72 hours respectively), and changed to a xylene solution (48 hours). The samples were then infiltrated in pure methyl methacrylate monomer mixed with α -azo-iso-butyronitrile under vacuum and polymerised to PMMA at 30°C . Tibia blocks were cut transversally at the mid-diaphysis and the cross-sections polished with graded silicon carbide papers (from P800 to P4000), and cleaned ultrasonically with distilled water between polishing steps.

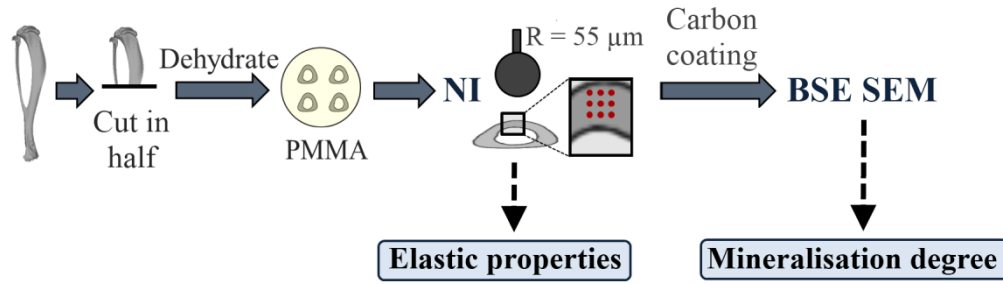


Figure 6.10: Tissue characterisation protocol. Tibiae were cut in half and embedded in PMMA. Elastic properties were measured via nanoindentation with a sphere-tip ($R = 55 \mu\text{m}$). Samples were carbon coated to capture the mineralisation degree of the bone matrix via BSE SEM.

Nanoindentation (NI)

Nanoindentation was performed on mid-diaphyseal medial cross-sections of the PMMA-embedded tibiae using a TI700 UBI nanoindenter, following the same protocol as in Chapter 3 for spherical dry indentation of PMMA-infiltrated bone. Indentation tests were carried out with the specimen in dry condition using a $55 \mu\text{m}$ -radius sphere tip. A trapezoidal loading protocol was applied longitudinally to a maximum load of 8 mN with a rising time of 10 s and a holding of 30 s (Figure 3.9 in Chapter 3). Nine indents were made in each specimen with a minimum spacing of $15 \mu\text{m}$ between indents (Figure 6.10). Viscoelastic analysis was used to evaluate elastic properties (Oyen, 2005, 2006a, 2007), as previously described in Chapter 3 (Eq. 3.10-3.15). Briefly, the creep coefficients C_0 , C_i and the material time constants τ_i were obtained using a nonlinear least-square curve-fit function in MATLAB. The instantaneous G_0 and equilibrium G_∞ shear modulus were calculated from the creep coefficients and they were used to calculate the plane strain modulus, E' . Young's modulus E was computed assuming a Poisson's ratio of $\nu = 0.3$ in cortical bone, following:

$$E' = \frac{E}{(1 - \nu^2)} \quad [5.4]$$

Backscattered electron microscopy (BSE SEM)

After the indentations, the PMMA blocks were repolished and carbon coated. Samples were analysed using EVO®MA10 scanning electron microscope (Zeiss UK Ltd, UK) operated at 20 kV, with a beam current of 1.0 nA and at a working distance of 12 mm using monobromo and monoiodo dimethacrylate standards (Boyde et al., 1995; Vanleene et al., 2012). ImageJ (Schneider et al., 2012) was employed to plot the

combined histogram of the grey values of all the bones to identify the lower (A , 125) and upper (B , 235) bounds across histograms (Figure 6.11). The normalised histogram was obtained using Eq. 6.3 with the identified bounds A and B .

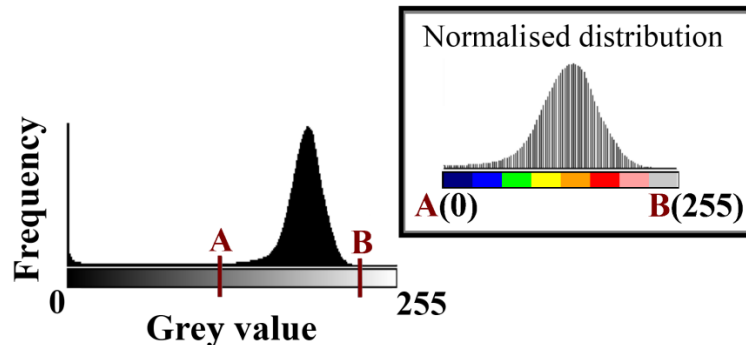


Figure 6.11: Combined histogram of grey values of all the bones, and the normalised combined histogram between A and B .

For visualisation purposes, the grey-level range of the normalised histogram was divided into eight equal size classes of different colours ranging from non-mineralised (A) to highly mineralised (B) bone matrix. The results reported hereafter are the ones corresponding to the normalised distribution in Figure 6.11.

6.3.1.3 Statistical Analysis

Mean values and standard deviations were calculated for the measured parameters. Independent t-tests were used to compare crystal size, mineral/matrix ratio, elastic properties and mean BSE intensity values of pathologic vs. healthy bone ($oim^{-/-}$ vs. $oim^{+/+}$; $PhosphoI^{-/-}$ vs. $PhosphoI^{+/+}$). Equality of variances was assumed when Levene's Test gave values of $p > 0.05$. Mann-Whitney U test was used in the cases where the data was not normally distributed according to the Shapiro-Wilk test. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS (v.21, SPSS Inc., Chicago, IL).

6.3.2 Results

Average crystal size

Pathologic bones had smaller crystal size than their controls (Figure 6.12). In brittle bones, the average crystal size decreased from 25.0 nm ($oim^{+/+}$) to 17.8 nm ($oim^{-/-}$) ($p < 0.001$). In ductile bones, it decreased from 22.9 nm ($PhosphoI^{+/+}$) to 21.6 nm ($PhosphoI^{-/-}$) ($p = 0.001$).

