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Mechanobiological Origins of Osteoporosis


A thesis submitted to the National University of Ireland as fulfilment of the requirements for the degree of Doctor of Philosophy

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Department of Biomedical Engineering
College of Engineering and Informatics
National University of Ireland, Galway

Supervisor of Research: Dr. Laoise M. McNamara
Abstract

The osteocyte is believed to act as the primary sensor of mechanical stimulus in bone, controlling signalling for bone growth and resorption in response to changes in the mechanical demands placed on bones throughout life. Alterations in local bone tissue composition and structure arising during osteoporosis likely disrupt the local mechanical environment of these mechanosensitive bone cells, and may thereby initiate a mechanobiological response. However, due to the difficulties in directly characterising the mechanical environment of bone cells in vivo, the mechanical stimuli experienced by osteoporotic bone cells are not known. The global aim of this thesis is to discern the in vivo mechanical environment of the osteocyte, both in healthy bone tissue and during the disease of osteoporosis.

The first study of this thesis involved the development of 3D finite element models of osteocytes, including their cell body and the surrounding pericellular matrix (PCM) and extracellular matrix (ECM), using confocal images of the lacunar-canalicular network. These anatomically representative models demonstrated the significance of geometry for strain amplification within the osteocyte mechanical environment. A second study employed fluid-structure interaction (FSI) modelling to investigate the complex multi-physics environment of osteocytes in vivo. These models built upon the anatomically representative models developed in the first study, and FSI methods were used to simulate loading-induced interstitial fluid flow through the lacunar-canalicular network. Interestingly, the in vivo mechanical stimuli (strain and shear stress) predicted using these computational approaches were above thresholds known to elicit osteogenic responses from osteoblastic cells in vitro, and thereby provide a novel insight into the complex multi-physics mechanical environment of osteocytes in vivo.

The third study of this thesis sought to experimentally characterise the strain environment of osteoblasts and osteocytes under physiological loading conditions in healthy and osteoporotic bone, using a rat model of osteoporosis. A custom-designed loading device compatible with a confocal microscope was constructed to apply strains to fluorescently stained femur samples from normal and ovariectomised rats. Confocal imaging was performed simultaneously during loading and digital image
correlation techniques were used to characterise cellular strains from the images acquired. These results suggested that the mechanical environment of osteoblasts and osteocytes is altered during early-stage osteoporosis, and it is proposed that a mechanobiological response restores the homeostatic mechanical environment by late-stage osteoporosis. A final study applied these results as inputs for the developed computational models to investigate whether changes in tissue properties, lacunar-canalicular architecture or amplification mechanisms during osteoporosis could explain the altered mechanical stimulation of osteocytes observed. The findings of this study shed new light on the osteocyte mechanical environment in both healthy and osteoporotic bone, elucidating a possible mechanobiological relationship between increases in strain stimulation of the osteocyte and subsequent increases in mineralisation of bone tissue as key events in the progression of osteoporosis.

Together, the studies in this thesis provide a novel insight into the closed mechanical environment of the osteocyte. Using both computational and experimental methods, the mechanical stimuli that osteocytes experience under physiological loading in vivo, in both healthy and osteoporotic bone, were elucidated. In particular, the research in this thesis provides a missing mechanobiological link in the temporal development of post-menopausal osteoporosis, and the information gained from this body of work may inform future treatments for osteoporosis.
Publications

Journal Articles
The following publications have arisen from the work presented in this thesis:

- Verbruggen SW, Vaughan TJ, McNamara LM “Mechanisms of osteocyte stimulation in health and osteoporosis”, In Preparation

Conference Presentations
Peer Reviewed International Conferences:

- Podium and poster presentations at the Annual Meeting of the Orthopaedic Research Society, San Antonio, TX, USA. January 2013
- Podium and poster presentation at the 21st Annual Symposium on Computational Methods in Orthopaedic Biomechanics, San Antonio, TX, USA. January 2013
- Podium presentation at the European Congress on Computational Methods in Applied Sciences and Engineering, Vienna, Austria. September 2012
• Podium presentation at the American Society of Mechanical Engineers
  Summer Bioengineering Conference, Puerto Rico, USA. July 2012
• Poster presentation at the Annual Meeting of the Orthopaedic Research
  Society, San Francisco, CA, USA. January 2012
• Podium and poster presentation at the 20th Annual Symposium on
  Computational Methods in Orthopaedic Biomechanics, San Francisco, CA,
  USA. January 2012
• Podium presentation at the 21st International Workshop on Computational

Peer Reviewed National Conferences:
• Podium presentations at the 17th, 18th and 19th Annual Conference of the
  Section of Bioengineering of the Royal Academy of Medicine in Ireland,
• Podium presentation at the 15th Annual Sir Bernard Crosslands Symposium,
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# Nomenclature

## Roman Letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$E$</td>
<td>Young’s Modulus (Pa)</td>
</tr>
<tr>
<td>$I$</td>
<td>Moment of inertia (kg m$^2$)</td>
</tr>
<tr>
<td>$EI$</td>
<td>Flexural rigidity (N m$^2$)</td>
</tr>
<tr>
<td>$K$</td>
<td>Bulk modulus (Pa)</td>
</tr>
<tr>
<td>$G$</td>
<td>Shear modulus (Pa)</td>
</tr>
<tr>
<td>$r$</td>
<td>Radius (m)</td>
</tr>
<tr>
<td>$t$</td>
<td>Time (s)</td>
</tr>
<tr>
<td>$Ca$</td>
<td>Calcium</td>
</tr>
<tr>
<td>$S^f$</td>
<td>Solution of fluid domain</td>
</tr>
<tr>
<td>$S^s$</td>
<td>Solution of solid domain</td>
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</table>

## Greek Letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\varepsilon$</td>
<td>Strain</td>
</tr>
<tr>
<td>$\mu \varepsilon$</td>
<td>Microstrain ($\varepsilon \times 10^{-6}$)</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Normal stress (Pa)</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Shear stress (Pa)</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Shear modulus (Pa)</td>
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## Acronyms

<table>
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<th>Description</th>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Bone multi-cellular unit</td>
</tr>
<tr>
<td>CFD</td>
<td>Computational fluid dynamics</td>
</tr>
<tr>
<td>DIC</td>
<td>Digital image correlation</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FE</td>
<td>Finite element</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FSI</td>
<td>Fluid-structure interaction</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomised animal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered solution</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericellular matrix</td>
</tr>
<tr>
<td>PCS</td>
<td>Pericellular space</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>qBEI</td>
<td>quantitative backscatter electron imaging</td>
</tr>
<tr>
<td>SHAM</td>
<td>Sham-operated animal</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UHVEM</td>
<td>Ultra-high voltage electron microscopy</td>
</tr>
<tr>
<td>ZNCC</td>
<td>Zero-mean normalised cross-correlation</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
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Chapter 1

Introduction and Objectives

1.1 Bone Cell Mechanobiology

Bone is a dynamic and adaptive material, which actively remodels itself in response to the mechanical demands imposed by physical activity throughout life. This adaptive nature is crucial to bone’s physiological function, allowing the skeleton to survive under a variety of loading conditions and repair itself in response to damage. Central to this adaptive behaviour are osteoblast and osteocyte cells, which are known to be highly mechanosensitive and work in concert by signalling to osteoclast and osteoblast cells to optimise bone structure (Birmingham et al. 2012b; Jahani et al. 2012; Klein-Nulend et al. 1995b; Mullender et al. 2004b; Owan et al. 1997; Smalt et al. 1997; You et al. 2000).

Numerous in vitro cell culture studies have demonstrated the ability of osteoblast and osteocyte cells to sense external loading, such as fluid flow and matrix stretch, and respond through the production of biochemicals and proteins associated with osteogenesis (Ajubi et al. 1996; Klein-Nulend et al. 1995a; Klein-Nulend et al. 1995b; Owan et al. 1997; Pitsillides et al. 1995; Smalt et al. 1997; Westbroek et al. 2000; You et al. 2000). Osteocytes are believed to be the most mechanosensitive bone cells (Ajubi et al. 1996; Klein-Nulend et al. 1995b; Westbroek et al. 2000) and are connected by a network of highly mechanosensitive dendritic cell processes (Adachi et al. 2009b; Burra et al. 2010; Klein-Nulend et al. 2013; Wu et al. 2011). Due to this ubiquitous distribution throughout bone, osteocytes are thought to act as a network of strain sensors, monitoring the mechanical environment throughout bone tissue orchestrating adaptive responses of bone to mechanical loading (Bonewald
2002; Cowin et al. 1991; Lanyon 1993). A recent in vitro study has shown that osteocytes are more influential than osteoblasts in stimulating osteogenesis in mesenchymal stem cells (MSCs) (Birmingham et al. 2012b). However, the location of osteocytes deep within the bone matrix represents a significant challenge experimentally and, as such, the in vivo mechanical environment of the osteocyte remains to be characterised.