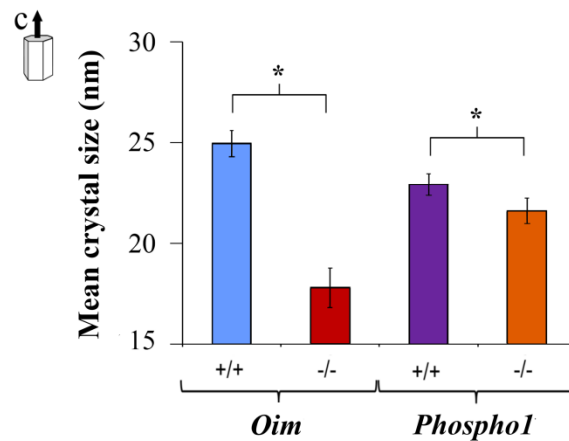


Figure 6.12: Means/standard deviations of the average apatite crystal size in the c-direction. Pathologic bones had smaller crystal size. (* $p \leq 0.001$)

Bulk mineral content

The percentages of dry weight associated with organic content, mineral phase and carbonate content are shown in Table 6.1. The weight loss associated with moisture (between 0-200°C) might be influenced by sample preparation, which is why the percentages were normalised to dry weight. Brittle *oim*^{-/-} and ductile *Phospho1*^{-/-} bones exhibited increased organic content ($p < 0.001$) compared to their controls. No differences were found in the loss of carbonate content to 800°C.

Table 6.1: Means and standard deviations (in brackets) of dry weight % of organic (200-600°C), mineral (at 600°C) and carbonate content (600-800°C) for *oim* and *Phospho1* wild-type (+/+) and pathologic (-/-) femora powder. (* $p < 0.05$)

		Wild-type vs. Pathologic		p-value
		+/+	-/-	
<i>Oim</i>	Organic (wt %)	27.5 (1.0)	37.0 (2.6)	<0.001*
	Mineral (wt %)	72.5 (1.0)	63.0 (2.6)	<0.001*
	Carbonate (wt %)	1.7 (0.2)	1.8 (0.2)	0.301
<i>Phospho1</i>	Organic (wt %)	25.1 (0.7)	29.4 (1.2)	<0.001*
	Mineral (wt %)	74.9 (0.8)	70.6 (1.1)	<0.001*
	Carbonate (wt %)	1.4 (0.2)	1.5 (0.2)	0.628

Representative TGA curves of weight loss with temperature and average mineral to matrix ratios are plotted in Figure 6.13. Mineral/matrix ratios, calculated as the percentage of dry mass remaining at 600°C (mineral) divided by the mass of organic matter (mass loss between 200°C-600°C) indicated that both pathologic bones had smaller mineral/matrix ratio compared to their wild-type controls ($p < 0.001$).

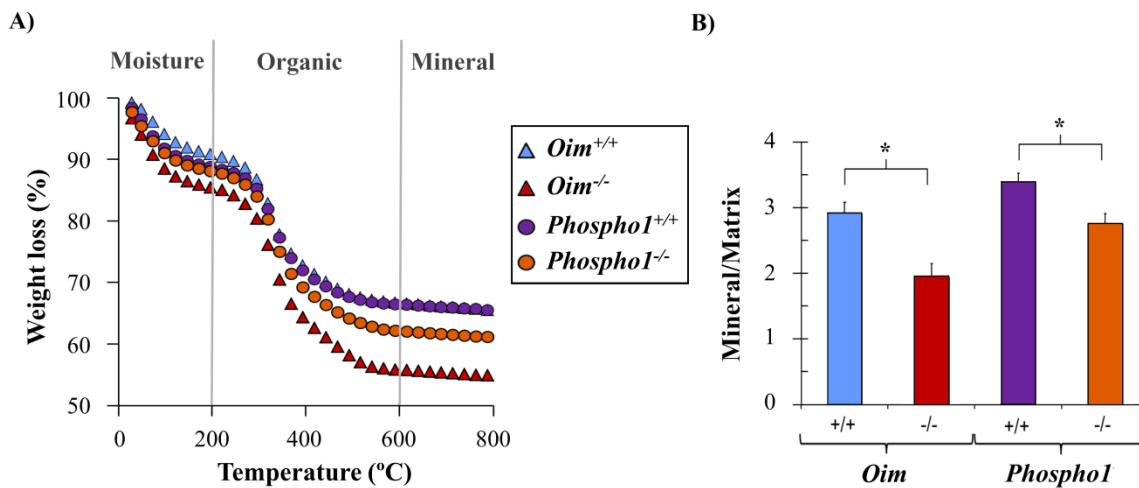


Figure 6.13: A) Representative TGA curves of femur powder heated to 800°C. B) Bulk mineral to matrix ratios, with pathologic bones showing smaller ratios. (* $p < 0.001$)

Conversion to HA and TCP

Figure 6.14 represents XRD patterns of *oim* and *Phospho1* bone powder in three stages: unheated, after TGA to 800°C, and after the heat treatment to 1200°C. The XRD spectra of unheated bones could not be distinguished among all four mouse models due to low crystallinity and peak overlap. XRD after thermal treatment induced an increase in crystallinity for all the samples.

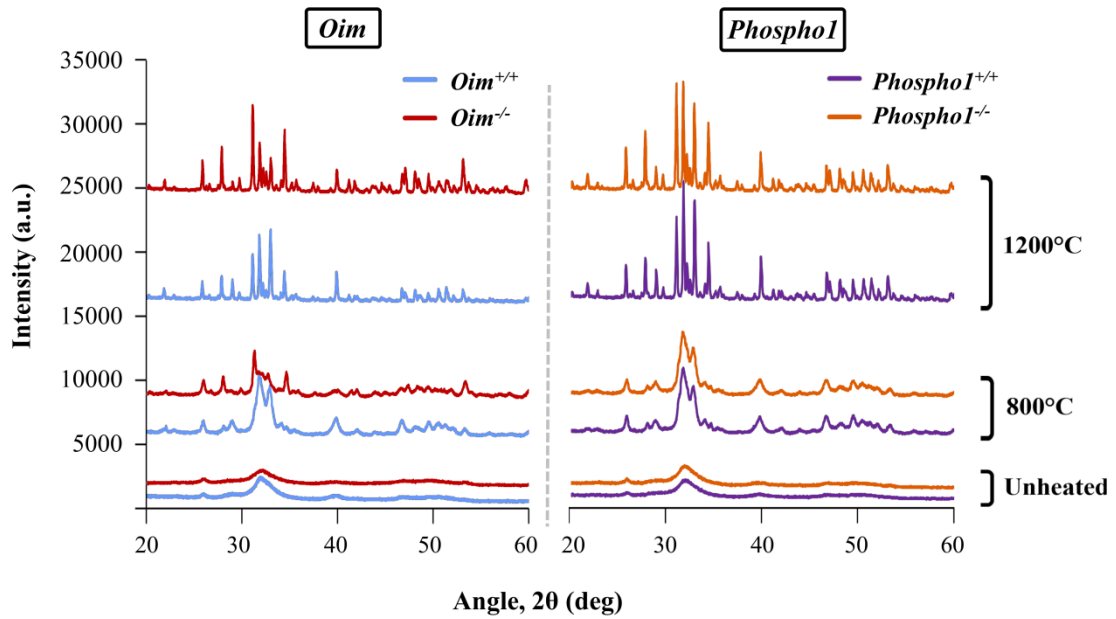


Figure 6.14: XRD spectra of *oim* (left) and *Phospho1* (right) bones before TGA (unheated) and after heating to 800°C and 1200°C. The mineral becomes more crystalline with temperature.

XRD spectra of previously heated samples revealed differences in the amounts of HA and TCP between the bones. Figure 6.15 shows representative diffraction patterns of the samples previously heated to 1200°C, where the peaks corresponding to HA and TCP are identified. The heat treatment induced a bigger mass conversion to TCP for pathologic bones heated to 1200°C ($42 \pm 3\%$ in *oim*^{-/-}, and $31 \pm 1\%$ in *Phospho1*^{-/-}) compared to wild-type controls ($25 \pm 3\%$ in *oim*^{+/+}, and $25 \pm 1\%$ in *Phospho1*^{+/+}). It must be noted that only half of the bones were heated to 1200°C. Nevertheless, the same trend was found in the bones heated to 800°C, with pathologic bones having a bigger fraction of TCP mass ($44 \pm 6\%$ in *oim*^{-/-}, and $23 \pm 1\%$ in *Phospho1*^{-/-}) than their wild-type controls ($18 \pm 1\%$ in *oim*^{+/+} and *Phospho1*^{+/+}).

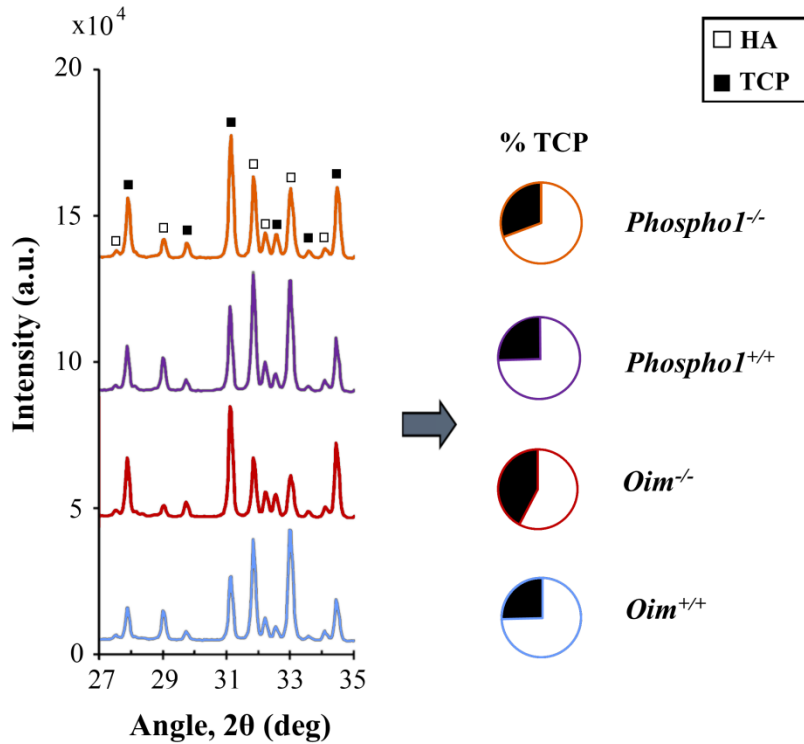


Figure 6.15: XRD patterns of *oim* and *Phospho1* bone powder after being heated to 1200°C, and the average percentages of TCP. Pathologic samples showed a higher conversion to TCP.

Tissue elastic properties

Means and standard deviations of elastic properties of *oim* and *Phospho1* tibiae are summarised in Table 6.2. The values of plane strain modulus E' , instantaneous shear modulus G_0 , and infinite shear modulus G_∞ were significantly smaller in pathologic bones compared to wild-type controls. No significant differences were found in the extent of viscoelasticity, f . Pathologic bones had smaller Young's modulus than their controls ($p < 0.001$ for *oim*^{-/-}, $p = 0.017$ for *Phospho1*^{-/-}) (Figure 6.16).

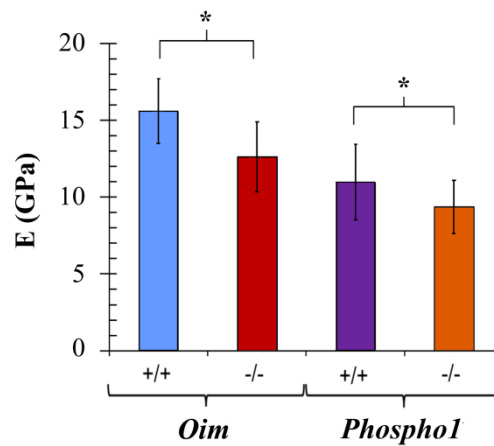


Figure 6.16: Means and standard deviations of Young's modulus E , with pathologic bones exhibiting reduced Young's modulus. (* $p < 0.05$)

Table 6.2: Means and standard deviations (in brackets) of elastic properties of PMMA-embedded *oim* and *Phospho1* tibiae, indented with a sphere in dry conditions. E' is the plane strain modulus; G_0 is the instantaneous shear modulus and G_∞ corresponds to the shear modulus at infinite time; $f = G_\infty/G_0$ represents the elastic fraction (viscous $0 \leq f \leq 1$ elastic). (* $p < 0.05$)

		<i>Wild-type vs. Pathologic</i>		p-value
		+/+	-/-	
<i>Oim</i>	E' (GPa)	17.1 (2.3)	13.9 (2.5)	<0.001*
	G_0 (GPa)	4.27 (0.58)	3.47 (0.63)	<0.001*
	G_∞ (GPa)	2.77 (0.54)	2.35 (0.53)	0.004*
	$f = G_\infty/G_0$	0.65 (0.10)	0.68 (0.09)	0.487
<i>Phospho1</i>	E' (GPa)	12.0 (2.7)	10.3 (1.9)	0.014*
	G_0 (GPa)	3.01 (0.68)	2.57 (0.48)	0.016*
	G_∞ (GPa)	2.03 (0.40)	1.64 (0.33)	<0.001*
	$f = G_\infty/G_0$	0.68 (0.05)	0.65 (0.09)	0.440

Degree of mineralisation of bone matrix

Oim^{-/-} bones were more mineralised and *Phospho1*^{-/-} were less mineralised than their wild-type controls (*oim*^{+/+} and *Phospho1*^{+/+}), as shown in Figure 6.17.