While whole bones are known to suffer damage at strain levels of greater than 3,500 µε (Carter et al. 1987; Mosley 2000), individual bone cells have been shown in vitro to require at least 10,000 µε to elicit an osteogenic response (Burger and Veldhuijzen 1993; You et al. 2000). This represents a paradox, as it implies that bone cells might not be sufficiently stimulated in vivo during normal physiological loading conditions. Previous theoretical and computational models have explained this paradox, by proposing that strain amplifying elements exist within the local osteocyte environment (Han et al. 2004; Rath Bonivtch et al. 2007; Wang et al. 2007; You et al. 2001b; Verbruggen et al. 2012). However these models have not incorporated the effect of the intricate architecture of the lacunar-canalicular network, which is tortuous and highly variable (Ferretti et al. 1999; Kusuzaki et al. 2000; Marotti et al. 1985; Sugawara et al. 2005). Therefore, the first research hypothesis of this thesis is “Mechanical loading to the osteocyte is amplified by the native geometry of the osteocyte environment.”

Fluid flow has repeatedly been shown to be highly stimulatory to bone cells (Owan et al. 1997; Smalt et al. 1997; You et al. 2000), in particular osteocytes, which are the most responsive cell type to fluid flow (Ajubi et al. 1996; Klein-Nulend et al. 1995b; Westbroek et al. 2000; Pitsillides et al. 1995). For this reason loading-induced fluid flow through the lacunar-canalicular network is thought to be the primary mechanical stimulus to osteocytes, having been both predicted theoretically and observed experimentally (Han et al. 2004; Knothe Tate and Knothe 2000; Knothe Tate et al. 1998a; Knothe Tate et al. 1998b; Knothe Tate et al. 2000; Piekarski and Munro 1977; Wang et al. 2000; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994). Previous attempts to model this fluid flow computationally have used mostly idealised geometries of portions of the cells (Anderson et al. 2005; Anderson and Knothe Tate 2008; Kamioka et al. 2012) using computational fluid dynamics approaches. However no previous method has captured the complex
interplay between the extracellular bone matrix, the interstitial fluid and the osteocyte cell body. Therefore the second research hypothesis of this thesis is “Loading-induced interstitial fluid flow significantly contributes to the mechanical stimulation of osteocytes in vivo.”

Osteoporosis is a prevalent disease known to lead to long-term degeneration in both the macroscopic material properties and micro-architecture of bone (Bourrin et al. 2002; Compston et al. 1989; Ederveen et al. 2001; Geusens et al. 1996; Parfitt 1987). This loss of bone mass occurs with age (Wendlová and Pacáková 2007), and is exacerbated by post-menopausal oestrogen deficiency (Falahati-Nini et al. 2000; Riggs et al. 2002). Indeed oestrogen deficiency is a primary cause of osteoporosis, and causes sudden and rapid bone loss at the onset of osteoporosis resulting in micro-structural changes in bone strength, mass and mineral density (Brennan et al. 2011a; Brennan et al. 2011b; Brennan et al. 2012; Compston et al. 1989; Lane et al. 1998; McNamara et al. 2006; Parfitt 1987). These changes in tissue properties likely alter the mechanical environment sensed by bone cells, and may stimulate mechanobiological responses, which could play a role in the macroscopic changes in bone mechanics during osteoporosis. However, as the mechanical environments of osteoblasts and osteocytes have not been observed directly in situ, the mechanobiology of these bone cells in disease remains poorly understood. The third hypothesis of this thesis is “Rapid bone loss at the onset of osteoporosis increases mechanical stimulation of osteoblasts and osteocytes.”

Interestingly, recent experimental studies have observed that bones experiencing long-term oestrogen deficiency exhibit increased mineral content and elastic modulus at the tissue level (Brennan et al. 2011a; Brennan et al. 2011b; Busse et al. 2009; McNamara et al. 2006). Additionally, an increase in trabecular thickness, strength and stiffness occurs during long-term osteoporosis (Brennan et al. 2012; McNamara et al. 2006; Waarsing et al. 2004; Waarsing et al. 2006). These changes might suggest that a mechanobiological response is occurring, in which bone cells are altering local bone mineral content in an attempt to return their mechanical environment to a homeostatic state. Therefore, the fifth hypothesis of this thesis is “A compensatory mechanobiological response by osteocytes to increased loading results in subsequent alterations in local tissue mineralisation and stiffness in late-stage osteoporosis”.

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As well as the macroscopic changes in bone tissue properties during osteoporosis, it has been demonstrated that the lacunar-canalicular network in humans with osteoporosis is disorganised, with a more tortuous canalicular anatomy (Knothe Tate et al. 2002; Knothe Tate et al. 2004). These changes during osteoporosis might further affect the mechanical stimulation of the osteocyte in vivo. Similarly, the lacunar and canalicular walls have been observed to be rougher, with loose collagen fibrils and matrix debris, in an oestrogen deficient rat model of osteoporosis (Sharma et al. 2012). It has been postulated that this degradation could disrupt the attachments connecting the cell processes to the canalicular wall (Sharma et al. 2012), which could in turn affect osteocyte mechanosensation and viability (Lanyon and Skerry 2001; Plotkin et al. 2005). Together these complex changes would be expected to alter the mechanical stimulation of the osteocyte, and thus the final hypothesis of this thesis is “The extracellular environment alters the mechanical stimulation of osteocytes during osteoporosis.”

1.2 Objectives and Hypotheses

The global objective of this thesis is to characterise the mechanical environment of osteoblasts and osteocytes under physiological loading conditions in healthy and osteoporotic bone. The primary specific objective of this research is to delineate the normal mechanical environment of these bone cells. The secondary specific objective is to determine whether this mechanical environment is altered during osteoporosis. The third specific objective is to derive an understanding of how the mechanical environment of the osteocyte plays a role in bone mechanobiology during the development of osteoporosis. To address these objectives, five hypotheses have been defined, each of which will underpin the research of Chapters 4-7 of this thesis.

Hypothesis 1: Mechanical loading to the osteocyte is amplified by the native geometry of the osteocyte environment

Hypothesis 2: Loading-induced interstitial fluid flow significantly contributes to the mechanical stimulation of osteocytes in vivo
Hypothesis 3: Rapid bone loss at the onset of osteoporosis increases mechanical stimulation of osteoblasts and osteocytes

Hypothesis 4: A compensatory mechanobiological response by osteocytes to increased loading results in subsequent alterations in local tissue mineralisation and stiffness in late-stage osteoporosis

Hypothesis 5: The extracellular environment alters the mechanical stimulation of osteocytes during osteoporosis

By testing each of these hypotheses, the research questions outlined above can be answered and the proposed research will deliver significant advances in the understanding of bone mechanobiology during normal physiology and at the onset of the disease of osteoporosis.

Confocal imaging and image processing software will be employed to generate anatomically representative geometries of osteocytes, which will then be used to generate 3D finite element models of osteocytes to predict the mechanical environment of osteocytes in vivo (Hypothesis 1). In order to capture the complex interplay between bone matrix, interstitial fluid and the cell in the lacunar canalicular network, these geometries will be used to conduct fluid-structure interaction analyses and determine the effects of loading-induced fluid flow on osteocytes in vivo, and thereby further delineate the mechanisms by which osteocytes are stimulated in both healthy and osteoporotic bone tissue (Hypotheses 2, 5).

Finally, a novel loading device will be designed, allowing simultaneous loading and confocal imaging of individual bone cells in rat femur explants. These image series will then be analysed using digital image correlation (DIC) techniques, allowing the strain distribution within the cells to be characterised. Using an ovariectomised rat model of post-menopausal osteoporosis, the strain in bone cells under macroscopic loading will be compared, for both healthy and osteoporotic bone (Hypotheses 3 and 4). The results of this study will then act as inputs for the computational models, to further elucidate the relationship between bone cell mechanobiology and osteoporosis (Hypothesis 5).
1.3 Thesis Structure

This thesis comprises the work completed for the duration of the candidate’s PhD studies. First, a thorough review of the literature is presented in Chapter 2, detailing the function of bone, remodelling theories, and the current knowledge of bone cell mechanobiology in both healthy and osteoporotic bone. Next, the theory involved in the computational modelling and image analysis is outlined in Chapter 3. The effect of extracellular geometry on osteocyte stimulation and strain amplification is described in Chapter 3, testing hypothesis 1. Fluid-structure interaction models of the osteocyte mechanical environment are developed in Chapters 4 and 7, using both anatomically representative and idealised models to test hypotheses 2 and 5. Chapter 6 investigates hypotheses 3 and 4 by simultaneously loading and imaging bone samples which have been exposed to both short- and long-term oestrogen deficiency. Each of Chapters 4-7 contains a detailed description of the methods in each study and comprehensive discussions of the results. A summary of the main findings of the thesis is contained in Chapter 8, placing them in the context of current bone cell mechanobiology and osteoporosis research, along with recommendations for future research in these fields.
Chapter 2

Literature Review

2.1 Bone

2.1.1 Bone Function and Composition
Bone is the primary component of the skeleton, which provides a support framework and protection for internal organs, and allows movement in concert with muscles, tendons and ligaments. Bone is capable of efficiently performing these functions due to its high strength to weight ratio, arising from its unique composition including a stiff mineral phase and a softer organic phase (Currey 1984). Bone tissue is composed of approximately 65% mineral hydroxyapatite crystals (Fratzl et al. 2004) and 35% organic molecules. This organic phase is largely composed of primarily type I collagen (Miller and Parker 1984; Miller et al. 1969), but also contains non-collagenous proteins, such as osteopontin, osteocalcin, osteonectin and bone sialoprotein, which provide bonds between collagen fibrils and facilitate bone mineralisation (Roach 1994). This composition provides bone with its load-bearing capacity while also ensuring a degree of flexibility in bending.