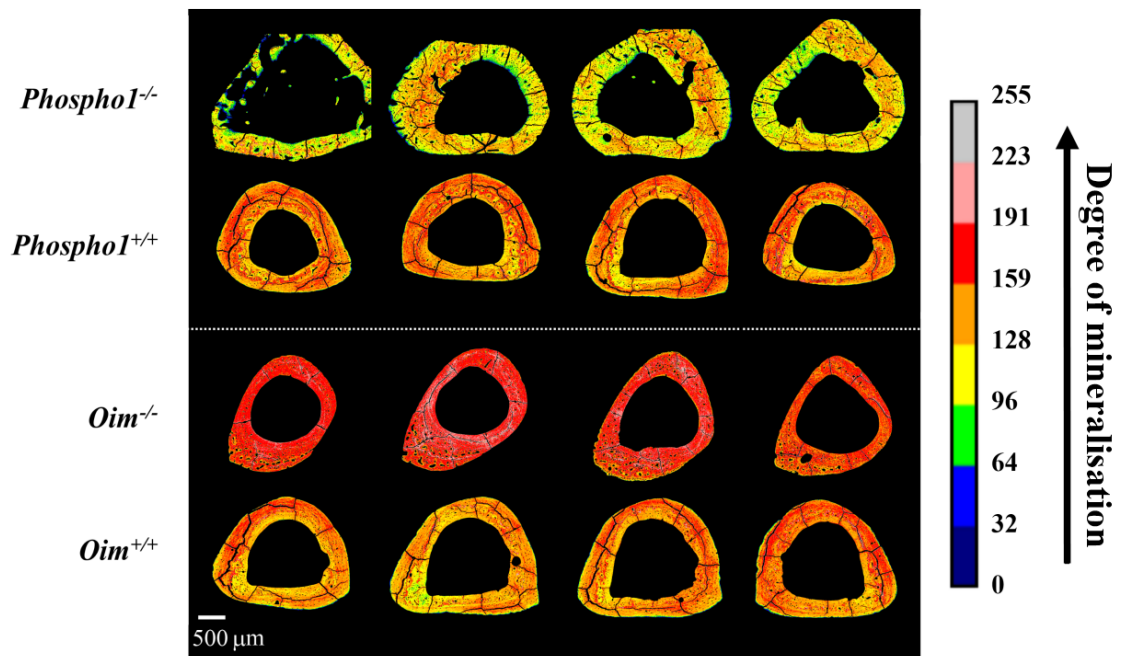


Figure 6.17: Normalised BSE SEM maps of tibial cross sections. *Phospho1*^{-/-} were hypomineralised, while *oim*^{-/-} were hypermineralised compared to their respective controls.

Combined histograms were plotted per strain in order to compute average pixel distributions (Figure 6.18). The mean grey value was significantly smaller for *PhosphoI*^{-/-} bones (117 ± 28) and bigger for *oim*^{-/-} (168 ± 29) when compared to *PhosphoI*^{+/+} (143 ± 26 , $p < 0.001$) and *oim*^{+/+} (139 ± 27 , $p < 0.001$) respectively.

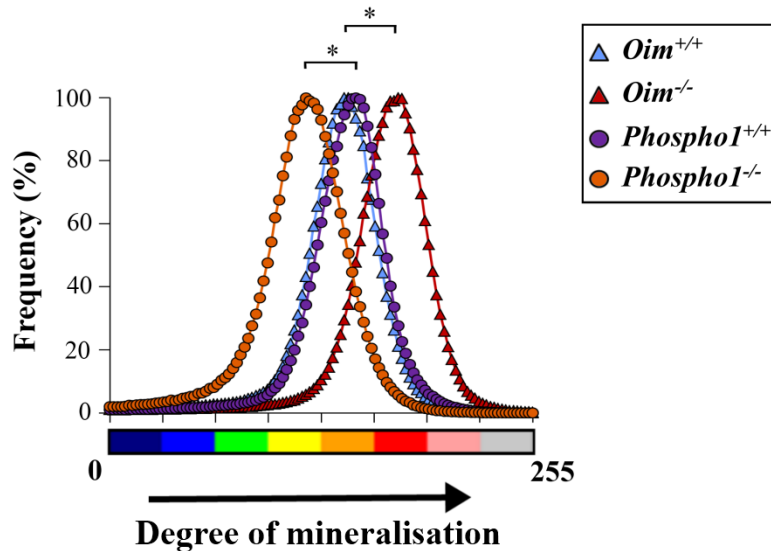


Figure 6.18: Combined histograms of pixel values for each strain from non-mineralised (0) to high mineralisation (255). Bone matrix was hypomineralised in *PhosphoI*^{-/-} and hypermineralised in *oim*^{-/-}, as calculated from the mean grey values of the histograms. (* $p < 0.001$)

6.3.3 Discussion

This chapter explored the mineral phase of brittle *oim*^{-/-} and ductile *PhosphoI*^{-/-} bones. Despite their extremely different mechanical behaviour at the macro-scale, the mineral of *oim*^{-/-} and *PhosphoI*^{-/-} bones displayed some similar trends. Both pathologic bones had smaller mineral crystal size, less bulk mineral to matrix ratio and bigger thermal conversion to TCP than controls. However, *oim*^{-/-} bones were hypermineralised, while *PhosphoI*^{-/-} were hypomineralised. Despite the differences in the degree of mineralisation of the bone matrix, both brittle and ductile bones had reduced tissue elastic moduli.

At the mineral level, average size of *oim*^{-/-} and *PhosphoI*^{-/-} apatite crystals along the c-axis was reduced (Figure 6.12). This might be a consequence of the disrupted collagen fibril template in brittle bones (Vanleene et al., 2012) and altered mineral formation pathway due to lack of phosphate in ductile bones (Roberts et al., 2007; Stewart et al., 2006). Transmission electron microscopy (TEM) and small angle X-ray

scattering (SAXS) have shown that apatite crystals of *oim*^{-/-} bone have significantly smaller thickness and are packed more tightly in a less organised manner when compared to *oim*^{+/+} (Fratzl et al., 1996; Grabner et al., 2001; Vanleene et al., 2012). Smaller crystals have also been measured in children with a severe form of osteogenesis imperfecta (Vetter et al., 1991). In the current study it was found that, although the differences were not as pronounced as in brittle bone, *PhosphoI*^{-/-} bones also had significantly reduced average mineral crystal size. Variations in the mineral crystal size have previously been found in bones exhibiting altered mechanical properties (Boskey, 2003), as the crystal size might influence how bone, as a composite material, responds to load. The current study shows that a reduction in the average crystal size is associated with both brittle *oim*^{-/-} and ductile *PhosphoI*^{-/-} bone.