2.1.2 Hierarchical Structure of Bone
Two distinct types of bone structure are discernible in vivo (see Figure 2.1). Cortical bone is formed as a dense outer shell consisting of highly calcified tissue and, as it represents approximately 80% of the bone mass and dictates the primary structure of most bones, it is largely responsible for providing support and protection to the human body (Buckwalter et al. 1995; Currey 1984; Favus and Christakos 1996).
Trabecular bone is found within this outer cortical structure and forms supportive struts which act with the cortical shell to distribute high stresses throughout the bone structure (Lemaire et al. 2004).

Figure 2.1: Diagram of the hierarchical structure of bone, illustrating (a-b) the composite nature of bone and the bone microstructure organised into (c) osteons and (d) trabeculae. These organisational units respectively form the basic constituents of (e) the cortical and trabecular macrostructure of bone (Vaughan et al. 2012)

The structure of bone is hierarchically organised into functional units over multiple scales, so that micro-architecture is optimised to bear high loads experienced in everyday situations. At the micro-scale, cortical bone is composed of functional units known as osteons. These are cylindrical structures composed of concentric rings of osteocytes orientated about a vascular Haversian canal (see Figure 2.2). This compact network facilitates mechanosensation, communication and nutrient supply to bone cells, whilst providing the basic structural unit for cortical bone (Eriksen et al. 1994). Indeed, the osteon is thought to give structural support to bone by slowing crack growth through their outer cement lines (Burr et al. 1988).

Trabecular bone is also composed of structural units called trabecular packets, or hemiosteons (Cowin 2001). While these packets are similar in size to osteons, they are highly porous and do not have a vascular supply (Parfitt 1983), instead receiving nutrients and mechanical stimulus from the surrounding bone marrow (Coughlin and Niebur 2012b; De Bruyn et al. 1970). Trabecular bone struts are thought to align
themselves along the trajectories of weight-bearing forces, providing optimised mechanical support for minimal bone mass (Wolff 1986).

**Figure 2.2**: (A) Cross-section of cortical bone showing the osteons (Royce and Steinmann 2003), with (B) osteons, osteocytes and canaliculi indicated in a diagram (Gray and Standring 2008)

### 2.1.3 Bone Porosity and Fluid Flow

Bone in characterised by a number of different porosities across multiple scales (see Figure 2.3); the trabecular porosity within the bone marrow cavity (Arramon and Cowin 1998), the vascular porosity resulting from the network of blood vessels spread throughout bone (Zhang et al. 1998), and the lacunar-canalicular porosity which houses more than 90% of bone cells (Cowin 2001). The trabecular porosity consists of the space between trabecular struts, known as the medullary cavity, which contains the bone marrow. This marrow displays viscosities up to two orders of magnitude greater than fluid in other bone porosities (Bryant 1983; Bryant 1988; Hrubá et al. 1988), and results in a unique mechanical environment that has been shown to be stimulatory to various bone cell types residing in the marrow (Birmingham et al. 2012a; Coughlin and Niebur 2012b). The vascular porosity contains blood vessels at pressures largely similar to those found throughout the vasculature (Cowin 2001), and is highly permeable compared to the other porosities (Zhang et al. 1998). Finally, the lacunar-canalicular porosity contains interstitial fluid, which bathes the osteocytes within the system and has an ionic composition similar to plasma or salt water (Aukland 1984). Experimental tracer studies have
shown that mechanical loading of the bone matrix surrounding this porosity generates a pressure differential that drives fluid through the lacunar-canalicular network (Knothe Tate and Knothe 2000). Further tracer studies have recently observed interstitial fluid velocities of approximately 60 µm/s within individual canaliculi under applied mechanical loading (Price et al. 2011).

These porosities contribute to the mechanical behaviour of bone, by dictating the time-dependent response to loading, which is related to the time and energy required to drive fluid through these porosities (Cowin 1999). While the more permeable vascular porosity can dissipate a loading-induced build-up of pressure rapidly through the vascular network, the lacunar-canalicular porosity requires a relaxation time that is three orders of magnitude greater (Zhang et al. 1998). The combination of the mechanical effects of these different porosities contribute to the ability of bone to absorb high loads (Cowin 1999), but importantly also generate interstitial fluid flow around osteocytes (Knothe Tate and Knothe 2000). This fluid flow facilitates nutrient supply (Knothe Tate et al. 1998a; Rho et al. 1998) and is also believed to play an important role in the mechanobiological behaviour of bone (Burger et al. 1998).

**Figure 2.3:** Diagram of an osteon, the primary structural unit of bone, with the concentric locations of osteocytes shown (Vaughan et al. 2013b)
2.2 Bone Cells

Numerous cell types reside in bone tissues, and act in concert to maintain bone. These cells largely develop from two lineages: the osteogenic lineage and the monocyte-macrophage lineage. Their development is illustrated in Figure 2.4 and described in the following sections.

![Figure 2.4: Differentiation of cell types along (A) the osteogenic lineage and (B) the osteoclastic lineage (Wang et al. 2012)](image)

Encased within the cortical bone and surrounding the trabeculae is the bone marrow, the source of osteoprogenitor cells, which differentiate into osteoblasts and ultimately osteocytes (Okazaki et al. 2002). The vasculature within the bone tissue itself acts as a source of multinucleated cells and monocytes, which can develop into osteoclasts (Udagawa et al. 1990). The morphological differences between these cells types can be seen in Figure 2.5.
2.2.1 MSCs and Osteoprogenitors

Mesenchymal stem cells (MSCs) are multipotent cells that have the potential to differentiate into numerous cell types. While they play a key role in early skeletal development, they can also be found in mature adult bone and are present in the periosteum, endosteum and bone marrow. The MSCs exhibit a basic cell morphology, defined by a small cell body containing a large, round nucleus, and are capable of altering their morphology in response to their local environment (McNamara 2011). When osteogenesis is stimulated MSCs begin to proliferate, influenced by growth factors, cytokines and physical stimuli to become osteoprogenitor cells. These cells have committed to the osteochondral lineage and are now only capable of differentiating along the chondrogenic (cartilage) or osteogenic (bone) pathways. Morphologically larger than MSCs, osteoprogenitors can be influenced by growth factors such as bone morphogenic proteins (BMPs) to differentiate further into osteoblasts and ultimately into osteocytes (Okazaki et al. 2002).

2.2.2 Osteoblasts

Osteoblasts are bone forming cells that are responsible for the production of bone tissue from birth, and also the renewal of bone tissue in modelling and remodelling (Section 2.3.2) throughout life. As described above, osteoblasts differentiated from osteoprogenitor cells, which are present in bone marrow and form part of the same osteoblastic lineage as osteocytes (see Figure 2.4A) (Okazaki et al. 2002). Displaying a cuboidal geometry, the osteoblast possesses a large Golgi apparatus and rough endoplasmic reticulum when active (Cowin 2001), and organelles that play a key role in the cell’s primary function of synthesis of osteoid (Hammond and Helenius 1995; Rothman 1994). The deposition and subsequent mineralisation of this new bone matrix (Ducy et al. 2000) forms a key part of the bone remodelling cycle (Parfitt 1994). Osteoblasts actively control this process by deposition of fibronectin (Gronowicz and Derome 1994; Moursi et al. 1996; Owen et al. 1990), which is responsible for mineralisation, matrix formation and osteoblast differentiation (Gronowicz and Derome 1994; Moursi et al. 1996).
Quiescent osteoblasts on resting bone surfaces are known as bone-lining cells, displaying a flattened, elongated geometry and lying upon an organic collagen matrix approximately 1-2 µm thick (Manolagas 2000). These cells have been observed to form bone in response to both biochemical signals (Dobnig and Turner 1995; Merz and Schenk 1970; Miller and Jee 1987) and mechanical strain (Lanyon 1993, 1996), suggesting involvement in maintaining bone homeostasis (Miller and Jee 1987; Miller et al. 1980; Talmage 1969). Furthermore, osteoblasts are thought to aid bone resorption by removing the organic collagen layer and contracting to allow osteoclasts to access the mineralised bone tissue (Favus and Christakos 1996; Puzas and Lewis 1999).

![Figure 2.5: TEM image of an osteocyte, osteoblast and osteoclast, with the ruffled border of the osteoclast indicated (red arrow). Reproduced with permission of Dr. L.M. McNamara](image)

**2.2.3 Osteoclasts**

Osteoclasts, derived from mononuclear precursor cells in the hematopoietic vascular channels in bone (Udagawa et al. 1990), are giant multi-nucleated cells that can range in diameter from 20-100 µm (Roodman 1996). The main function of these cells is to break down and resorb bone matrix, and as such they are usually found in temporary cavities on the bone surface known as Howship’s lacunae during the bone remodelling process (Watanabe et al. 1995). Osteoclast resorption is initiated by the activity of bone-lining cells, which retract from their location on the bone surface to provide access for the osteoclast to attach to the bone matrix (Favus and Christakos 1996). The osteoclast then forms a sealing zone (Salo et al. 1997), which is a closed microenvironment (Teitelbaum 2000) controlled by the cell and rendered highly
acidic to digest bone tissue (Väänänen and Horton 1995). This is facilitated by a remarkable morphological feature known as the ruffled border (see Figure 2.5), a complex folding of the cell’s plasma membrane at the bone interface, providing an increased surface area through which acids and proteolytic enzymes are secreted to degrade the bone matrix (Palokangas et al. 1997). Minerals and ions resulting from the breakdown of bone are released into the extracellular environment by the osteoclasts, thereby maintaining homeostasis under alternating mechanical loads (Bilezikian et al. 2008).

### 2.2.4 Osteocytes

Osteocytes are distributed abundantly throughout bones encased within the bone matrix, and represent the terminal differentiation of the osteogenic lineage (Frost 1960a). Differentiation of osteoblasts into osteocytes occurs during deposition of new bone matrix as part of the bone remodelling process (Cowin 2001). Organised concentrically around blood vessels within osteons (see Figure 2.6), osteocytes are uniquely placed to sense mechanical loading.

![Confocal scan of osteocyte network](image)

**Figure 2.6:** Confocal scan of osteocyte network, with aspects of its mechanical environment highlighted
The cells extend a network of dendritic processes outwards through a system of passages known as canaliculi (see Figure 2.6), to contact and communicate with other osteocytes via gap junctions (Li et al. 2000; Xia et al. 2010). These canaliculi are believed to provide a vital system for nutrient supply and waste disposal to the cells (Cowin 2001), as well as allowing transduction of biochemical signals to other cells, both in the matrix and on the bone surface (Lanyon 1998; Turner et al. 1998). A mesh-like pericellular glycocalyx surrounds the osteocyte, tethering it to the extracellular matrix (You et al. 2001b), while punctate integrin attachments between the cell processes and the matrix are present in the canaliculi (McNamara et al. 2009). Osteocytes reside within a complex mechanical environment, experiencing mechanical loading during exercise through both direct strain in the bone matrix (Huiskes et al. 2000) and interstitial fluid flow which is driven through the lacunar-canalicular network (Cowin et al. 1995).

2.3 Bone Growth and Adaptation

2.3.1 Bone Growth

Bone formation occurs both during embryonic development and throughout life, adapting the geometry of bones in response to changes in mechanical loading or replacing aged or damaged bone. The bone formation process can occur via two specific pathways: endochondral ossification and intramembranous ossification. Both of these processes involve the initial deposition of an organic matrix, osteoid or cartilage, which is then mineralised over time to produce the composite bone matrix.

2.3.1.1 Endochondral Ossification

Endochondral ossification is the process involved in embryonic bone formation, and is initiated by MSCs creating a cartilage template upon which bone is formed (Ortega et al. 2004). This is performed by differentiation of MSCs into chondroblasts, which then secrete a matrix comprised of collagen and proteoglycans. Further differentiation of these chondroblasts into chondrocytes results in the secretion of biochemicals and growth factors that initiate mineral deposition and promote vascularisation of the template. Endothelial cells on the lining of these
vessels produce essential growth factors that control the recruitment, proliferation, and differentiation of osteoblasts (Sumpio et al. 2002), emphasising the importance of vascularisation for bone formation (Collin-Osdoby 1994; Gerber and Ferrara 2000). This formation process through endochondral ossification is on-going throughout childhood, particularly in the epiphyseal plate of long bones. New cartilage continues to be produced at this location during youth, which is replaced by bone, and thereby facilitates lengthening of bones.

### 2.3.1.2 Intramembranous Ossification

Embryonic bone formation can also occur by means of intramembranous ossification, which regulates the formation of non-long bones such as the bones of the skull and clavicle. Critically, the intramembranous process does not rely on the formation of a cartilage template. Rather, embryonic stem cells within mesenchymal tissue of the embryo, begin to proliferate and condense to form an aggregate of MSCs. This aggregate becomes surrounded by a membrane, and MSCs within the membrane begin to differentiate directly to osteoprogenitor cells and then to osteoblasts. These osteoblasts line the aggregate and secrete an extracellular matrix. Some of these osteoblasts become embedded within the newly formed matrix and, in this environment, they differentiate and form interconnecting cytoplasmic processes to become osteocytes. The cells on the outer surface form a periosteum, and mineralisation begins to occur to form rudimentary bone tissue that is populated by osteocytes and lined by active osteoblasts (Cowin 2001). This tissue eventually forms trabeculae, which then fuse to form woven bone. This woven bone is then remodelled over time to become lamellar bone, with concentric lamellae surrounding Haversian systems in what is known as an osteon (Chung et al. 2004; Cowin 2001; Kanczler and Oreffo 2008).

### 2.3.2 Bone Modelling and Remodelling

The dynamic adaptive nature of bone is fundamental to its ability to protect and support the body throughout life, allowing it to optimise its structure and composition to provide maximum strength for minimal mass.
Bone modelling is a process which facilitates growth and change in bone morphology, and largely occurs in childhood and adolescence. This occurs when bone resorption by osteoclasts simultaneous to bone formation by osteoblasts, but on different surfaces such that the dimensions of the bone are altered (Frost 1990a). While the bulk of bone modelling occurs during early life this process does continue in the adult skeleton, regulating both bone micro-structure and overall bone geometry in response to mechanical demands (Mosekilde 1990).

Bone remodelling is the process by which old bone is replaced with new tissue, enabling active repair of structural micro-damage and adaptation of microarchitecture to local stress conditions (McNamara 2011). The process of bone turnover begins at 2-3 years of age by replacing immature or primary bone present from infancy (Cowin 2001), and remodelling continues throughout life every 20 years for cortical bone and 1-4 years for trabecular bone (Parfitt 1983).

Bone remodelling, as the work of coupled osteoblasts and osteoclasts, is primarily a cell-controlled process (Parfitt 1994). Bone cells act in a coordinated fashion to remodel bone, in a cellular unit known as the bone multi-cellular unit (BMU) (Frost 1973). Within this unit osteoblasts and osteoclasts operate, whereby osteoclasts resorb unwanted/damaged bone to leave cavities, which are subsequently filled with new bone by osteoblasts (Parfitt 1994). This process, shown in Figure 2.7, occurs in six successive phases; resting, activation, resorption, reversal, formation and mineralisation (Cowin 2001).
Figure 2.7: Diagram showing the six phases of the bone remodelling cycle: resting, activation, resorption, reversal, formation and mineralisation (Hill 1998)

2.4 Bone Mechanobiology

Crucial to the adaptive and regenerative nature of bone is bone cell mechanobiology, the effect of physical forces on cells and their ability to convert these forces into biochemical signals (Machwate et al. 1995). There is much experimental evidence of bone mass adapting to different loading conditions, with net bone resorption occurring at low strains and net bone formation occurring at high strains (Carter 1984; Forwood and Turner 1995; Jee et al. 1991; Mosley and Lanyon 1998; O'Connor et al. 1982; Woo et al. 1981). This process is governed by the cells, osteoblasts, osteoclasts and osteocytes working in concert, and this is outlined further below. It is now known that many cells of the body have the ability to appraise their mechanical environment, by means of specific molecule or protein complexes known as mechanosensors, and communicate the need for adaptation by producing certain biochemical signals (mechanotransduction) to initiate an adaptive response in other cells when the mechanical environment is not favourable.
Mechanosensation, mechanotransduction and the mechanical environment of bone cells will be discussed in this section.