The bulk mineral content was reduced in both pathologic bones, which led to lower mineral/matrix ratios (Figure 6.13). In *PhosphoI*^{-/-} bones, the lower mineral/matrix ratio is in agreement with Raman experiments (Huesa et al., 2011). However, in *oim*^{-/-} bones, Raman and FTIR studies have measured increased (Carriero et al., 2014b; Vanleene et al., 2012) and decreased (Bart et al., 2014) mineral/matrix ratios. The discrepancy between Raman, FTIR and TGA in brittle bones is likely to be due to differences in how the organic and mineral contents are calculated: TGA provides a bulk measurement, whereas Raman and FTIR are local measurements. TGA measures the ratio of the total mineral content (including carbonated hydroxyapatite) to the total organic content (including bone matrix collagen, non-collagenous proteins, lattice water, blood vessels and all other organic content in osteocytes and other cells in blood vessel canal spaces) (Pramanik et al., 2012; Wang et al., 2010). In contrast, in FTIR and Raman the mineral content is represented only by the phosphate band and the organic content is limited to the collagen type I Amide I band (Feng and Jasiuk, 2011). In addition, Amide I band is affected by the structure and organisation of the collagen fibers (de Campos Vidal and Mello, 2011); hence, the mineral/matrix ratio calculated by spectroscopy is not purely a compositional measurement. TGA complements spectroscopy analyses indicating that despite the differences in the local Phosphate/Amide-I ratio found in the literature (Bart et al., 2014; Carriero et al., 2014b; Huesa et al., 2011; Vanleene et al., 2012), there was a decrease in the bulk mineral/matrix ratio in both pathologies. The increase in the total organic fraction in pathologic bone is associated not only with altered collagen, but also with non-

collagenous proteins and blood vessels, which are expected to influence the fracture behaviour of bone (Abdel-Wahab et al., 2012; Buehler, 2008; Miller et al., 2007a; Tang and Vashishth, 2011).

After the heat treatment, all bones evolved to a biphasic mixture of HA and TCP but pathologic samples showed a higher conversion to TCP (Figure 6.15), indicating chemical deviations from the stoichiometric HA. The increased conversion to TCP, with a lower Ca/P ratio than HA, suggests that the mineral of *oim*^{-/-} and *PhosphoI*^{-/-} bones had lower Ca/P ratio than their controls. These results are limited by the reduced number of samples heated to each of the temperatures (three per strain to 800°C and three to 1200°C). However, the results are consistent for both temperatures and in agreement with previous literature. Brittle bones exhibited the highest deviation from stoichiometric apatite. Phillips et al. (Phillips et al., 2000) measured a reduction of Ca/P in *oim*^{-/-} femora using neutron activation analysis. Reductions in Ca/P ratio have also been reported in human osteogenesis imperfecta bone (Sarathchandra et al., 1999) and in osteoporotic bone (Kourkoumelis et al., 2012; Tzaphlidou, 2008). Results from the current study further indicate that pathologic bone mineral tends to be less stoichiometric (Rogers and Daniels, 2002; Wang et al., 2003). Overall, heating the samples to different temperatures prior to XRD measurements brings out differences in composition of pathologic bones not readily evident from XRD without heat treatment.

A notable difference in the tissue properties of brittle and ductile bones in the present experiments resides in the mineralisation of the tissue (Figure 6.18). Since the aim of this chapter was to compare mineralisation degrees between bones within the same study, mineralisation was left in terms of normalised BSE SEM grey values, instead of translating these values to mineral density (Boyde et al., 1995). This was done with the purpose of avoiding the many assumptions required to convert grey values to mineral density (Vanleene et al., 2012). Mineralised matrix of *oim*^{-/-} bone was more mineralised, which is in agreement with previous BSE SEM studies on *oim*^{-/-} and human OI bone (Grabner et al., 2001; Vanleene et al., 2012). In contrast, as expected from the lack of PHOSPHO1 enzyme and suggested from previous studies (Yadav et al., 2011), *PhosphoI*^{-/-} bones were less mineralised than controls.

Tissue elastic modulus was reduced in brittle and ductile bones (Figure 6.16), indicating that in pathologic bones, mineral density does not necessarily correlate with

modulus (Oyen et al., 2008; Vanleene et al., 2012). The measured elastic values (Table 6.2) are in agreement with previously reported nanoindentation data (Bembey et al., 2006a; Oyen et al., 2012; Rodriguez-Florez et al., 2013). Young's modulus was reduced by 19% in *oim*^{-/-} bones, which is in accordance with results from ultrasound critical-angle reflectometry (Mehta et al., 2009) and sharp Berkovich nanoindentation (Vanleene et al., 2012). Huesa et al. (Huesa et al., 2011) measured the elastic properties of young *PhosphoI*^{-/-} tibiae using sharp indentation and reported a decrease of 11% in their elastic modulus, close to the 15% reduction found in the current study.

When combining results from TGA and BSE SEM, interesting conclusions can be drawn. In *PhosphoI*^{-/-} bones, TGA measured less mineral content and this mineral was loosely packed, as inferred from smaller grey intensity values which indicated lower mineral density of the mineralised matrix. In brittle bones TGA also measured less mineral content, but in contrast, since this mineral was more tightly packed (Vanleene et al., 2012), BSE SEM maps showed a higher degree of mineralisation. Colorimetric measurements of hydroxyproline have measured reduced collagen content in *oim*^{-/-} (Camacho et al., 1999; Yao et al., 2013). However, hydroxyproline was normalised by the mass of the bone powder (undemineralised). Thus, reductions in collagen content could also be due to greater mineralisation of the bone. Lattice water, which evaporates between 200-400°C, might contribute to the increased 'organic weight' loss, since the water in crystals might be lost more easily due to the small size of the crystals and the higher surface area (Wang et al., 2003) in pathologic bones. However, the differences in the organic weight loss also increased after 400°C ($p = 0.002$). This suggests that the increased organic fraction must be due to not only the lattice water, but also the increased non-mineralised matrix, non-collagenous proteins, and cell and blood vessel space, which are not visible in the SEM images. The differences between results derived from TGA and BSE SEM in both pathologic bones highlight the need to distinguish bulk bone mineral quantity (measured with TGA) from the extent of mineralisation of the bone matrix (by BSE SEM).