2.4.1 Mechanosensation

While osteocytes are thought to be the primary mechanosensors in bone, the precise mechanism by which they can sense mechanical strain is unknown. Osteocytes have been shown in vitro to be the most mechanosensitive bone cell type (Ajubi et al. 1996; Klein-Nulend et al. 1995a; Westbroek et al. 2000), particularly in response to fluid flow, and have also been shown to direct osteogenesis in other cell types (Birmingham et al. 2012b). This has reinforced the theory that osteocytes sense mechanical loading in the bone matrix and then orchestrate the adaptive bone remodelling response (Bonenwald 2002; Cowin et al. 1991; Lanyon 1993). Due to their presence deep within bone matrix, direct experimental observation of osteocytes in vivo has proven extremely challenging and, as such, the precise mechanical stimuli which they experience in vivo remain unknown.

Previous studies have proposed that deformation of the osteocyte lacuna (Rath Bonivtch et al. 2007), strain energy density in the bone tissue (Huiskes et al. 2000; Mullender et al. 1994), or loading-induced interstitial fluid flow in the osteocyte canalicular channels (Cowin et al. 1995) might stimulate the cell membrane. Experimental evidence has demonstrated an anabolic response in osteoblastic cells exposed to both fluid flow (Bakker et al. 2001; McGarry et al. 2005a; McGarry et al. 2005b) and matrix strain (Owan et al. 1997; You et al. 2000) in vitro. Multiple mechanisms for mechanosensation have been proposed, as shown in detail in Figure 2.8. The locations of some of these mechanisms with respect to the osteocyte are shown in Figure 2.9.
Figure 2.8: A diagram of the potential mechanisms by which an osteocyte may sense interstitial fluid flow (Thompson et al. 2012; You et al. 2001b): (A) deformation of the cell body causing strains at punctate integrin attachments to the extracellular matrix, (B) perturbation of tethering elements between the canalicular wall and the cell membrane and (C) bending of the primary cilium under flow.

It has been observed that punctate integrin attachments spaced along the canalicular wall exist, and appear to co-localise with projections of the extracellular matrix into the pericellular space (McNamara et al. 2009). It is thought that osteocytes may sense matrix strain and fluid-induced deformation in bone canaliculi via integrin-based (αVβ3) focal attachments between their cell processes and these ECM projections (see Figure 2.8A and Figure 2.9D, E) (McNamara et al. 2009; Wang et al. 2007). Another mechanism by which the osteocyte has been hypothesised to sense fluid flow is via flow-induced drag force on tethering elements of the pericellular glycolcalyx (see Figure 2.8B and Figure 2.9B), which attaches the cell to the lacunar-canonical wall (You et al. 2001b; You et al. 2004). This theory is reinforced by evidence that enzymatic removal of this glycocalyx in vitro reduces the ability of the osteocyte to respond via the PGE2 pathway (Reilly et al. 2003b).
Figure 2.9: Schematic representation of key mechanisms in the osteocyte environment implicated in mechanosensing (Adapted from (Schaffler et al. 2013)): (A) TEM image of an osteocyte process displaying the actin cytoskeleton (You et al. 2004); (B) TEM image of proteoglycan PCM tethering elements (black arrows) bridging an osteocyte cell process to the bony canalicular wall; (C) Fluorescent immunohistochemical staining showing that β1 integrins (white arrows) are located only on osteocyte cell bodies (McNamara et al. 2009); (D) TEM image demonstrating the discrete ECM projections from the canalicular wall that contact osteocyte processes; (E) Fluorescent immunohistochemical staining for β3 integrins (white arrows) are present in a punctate pattern along osteocyte processes, with similar periodicity and spacing pattern to ECM projections (McNamara et al. 2009)
It has been proposed that both of these extracellular attachments may act to amplify strain signals to the osteocyte (Han et al. 2004; McNamara et al. 2009; Pavalko and Burridge 1991; Wang et al. 2007; You et al. 2001b; You et al. 2004) through their connections with the cytoskeleton (Baik et al. 2013). This is particularly significant, as experimental studies have shown that osteocyte cell processes are significantly more mechanosensitive than the cell bodies (Adachi et al. 2009b; Burra et al. 2010; Klein-Nulend et al. 2013; Wu et al. 2011).

The primary cilia, a microtubule-based cellular structure, has recently been identified in bone tissue (Tonna and Lampen 1972). As these structures have been implicated as fluid flow sensors in other tissues, such as the kidney (Schwartz et al. 1997), it has been proposed that primary cilia may allow bone cells to sense strain-derived fluid flow (see Figure 2.8C) (Whitfield 2003; Whitfield 2008). This hypothesis has been supported by in vitro studies identifying primary cilia on osteoblast- and osteocyte-like cell lines in cell culture (Malone et al. 2007; Xiao et al. 2006), and demonstrating osteogenic responses under in vitro fluid flow (Malone et al. 2007).

2.4.2 Mechanotransduction

All cells in the osteoblastic lineage can transduce mechanical strain signals into biochemical cues for osteogenesis (Haj et al. 1990). It has also been observed that osteoblastic cells respond with biochemical signals to both direct matrix strain (Owan et al. 1997; You et al. 2000) and to fluid flow in vitro (Bakker et al. 2001; McGarry et al. 2005a; McGarry et al. 2005b), suggesting multiple mechanical mechanisms of bone cell stimulation in vivo. These mechanical stimuli are transduced into various biochemical cues that can orchestrate bone remodelling.

Intracellular calcium signalling is an early signalling event in bone mechanotransduction, and facilitates multiple important cell functions (Berridge et al. 2000; Zayzafoon 2006) as well as expression of bone matrix proteins such as osteopontin (You et al. 2001a). Osteopontin itself is a phosphorylated protein located in the cement lines of osteons and along walls of canaliculi (Devoll et al. 1997a; McKee and Nanci 1996b; McNamara et al. 2006; Nanci 1999; Sodek and McKee 2000). It is also believed to play a role in osteocyte mechanosensation by mediating
their attachment to surfaces, and its absence has been shown to change the response of bone to loading (Yoshitake et al. 1999). Nitric oxide (NO) is a biochemical signal that controls mechanically-stimulated bone formation in vivo (Chambers et al. 1998; Fox et al. 1996), with inhibition of this signal suppressing bone formation (Turner et al. 1996). Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) release has been shown to play a role in the adaptive response of bone to loading (Akamine et al. 1992), while blocking of this signal diminishes the osteogenic response of bone (Lucchinetti 2001). Indeed it has been confirmed that mechanical loading of bone cells results in increased expression of both NO and PGE\textsubscript{2} (Burger and Klein-Nulend 1999; Johnson et al. 1996; Klein-Nulend et al. 1998; McAllister and Frangos 1999; Mullender et al. 2004b; Smalt et al. 1997). Similarly, applied fluid shear stress in vitro has been shown to increase intracellular calcium, mineral production and other osteogenic biochemical signals such as PGE\textsubscript{2}, NO and osteopontin (Bakker et al. 2001; Batra et al. 2005; Donahue et al. 2003; McGarry et al. 2005a; Sikavitsas et al. 2003; Smalt et al. 1997; van den Dolder et al. 2003).

Osteocytes are the putative primary mechanosensory bone cell in vivo and respond to changes in mechanical strain (Bonewald 2002), demonstrating higher intrinsic sensitivity to loading than other osteogenic cells (Klein-Nulend et al. 1995b; Westbroek et al. 2000). However, the exact manner in which they are stimulated under mechanical loading in vivo remains unknown. As a result, several studies have compared the effect of strain with shear stress on bone cells in vitro. Shear stress applied to osteoblastic cells was found to rapidly up-regulate NO and PGE\textsubscript{2} release, while no effect was observed under mechanical strain (Smalt et al. 1997). Similarly, osteopontin expression has been shown to be more sensitive to shear stress than direct mechanical strain (Owan et al. 1997). However, mechanical strain has also been shown to increase both NO and intracellular calcium signalling in osteocytes (Adachi et al. 2009a; Ajubi et al. 1999; Zaman et al. 1999).

Previous studies comparing the mechanosensitivity of osteoblasts and osteocytes found that the position of the osteoblasts on the surface of bone made them less sensitive to changes in loading patterns, such as changes in loading direction of up to 20°, and therefore less likely to influence bone remodelling (Mullender and Huiskes 1997). Furthermore, osteocytes have been shown to be more responsive to fluid flow than osteoblasts in vitro, with respect to PGE\textsubscript{2} release (Klein-Nulend et al. 1995a).
Interestingly, recent in vitro experiments have observed the spreading of both NO and intracellular calcium signalling through networks of bone cells (Jing et al. 2013; Lu et al. 2012a; Lu et al. 2012b; Vatsa et al. 2007), demonstrating their ability to spread signals as a network. Moreover, osteocyte networks were found to be more mechanoresponsive in calcium signalling than osteoblast networks (Lu et al. 2012a), illustrating the importance of the osteocyte network for mechanosensation and signal transduction in bone remodelling.

### 2.4.3 Mechanical Environment

Despite the known importance of the osteocyte to mechanosensation and mechanotransduction in bone, its mechanical environment remains poorly understood. This is due to the osteocyte’s location deep within the bone tissue, making direct experimental observation without destructive interference with the environment challenging. Experiments have attempted to overcome this difficulty by focusing on the lacunar-canalicular network. High resolution imaging of this network under mechanical loading is limited to 2D imaging of lacunae on an exposed optical microscopy plane (Nicolella et al. 2005; Nicolella et al. 2001). This study demonstrated experimentally that applied strains at the whole bone level are amplified in the lacunar matrix (see Figure 2.10) (Nicolella et al. 2006). However, exposing the osteocyte mechanical system through sectioning alters the mechanical behaviour of the environment, and can introduce microcracks through destructive removal of material (Zioupos and Currey 1994). As loading-induced fluid flow is thought to be highly stimulative to osteocytes in vivo, fluorescent tracer studies have been performed to examine the fluid flow through the lacunar-canalicular network under mechanical loading (Knothe Tate et al. 1998a; Knothe Tate et al. 1998b; Price et al. 2011). This allows determination of the fluid flow in the environment surrounding the osteocyte under various conditions, most recently the average fluid velocity and predicted shear stresses within the system (Price et al. 2011).
2.5 Theoretical and Computational Analysis of Bone

2.5.1 Computational Modelling of Whole Bones

Due to the complex material properties and microstructure of bone, computational modelling techniques have been applied to develop a greater understanding of bone mechanical behaviour. Initially, bone was modelled as a homogenous continuum and was used to determine stresses in human bones including the femoral head (Brown and Ferguson 1978), the intervertebral disc (Belytschko et al. 1974) and the tibia (Hayes et al. 1978). The ability to predict the mechanical behaviour of orthopaedic systems without destructive interference led to its rapid adoption in the analysis of macro-scale bone treatment problems, such as bone-prostheses systems, fracture fixation devices and bone-implant interactions (Huiskes and Chao 1983). Computational modelling techniques have facilitated investigation of three major areas in orthopaedic research (Prendergast 1997): (1) design and pre-clinical analysis of prostheses/implants; (2) to investigate time-dependent mechanobiological adaptation tissues; and (3) to characterise the fundamental biomechanics of
musculoskeletal structures. The work of this thesis explores the fundamental mechanobiology of bone cells and hereafter this chapter will expand on current knowledge of the adaptive bone remodelling processes.

### 2.5.2 Adaptive Modelling of Bone

Modelling the adaptive nature of bone has largely been investigated either through bone remodelling algorithms, which seek to simulate the response of bone to modelling, and tissue differentiation algorithms, which attempt to mimic tissue and cellular differentiation in response to mechanical factors. These biological processes are intrinsically linked and as such, while dealt with separately here, information obtained from one method can inform the other.

#### 2.5.2.1 Bone Remodelling Algorithms

Multiple theories have been developed in an attempt to understand bone growth and its relationship with mechanical loading. Adaptive models specifically targeting bone were initially based on either strain stimuli (Cowin and Hegedus 1976; Huiskes et al. 2000) or microdamage stimuli (García-Aznar et al. 2005; Prendergast and Taylor 1994). Beginning with Wolff’s Law, which stated that healthy bone will adapt to the loads under which it is placed, a mechanostat theory was developed by Frost (Frost 1987b, a; Wolff 1986). Based on observed in vivo adaptive behaviour this hypothesis proposed a strain range of 200-1,500 µε, below which bone resorption occurs with bone hypertrophy occurring above this range (Frost 1990a, b; Frost 1997). This was further developed into an adaptive elasticity theory, which determined that mineral density, stiffness or geometry of bone adapts to return to a remodelling equilibrium strain state when deviations from normal loading conditions occur (Cowin and Hegedus 1976). This theory has been able to predict surface remodelling of bone (Cowin and Van Buskirk 1979), and has been expanded upon to successfully predict bone adaptation in both cortical (Beaupré et al. 1990b, a; Weinans et al. 1992) and trabecular bone (Carter et al. 1989; Huiskes et al. 2000), as well as in contact with prostheses (Huiskes et al. 1987).
More recently damage-adaptive laws have been developed based on microdamage stimuli (García-Aznar et al. 2005; Prendergast and Taylor 1994). These theories hypothesise that a homeostatic level of damage is always present in the form of microcracks in bone and that any deviation from this equilibrium state stimulates bone adaptation (Prendergast and Taylor 1994). Application of this theory showed physiological predictions of the remodelling response of bones to torsion loads (see Figure 2.11) (McNamara and Prendergast 2007; Prendergast et al. 1997).

While all of the above models have had success predicting some aspects of bone remodelling, it has been proposed that multiple control stimuli may exist (Carter et al. 1987). Therefore it was postulated that both microdamage and strain stimuli may orchestrate bone remodelling, in order to simultaneously maintain bone mass and prevent fracture (Prendergast and Huiskes 1995b). A recently developed bone remodelling algorithm incorporating both of these aspects, and in which damaged bone is removed when damaged above a critical level, predicted BMU behaviour (McNamara and Prendergast 2007). Most recently, damage and strain have been coupled alongside a spatial influence in order to better predict fracture in trabecular bone (Hambli et al. 2009). Further development of these theories will require a greater understanding of the mechanical stimuli sensed by bone cells in vivo, which this thesis aims to elucidate.
Figure 2.11: Bone remodelling algorithm of bone adaptation and strains in the tissue under various combinations of mechanical stimuli; (i) strain stimulus alone, (ii) micro-damage stimulus alone, (iii) a combination of strain and micro-damage and (iv) a stimulus of either strain or micro-damage (McNamara and Prendergast 2007)

2.5.2.2 Tissue Differentiation Algorithms

Bone remodelling behaviour is an extremely complex activity. While initial attempts were made to characterise this mechanobiological behaviour experimentally in a fracture callus (Goodship and Kenwright 1985), limitations on measurement of the changing stress environment within the fracture callus necessitated the use of computational modelling as a powerful analytical tool to quantify the mechano-regulation of musculoskeletal tissues (Mow and Huiskes 2005). Models were developed by Claes and Heigele, who attempted to quantitatively predict bone tissue formation by incorporation of the phenomena of intramembranous and endochondral ossification (Claes and Heigele 1999; Claes et al. 1998). Thus they proposed the occurrence of intramembranous bone formation at low Cauchy strains and compressive hydrostatic pressures (< 5% and < -0.15MPa respectively) and
endochondral ossification at greater magnitudes (< 15% and > -0.015MPa respectively), with all other conditions assumed to generate connective tissue or fibro-cartilage (see Figure 2.12) (Claes and Heigele 1999; Claes et al. 1998). Finite element models were generated to test these theories and, when compared to histological samples from in vivo experiments, demonstrated the efficacy of these quantitative boundary conditions (Claes and Heigele 1999; Claes et al. 1998). However these models predicted tissue composition from mechanical conditions at a specific point in time and could not simulate the development and progression of tissue differentiation with time.

![Diagram](image-url)

**Figure 2.12:** Mechano-regulation theory controlling tissue differentiation in a fracture callus (Claes and Heigele 1999)

A further set of bi-phasic mechano-regulatory computational models were generated by Prendergast and Huiskes, who employed poroelastic theory originally developed by Biot (Biot 1941) to demonstrate the importance of drag forces in interstitial fluid flow on differentiation along a specific pathway (Prendergast and Huiskes 1995a; Prendergast et al. 1997). This theory was then used to generate a tissue
differentiation algorithm dependent on maximum distortional strain and relative fluid velocity (Huiskes et al. 1997; Van Driel et al. 1998), which was then employed using an iterative finite element model to create the first simulation of the progression of tissue differentiation over time. Later, the incorporation of cells in computational models allowed inclusion of the effect of loading on cell migration, proliferation and apoptosis (Lacroix et al. 2002), as well as calculation of bone resorption (see Figure 2.13A).

**Figure 2.13:** Tissue differentiation algorithms controlled by (A) tissue shear strain and interstitial fluid flow (Lacroix et al. 2002), and (B) oxygen tension and substrate stiffness (CC: calcified cartilage) (Burke and Kelly 2012)

Tissue differentiation algorithms have continued to be developed, investigating the effects of other physiological processes on mechanoregulation. Later developments incorporated the effects of both cell proliferation and cell death (Kelly and Prendergast 2005), successfully predicting cellular differentiation along different pathways (Shapiro et al. 1993). The most recent iterations have represented vasculogenesis using an oxygen tension gradient (see Figure 2.13B) (Burke and Kelly 2012), showing the oxygen availability is a key factor in bone remodelling (Burke et al. 2013).
2.5.3 Modelling of the osteocyte environment

Previous numerical simulations of the osteoblast-osteocyte network have shown that osteocytes, due to their ubiquitous distribution throughout bone, are uniquely placed to act as mechanosensors (Mullender and Huiskes 1997). However, this distribution also results in their location deep within bone tissue. As their mechanical environment is difficult to directly investigate experimentally, researchers have turned to theoretical, numerical and computational modelling techniques to characterise the mechanical stimuli that they sense in vivo. These have been largely concerned with two important aspects of the osteocyte mechanical environment: the structural behaviour of solid components such as the ECM and the dynamics of the fluid within the lacunar-canalicular network. The development of each of these analyses is described here.