To conclude, although the consequences of these two defects are very different at the macro-scale, the current study demonstrated that both pathologies had smaller apatite crystals which were less stoichiometric than healthy bone mineral, and showed that pathologic bones had a lower weight % of bulk mineral content. In contrast, the extent of mineralisation of the bone matrix was different for *oim*^{-/-} and *PhosphoI*^{-/-} bones, as

brittle bones were hypermineralised, while ductile bones were hypomineralised. Despite these differences in the mineralisation, the tissue elastic modulus was reduced in both pathologies. This emphasizes that mineralisation is not the only determinant of tissue elastic moduli, and suggests that deviations in the size, composition and organisation of bone mineral affect bone micro-mechanics.

6.4 Conclusions

In this chapter, the altered mechanics of brittle *oim*^{-/-} and ductile *Phospho1*^{-/-} bones were explored by analysing the implications of these pathologies in the mineral. Despite differences in the degree of the mineralisation of the bone matrix (*oim*^{-/-} bones were hypermineralised, while *Phospho1*^{-/-} bones were hypomineralised), **deviations from the normal crystal size, composition, and structure were correlated with reduced mechanical integrity of bone** in both pathologies.

In conclusion, although *oim*^{-/-} and *Phospho1*^{-/-} bones represent the two extremes of whole bone toughness, their mineral properties have many similar characteristics. This highlights the need of analysing the organic content, as well as the interaction between the mineral and collagen in order to gain a deeper understanding of the differences in the bone building blocks between brittle and ductile bone.

7 OUTCOMES, CONTRIBUTIONS AND FUTURE WORK

This chapter summarises the main outcomes of this thesis, drawing together the insight obtained from the experimental and computational techniques employed to provide a greater understanding of cortical bone mechanics at the micro- and nano-scale. Concluding remarks are provided and recommendations for further work in the field of bone mechanics are discussed.

7.1 Outcomes and Contributions to the Field of Bone Mechanics

The goal of this thesis was to characterise cortical bone at the micro- and nano-scale employing various techniques applied to mouse models. This section highlights the key outcomes of each chapter and their significance for the field of bone mechanics.

Chapter 3- Nanoindentation to Measure Tissue Mechanics

Chapter 3 provided the first systematic study where the effects of sample preparation, testing parameters and analysis options for examining the nanoindentation properties of the same bones have been investigated. This study concluded that:

- ✓ *Nanoindentation values of bone are dependent on the hydration state of the sample, indenter probe geometry and data analysis method.*

Results highlight the need of specifying the employed protocol when reporting nanoindentation properties of bone. In Chapter 3, three possible data analysis methods were described and the use of the viscoelastic approach was recommended for future experiments. (Rodriguez-Florez et al., 2013).

Chapter 4- Nanoindentation to Measure Permeability: Application in Ageing

In Chapter 4, nanoindentation was used to measure mouse bone permeability. This study provided a first experimental approximation of the value of lacunar-canalicular permeability in non-osteonal mouse bone. Obtaining experimental estimates of lacunar-canalicular permeability has special interest for developing computational models of bone mechano-adaptation, as shown by recent poroelastic FE models of mouse cortical bone (Pereira and Shefelbine, 2013).

Similar to Chapter 3, the quantitative values obtained in this study depend on the sample preparation and testing options and the assumptions made in the poroelastic analysis. Nevertheless, such limitations do not affect the reported age-related trends. Results indicated that:

- ✓ *Lacunar-canalicular permeability decreases from young to skeletally mature bone with no further significant change from skeletally mature to old bone.*

As the mechanical competence of bone deteriorates with age, understanding age-related changes in permeability will contribute to elucidate the relationship between interstitial fluid flow and whole bone mechanics.

The nanoindentation measurements of permeability in Chapter 4 were done using spherical tips of different sizes, which induced different contact sizes in bone tissue. This demonstrated that:

- ✓ *Nanoindentation with varying contact sizes is capable of capturing different levels of permeability in bone.*

This result suggests that the differences in lacunar-canalicular permeability values found in the literature (Cardoso et al., 2013), might be reflecting the multi-scale nature of permeability in bone. Overall, characterising the permeability of bone following the nanoindentation protocol presented in Chapter 4 will help understand alterations in mechano-adaptation. (Rodriguez-Florez et al., 2014a).

Chapter 5- Effects of Micro-porosity on Bone Mechanics

Chapter 5 assessed the use of Abaqus-XFEM to investigate the influence of cortical bone micro-porosity on crack initiation and propagation at the micro-scale.

- ✓ *A novel method of assigning multiple enrichment regions was proposed to propagate cracks through holes in Abaqus-XFEM.*

Previous Abaqus-XFEM studies of bone micro-porosity have not contemplated the consecutive initiation of new micro-cracks as the main crack advances. When the interest of the study is to understand the behaviour of crack initiation and growth at the micro-scale, Chapter 5 demonstrated that assigning material properties to voids to make the cracks propagate through pores is not a valid technique in Abaqus-XFEM.

The multiple enrichment region technique was employed in 2D vascular porosity models of the mouse model of osteogenesis imperfecta, *oim*^{-/-}. Results in Chapter 5 point that:

- ✓ *Intra-cortical porosity affects the propagation of the crack at the tissue level, as cracks grow following the vascular canals. Results suggest that the increased vascular porosity found in *oim*^{-/-} bone contributes to its brittleness.*

Although bisphosphonates are widely used to increase bone density and reduce the fracture risk in patients with osteogenesis imperfecta, there are several questions about the efficiency of such drugs and their consumption in children (Dwan et al., 2014; Shapiro et al., 2013). If vascular porosity is found to be a significant contributor of bone brittleness, new therapies could be designed to target porosity. In addition, combined by in-vivo imaging techniques, evaluating vascular porosity could be used in the future for fracture risk assessment.

Chapter 6- Altered Mechanics in Pathologic Bone: Implications in the Mineral

Chapter 6 evaluated the mineral properties of brittle *oim*^{-/-} and ductile *PhosphoI*^{-/-} bones combining various characterisation techniques traditionally employed in Materials Science (nanoindentation, BSE SEM, XRD, TGA) to comprehend the implications of these two pathologies in the mineral phase. This study demonstrated that:

- ✓ *Brittle and ductile bones have smaller and less stoichiometric apatite crystals and reduced bulk mineral content than their wild-type controls.*
- ✓ *At the tissue level, brittle bones are hypermineralised, while ductile bones are hypomineralised. However, tissue elastic moduli are reduced in both pathologies.*

These results suggest that mineralisation is not the only determinant of tissue elastic moduli, and deviations in the size, composition and organisation of bone mineral affect bone micro-mechanics. Hence, mineral density should not be the only indicative of fracture risk assessments. Chapter 6 highlights the need of exploring the organic content and the interaction between the collagen and mineral to gain a deeper comprehension of bone toughness. The knowledge gained by studying the ramification of molecular defects throughout bone's hierarchical structure will lead to the development of drugs that target the nano- and micro-scale processes, composition and structure in bone instead of bone mineral density only. (Rodriguez-Florez et al., 2014b).

7.2 Concluding Remarks

Overall, this thesis has investigated mechanical, structural and compositional properties of cortical bone at the micro- and nano-scale and contributed to a deeper understanding of bone material by:

- I. providing advancements in techniques used to probe bone at the micro- and nano-scale; and
- II. bringing insight into the effects of ageing and skeletal pathologies (osteogenesis imperfecta and rickets) in the mechanical integrity of bone at different hierarchical levels.

The contributions of this thesis to the field of bone mechanics have brought new research questions that will ultimately enable a multi-scale interpretation of bone mechanics. A better comprehension of hierarchical bone mechanics will lead to future improvements in preventive and therapeutic treatments for skeletal diseases, as well as to better designs of implants and replacement materials.

7.3 Future Perspectives

The studies conducted throughout this thesis opened new questions and possibilities for future research. This section suggests some of the areas that could be investigated to deepen the current understanding of bone mechanics.

Nanoindentation to Map Bone Properties

In this thesis nanoindentation was used 1) to compare different preparation, testing and analysis protocols (Chapter 3); 2) to analyse the poroelastic properties of young and aged bone (Chapter 4); and 3) to evaluate the tissue elastic properties of brittle and ductile bones (Chapter 6). In these projects, the nanoindentation properties of all the indents in each bone were averaged to obtain a mean value of tissue elastic modulus, permeability etc. per bone. However, nanoindentation can also be used to map changes in the mechanical properties within the bone (Doubé, 2010; Feng et al., 2012; Gupta et al., 2005; Pathak et al., 2012).

Chapter 6 concluded that the average tissue elastic modulus of brittle *oim*^{-/-} and ductile *Phospho1*^{-/-} bone was reduced, although the average degree of mineralisation of the bone matrix differed in both pathologies. In computational models where bone heterogeneity is taken into account, the mineralisation degree is often translated to elastic modulus (Helgason et al., 2008; Kopperdahl et al., 2002; Xin et al., 2013). However, as suggested in Chapter 6, this might not be adequate in certain skeletal pathologies where bone building blocks are altered. The BSE intensity maps obtained in this thesis (Figure 7.1) could be used to choose indentation sites for nanoindentation (Doubé, 2010) and further demonstrate whether there is any correlation at all between the local BSE intensity value and tissue elastic modulus in *oim*^{-/-} and/or *Phospho1*^{-/-} bones.

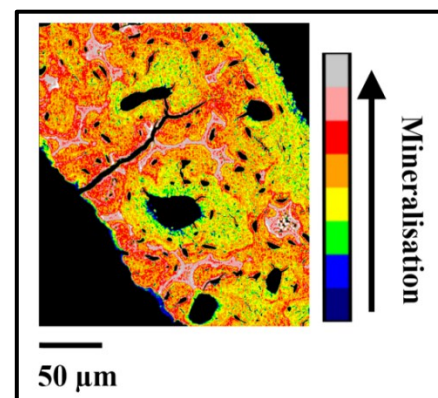


Figure 7.1: BSE SEM image of zoomed *Phospho1*^{-/-} tibia cross section.

Permeability and Intra-cortical Porosity with Age

Chapter 4 evaluated changes in lacunar-canalicular permeability with age. Bone permeability is expected to be related to intra-cortical porosity (Anderson et al., 2008;

Cardoso et al., 2013; Schneider et al., 2010). However, age-related changes in non-osteonal bone porosity have not been measured. Nano-CT techniques provide the means of capturing vascular and lacunar porosity of cortical bone in 3D. Nano-CT scans can be processed to obtain morphological information about the vascular canals and osteocyte lacunae, as described by (Carriero et al., 2014a). Figure 7.2 shows the 3D scans of 7 month-old B6 tibiae and an example of the morphological information that could be extracted from such analysis (i.e. lacunar volume distribution).

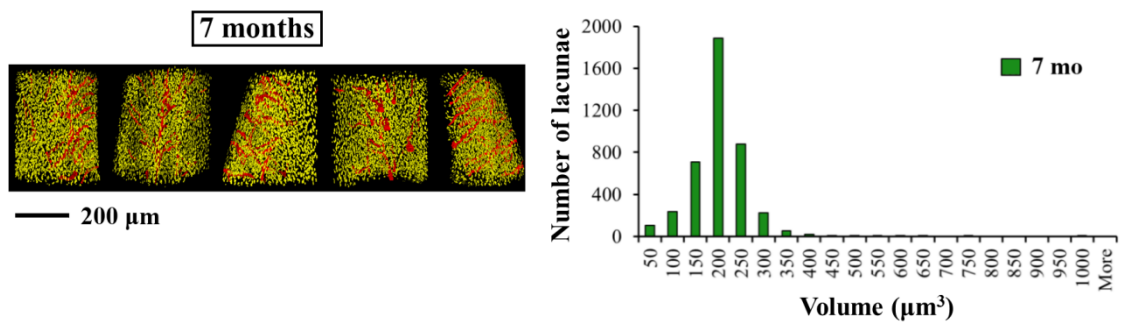


Figure 7.2: Nano-CT scans of the lacunar and vascular porosities of a 7 month-old B6 mouse tibia at the mid-diaphysis. Lacunar volume distribution is shown on the right.

XFEM to Assess the Influence of Bone Micro-structure on Crack Propagation

In Chapter 5 the multiple enrichment region method was suggested for future XFEM analysis of crack growth through bone's porosity in Abaqus. In order to verify this method, crack propagation through holes should be evaluated experimentally. Ongoing research consists of performing notched 3-point bending experiments to acrylic square rods with different configuration of holes in order to compare the experimental and computational force-displacement curves.