2.5.3.1 Theoretical Modelling of the Osteocyte Environment

Mathematical modelling has been applied at the osteonal level to model fluid pressure relaxation (Wang et al. 1999), poroelasticity (Smit et al. 2002) and tracer transport experiments (Wang et al. 2000) within an osteon under cyclic loading. Theoretical models specific to the osteocyte were developed to investigate the canalicular environment, in particular the effect of loading-induced fluid flow along the cell process. Initially, these models predicted the occurrence of pressure-driven interstitial fluid flow under applied global matrix strain (see Figure 2.14A) (Cowin et al. 1995), and resulting shear stresses and streaming potentials on the cell process membrane (Weinbaum et al. 1994; Zeng et al. 1994). Further development led to the inclusion of an internal actin cytoskeleton in the cell process (see Figure 2.14B), with tethering elements anchoring it to the surrounding ECM (You et al. 2001b). This resulted in greatly amplified drag forces and strains to the cell process (You et al. 2001b). A refined three-dimensional theoretical model was then generated, using contemporary experimental data on the flexural rigidity of the PCM tethering elements (You et al. 2004) to further test these results (see Figure 2.14C), and similarly found strain amplification occurring as a result of these tethering elements (Han et al. 2004). The most recent iteration of these models incorporated projections of the ECM (see Figure 2.14D), which had recently been identified and shown to
colocalise with proposed integrin attachments (Wang et al. 2007). Inclusion of these attachments in the model resulted in predicted strains an order of magnitude greater than those induced by the PCM tethering elements alone (Wang et al. 2007).

Figure 2.14: The development of theoretical models of the canalicular environment by Cowin, Weinbaum and coworkers (Cowin et al. 1995; Han et al. 2004; Wang et al. 2007; You et al. 2001b)

2.5.3.2 Structural Modelling of the Osteocyte Environment
Computational modelling has also been used as a tool to characterise the osteocyte mechanical environment (see Figure 2.15). Finite element models at the level of Haversian systems have been applied to investigate microdamage in bone, determining that the accumulation of damage results in changes in strain which could be sensed by the osteocyte (Prendergast and Huiskes 1996). Homogenisation theory has been employed using finite element models to determine the strains in individual lacunae under applied trabecular tissue level strains, showing an amplification occurring (McCreadie and Hollister 1997). Idealised finite element models have also been used to investigate the strain in osteons and Howship’s lacunae during
remodelling, and the action of osteoblasts and osteoclasts during remodelling (Smit and Burger 2000). Discretised osteon models were also developed to investigate localised strain fields under applied osteonal loads, predicting the strain fields around hollow lacunae (Lenz and Nackenhorst 2004).

![Figure 2.15](image)

**Figure 2.15:** Idealised finite element models of (A) strains within an osteon with discrete osteocyte elements (Lenz and Nackenhorst 2004), (B) strains within a Howship’s lacuna (Smit and Burger 2000), (C) strains within the lacunar matrix (Rath Bonivtch et al. 2007) and (D) strains within osteocytes derived from osteon-level strains (Vaughan et al. 2013b)

The first complete three-dimensional idealised finite element model of a whole osteocyte lacuna was later developed, and predicted that a strain amplification factor of 1.26 to 1.52 occurred for an applied global strain of 2,000 µε, increasing to 3 with the inclusion of canaliculi in the simulations (Rath Bonivtch et al. 2007). However, both the analytical studies and this computational approach employed idealised geometries, which do not accurately represent osteocytes in vivo. Information on the mineral and organic composition of bone determined at the nano-structural level has also been applied using finite element techniques to determine the stiffness values.
across multiple scales, allowing characterisation of the microstructural properties of osteons and trabeculae at various mineral volume fractions (Vaughan et al. 2012). Recently, multi-scale modelling techniques have been applied alongside periodic boundary conditions to determine that an inhomogeneous strain field results in osteocytes experiencing a range of microstructural strains under the same loading, depending on their location relative to the microstructural porosities (e.g. Volkmann Canals) (Vaughan et al. 2013b). Moreover, it was found that lamellar orientation can have a significant effect on strain experienced at the cellular level (Vaughan et al. 2013b).

### 2.5.3.3 Fluid Modelling of the Osteocyte Environment

Initially bone was treated as a biphasic continuum, with the application of Biot’s poroelastic theory (Biot 1941; Biot 1955), and these studies predicted that pressure gradients resulting from mechanical loading generated fluid flow around the osteocyte (Piekarski and Munro 1977). Analytical models of idealised osteocyte canaliculi under load-induced fluid flow have been developed and applied to predict the in vivo range for shear stress (0.8-3 Pa) and deformation of osteocyte cell membranes (Han et al. 2004; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994). With the development of tracer transport experiments (Knothe Tate and Knothe 2000) mathematical models were developed to explain the movement of solute through bone despite cyclic fluid movement under loading (Wang et al. 2000), generating much debate about the primary mechanisms of loading-induced fluid flow through the lacunar-canalicular network (Knothe Tate 2001; Wang et al. 2001). Recent numerical models have been developed to determine the effect of the pericellular matrix on flow through the canaliculus, investigating the permeability (Anderson et al. 2008; Lemaire et al. 2012b; Lemonnier et al. 2011), fluid movement (Lemaire et al. 2012a; Lemaire et al. 2006), and electro-chemo-mechanical effects (Lemaire et al. 2008; Sansalone et al. 2012). It has also recently been determined that interstitial fluid viscosity and pericellular matrix permeability are the dominant parameters affecting flow in the lacunar-canalicular network (Sansalone et al. 2012).

Computational finite element modelling techniques have also been applied to idealised models of the lacunar-canalicular system and have predicted abrupt
changes in the drag forces within the canaliculi arising from changes in geometry or proximity to bone microporosity and the Haversian canals (Mak et al. 1997). Similar techniques were applied to characterise loading-induced fluid flow at the level of whole bones (Steck et al. 2003). One study investigated the fluid environment of an idealised osteocyte and predicted high shear stresses within the canaliculi whereas the osteocyte cell body is primarily exposed to hydrodynamic pressure (Anderson et al. 2005). Recent studies identified projections of the extracellular matrix into the pericellular space around the canaliculi (McNamara et al. 2009), and computational studies which incorporate 3D approximations of realistic 2D geometries have suggested that such projections amplify the fluid shear stimulus to the osteocyte (see Figure 2.16a and b) (Anderson and Knothe Tate 2008). Ultra high voltage electron microscopes (UHVEM) have been used to develop highly detailed computational models of 80 nm long sections of osteocyte canaliculi (see Figure 2.16c), and these have been applied to predict that the three-dimensional geometry of the canalicular space greatly affects the velocity of fluid flow around the osteocyte cell processes (Kamioka et al. 2012).

![Computational fluid dynamics models of 3D representative of osteocyte canaliculi, showing (a) axial velocity and (b) shear stress (Anderson and Knothe Tate 2008) and (c) velocity (Kamioka et al. 2012)](figure2.16.png)

**Figure 2.16:** Computational fluid dynamics models of 3D representative of osteocyte canaliculi, showing (a) axial velocity and (b) shear stress (Anderson and Knothe Tate 2008) and (c) velocity (Kamioka et al. 2012)

### 2.5.3.4 Current Computational Challenges

While the models described above provide important insight into the structural or fluid environments of the cell, they do not fully incorporate the intricate architecture of the lacunar-canalicular network surrounding the cell. Furthermore, these studies
model bone cells and tissues by using either solid mechanics approaches (where extracellular fluids were modelled using poroelastic assumptions) or fluid dynamics modelling, wherein all structures were assumed to be rigid. However, bone cells are actually complex materials composed of an elastic cell membrane that deforms under external fluid flow, and modelling of the osteocyte as a rigid body precludes investigation of cellular strains arising from fluid flow (Anderson et al. 2005; Anderson and Knothe Tate 2008; Kamioka et al. 2012). An example of a similarly complex and difficult to investigate environment is the bone marrow cavity, which involves a highly viscous fluid that flows at low velocities under compression of the bone in which it is encased. While detailed CFD analysis has been performed on this complex system previously (Coughlin and Niebur 2012b), fluid structure interaction techniques have recently been applied to determine the complex multi-physics interactions which occur in vivo (Birmingham et al. 2012a). Therefore fully simulating the complex mechanical behaviour of the osteocyte environment represents a most challenging multi-physics problem, which is too complex to solve analytically and requires a fluid-structure interaction (FSI) approach.