The protocol described in Chapter 5 (Section 5.5) could be used in models that include not only vascular porosity but also lacunar porosity, as shown in Figure 7.3. In the tested configuration, the main crack (in green) followed the vascular canals (in red), while the contribution of lacunae (in yellow) in the crack trajectory was minimal (Figure 7.3). This is in agreement with previous experimental studies of crack propagation in mouse bone (Voide et al., 2011). Since including lacunar pores in the XFEM model arises difficulties in the meshing and partitioning process while increasing the computational cost, future XFEM studies of bone intra-cortical porosity should first contemplate the influence of vascular canals only.

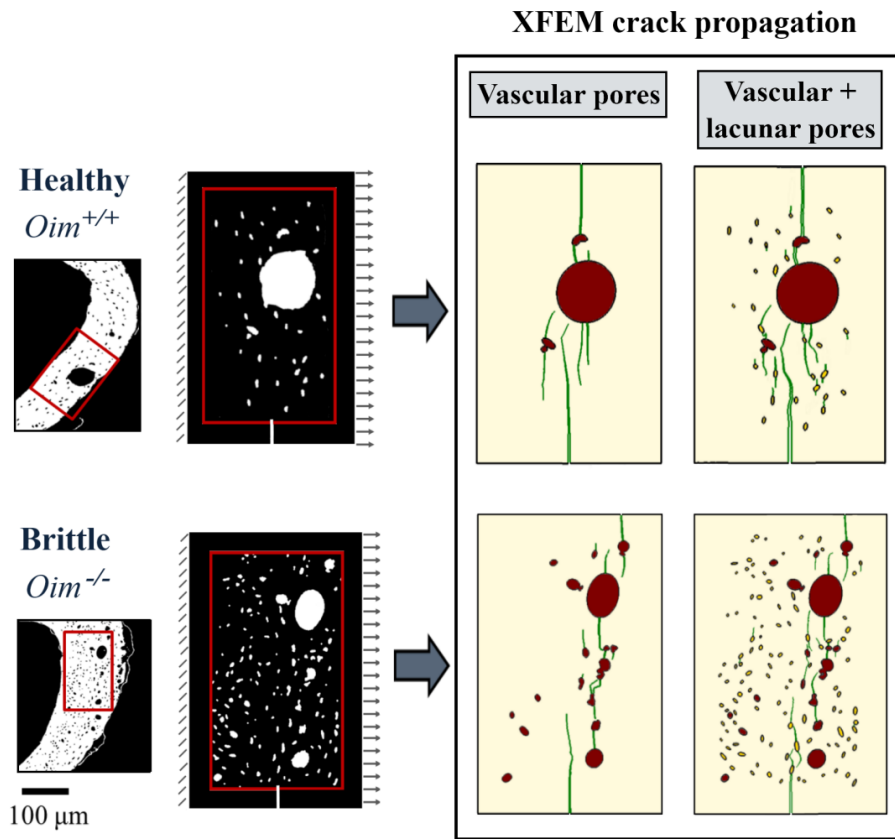


Figure 7.3: Synchrotron radiation-based CT images of the porosity of $oim^{+/+}$ and $oim^{-/-}$ bone can be used to generate 2D models of vascular and vascular+lacunar porosity. In this configuration, lacunar pores have a minimal effect on the path of crack propagation.

Investigation of the Organic Content in Brittle and Ductile Bone

Chapter 6 focused on analysing mineral properties of brittle and ductile cortical mouse bone. Since similar trends were found in the mineral of brittle $oim^{-/-}$ and ductile $Phospho1^{-/-}$ bone, future studies should examine alterations in the collagen composition, matrix architecture and collagen cross-links in order to identify differences between these two pathologies. At the micro-scale, a novel iodine vapour staining method can be applied to PMMA-embedded bones before the BSE SEM in order to capture osteoids, blood vessels and other unmineralised matrix (Boyde et al., 2014), which are not visible in the plain BSE SEM images shown in Chapter 6. Thermogravimetric analysis showed that brittle $oim^{-/-}$ bone have more bulk organic content than their controls, while BSE SEM images of the tibia cross sections indicated that $oim^{-/-}$ bones are hypermineralised. Hence, staining the bones following the technique proposed by Boyde et al. (2014) would help understand the reasons behind the increased organic content in brittle bones measured by TGA.

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9 APPENDIX

9.1 Testing Protocol for the Indentation of Hydrated Bone

This section provides a series of steps that were followed in this thesis when indenting fully hydrated bone.

Testing steps (based on a TI700 U nanoindenter, Figure 9.1)

1. Calibrate the system and stage as usual (Ubi® 1 User Manual).
2. Mount the metallic container - with the bones previously glued, and fluid covering about half of the height of the samples- onto the magnetic stage of the nanoindenter. It is important to keep the top surface of the samples free of fluid.

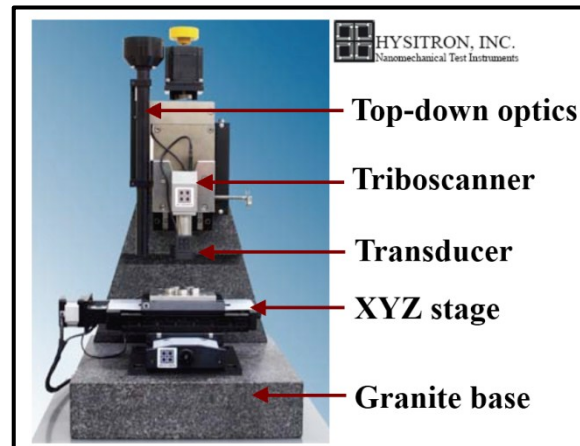


Figure 9.1: Hysitron TI700 U nanoindenter. Adapted from (Ubi® 1 User Manual)

1. Define the boundaries of all the samples mounted onto the stage in order set safety heights. As the indenter moves from left to right, keep the highest samples on the left.
2. Define the positions of the indents in each sample and set the grids or patterns to be followed automatically during the indentation process.
3. Fill the container with the rest of the fluid using plastic pippets until the samples have ~ 2 mm of fluid above them.
4. Switch off the lights in the nanoindenter, as once there is liquid in, it will not possible to see the samples with the microscope.

5. Perform a “Cal air indent” with the tip submerged ~ 2 mm in water. Usually this calibration is carried out in air, but in this case the “air” indent must be performed in water.
6. Start testing the samples according to the method previously defined in the automation by moving the indenter probe to the recorded positions.
7. Once the tests are finished, move the stage as far as possible from the indenter tip, turn off the nanoindenter and remove the container carefully.

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