2.6 Osteoporosis

2.6.1 Pathophysiology of Osteoporosis

Osteoporosis is a disease of the skeleton in which progressive degradation of both bone mass and bone microarchitecture occurs, with corresponding deterioration in bone mechanical strength. This leads to greater fragility of bones and a consequently higher risk of fracture. Osteoporosis occurs due to an imbalance in the rates of osteoblast and osteoclast activity, causing either disproportionate bone resorption or insufficient new bone formation, as reviewed recently by McNamara (McNamara 2011). This deterioration is most evident in trabecular bone, where resorption can lead to complete perforation of the trabeculae (Mosekilde 1990) and has been shown to reduce the overall mechanical integrity of bones (Aaron et al. 2000) (see Figure 2.17).
Figure 2.17: A scanning electron micrograph of (a) normal bone and (b) osteoporotic bone, with visible degeneration of trabecular struts visible in the diseased state (Ritchie et al. 2009)

The disease of osteoporosis can be classified clinically as primary osteoporosis, which occurs naturally in the body, or secondary osteoporosis, which results from the presence or treatment of pre-existing diseases such as anorexia nervosa, cystic fibrosis and diabetes mellitus type I (Lerner 2006). Primary osteoporosis can be further sub-divided into type I, which describes post-menopausal bone loss in women, and type II, which occurs as part of the natural ageing process. Post-menopausal type I osteoporosis is by far the most common form of the disease and is thought to arise due to deficient oestrogen production after the menopause (Falahati-Nini et al. 2000; Riggs et al. 2002), where a relatively short period of 4-8 years of disproportionate bone remodelling results in substantial trabecular bone loss. Type II osteoporosis sets in more slowly, occurring due to lower testosterone and oestrogen availability in old age and affecting both sexes (Riggs et al. 2002).

Primary osteoporosis becomes increasingly prevalent with age as bone mass, after plateauing up to approximately 30 years (Cowin 2001), begins to degrade slowly with as little as 70% remaining by 70 years (Wasnich 1999). Post-menopausal oestrogen deficiency has been shown to increase bone resorption in women (Riggs et al. 2002), and results in an increased likelihood of developing osteoporosis with approximately 40% of women over 50 at risk (Melton et al. 2005). This bone loss is irreversible, and leads to high risk of fracture for women with below-average initial
bone mass (Favus and Christakos 1996; Frost 1998; Frost 1999; Lindsay 1995; Tomkinson et al. 1997). Indeed, oestrogen levels have also been suggested to play a role in age-related bone loss in men (Favus and Christakos 1996), with up to 30% of men suffering an osteoporotic fracture in their lifetime (Randell et al. 1995). Significantly, sufferers are 50-100% more likely to sustain another separate fracture (Klotzbuecher et al. 2000), while mortality rates increase by 10-20% within a year of an initial fracture (Cummings and Melton 2002; Melton et al. 2005).

The primary concern during osteoporosis is the deterioration of the macroscopic mechanical properties of the whole bone, ultimately resulting in fracture or damage of the bone. Indeed, reductions in the elastic modulus and compressive strength have been observed in human osteoporotic bone at both the whole bone level (Hasegawa et al. 1993) and the trabecular level (Ciarelli et al. 2000; Sugita et al. 1999), when compared with normal bone. The decline in global mechanical behaviour associated with osteoporosis occurs due to a combination of changes in bone mass and bone quality, which together determine the mechanical behaviour of bone (Judex et al. 2003).

Bone mass is known to decrease following oestrogen deficiency (Lane et al. 1998), and as such, has been identified as a critical marker in the development of bone fractures. The standard quantitative measurement of the mass of a bone is bone mineral density (BMD) (Kanis et al. 2002), with a score below 2.5 standard deviations (T-score) of a young adult diagnosing osteoporosis (Kanis et al. 2003). However, BMD has been shown to be a poor predictor of bone fractures (Dempster 2000), with only 10-53% of post-menopausal fractures in patients over 65 having an osteoporotic BMD score (Garnero and Delmas 2004; Stone et al. 2003; Wainwright et al. 2005). Therefore, it is thought that assessment of the quality of the remaining bone tissue could explain this discrepancy.

There are two major determining factors of bone quality. The first is bone micro-architecture, the micro-scale organisation and morphology of both trabecular and cortical bone (McNamara 2010), and this is known to change dramatically due to bone loss during osteoporosis (Compston et al. 1989; Lane et al. 1998; Parfitt 1987). Interestingly, recent investigations of trabecular bone during oestrogen deficiency have observed gradual increases in trabecular thickness (Waarsing et al. 2004;
Waarsing et al. 2006) and apparent consolidation of trabecular architecture (Waarsing et al. 2006). This suggests a form of remodelling response to osteoporotic conditions, and incorporation of these disease-related changes in trabecular micro-architecture in computational models have improved fracture predictions by 13% compared to predictions based on BMD alone (Testi et al. 2001).

The second important determinant of bone quality is tissue composition, the ratio of the mineral phase, which governs the stiffness of the bone tissue, to the organic phase, which contributes to the strength and flexibility of bone. In particular, mineralisation of bone tissue is known to correlate strongly to bone strength and stiffness (Currey 1984) and therefore, observed changes in micro-structural tissue properties likely arise from altered mineral content (McNamara et al. 2006). In an animal model of osteoporosis it was shown that bone tissue from osteoporotic bone had a more heterogeneous mineral distribution than healthy bone, both within individual trabeculae (Brennan et al. 2011a; Ciarelli et al. 2003) and across different regions of the bone (see Figure 2.18) (Brennan et al. 2011a). This increased heterogeneity likely accounts for the observed osteoporotic changes in the micro-structural mineral content of bones, which has been shown to both increase (Boyde et al. 1998; Dickenson et al. 1981; McNamara et al. 2006; Zioupos and Aspden 2000) and decrease (Gadeleta et al. 2000) in diseased conditions. Changes in mineral content such as these may thus explain the fact that, despite a decrease in overall mineral density, there is an increase in the yield strength and elastic modulus of the remaining bone in osteoporosis of 40-90% when compared to controls (McNamara et al. 2006).
Figure 2.18: (a) Diagram of a proximal femur showing three different regions. (b) Increased mineral heterogeneity is across these regions in oestrogen deficiency is shown, with (c) the heterogeneous mineral distribution within an individual trabecular sample also visible (Brennan et al. 2011a)

The effects of oestrogen deficiency on osteoclasts are wide-ranging and have been explored in detail, and are indicative of the physiological processes that occur in cells during osteoporosis. Significantly, increases in the activation frequency of BMUs, and consequently bone turnover rate, have been observed during oestrogen deficiency (Brockstedt et al. 1993; Eriksen et al. 1999). While all bone cell types are known to possess oestrogen receptors (Braidman et al. 2001a), oestrogen acts through multiple pathways to affect the behaviour of different cell lineages. Oestrogen is known to have an inhibiting effect on bone resorption by reducing the formation of mature osteoclasts (Oursler et al. 1991). Therefore, oestrogen withdrawal gives rise to the recruitment of more osteoclasts (Rosen 2000) and a reduction in osteoclast apoptosis (Hughes et al. 1996), causing extended periods of resorption and subsequent bone perforation (Bell et al. 1996). Furthermore, oestrogen deficiency is known to result in increased apoptosis of both osteoblasts and osteocytes (Emerton et al. 2010; Kousteni et al. 2001; Tomkinson et al. 1997). This oestrogen deficiency-related apoptosis in osteocytes has also been suggested to result in hyper-mineralisation of the surrounding bone tissue (Boyde 2003; Frost 1960b; Kingsmill and Boyde 1998) and changes to the organisation of the osteocyte network (Knothe Tate et al. 2004; Sharma et al. 2012), which is thought to indicate a
reduction in bone production per osteoblast during osteoporosis (Mullender et al. 1996). Moreover, it has recently been observed that the effect of oestrogen on osteoclasts can in fact be regulated by osteoblasts (Michael et al. 2005), suggesting that the relationship between oestrogen deficiency and osteoporosis is dependent upon a complex multi-cellular network of behaviour that requires further study to elucidate fully (McNamara 2010).

2.6.2 Bone Cell Mechanobiology during Osteoporosis

The mechanobiology of bone cells during osteoporosis is poorly understood. However, as these cells regulate healthy bone remodelling in response to mechanical loading, it is likely that mechanotransduction in bone cells is crucial to understanding the pathophysiology and treatment of osteoporosis (Chen et al. 2010; McNamara 2010). It has been suggested that the presence of oestrogen allows the normal response of osteoblasts and osteocytes to mechanical loading (Lanyon 1996). This theory is reinforced by evidence that osteoblastic cells display defective responses to mechanical loading in vitro during oestrogen deficiency (Jessop et al. 2004; Sterck et al. 1998). Indeed, oestrogen deficiency has been shown to affect osteocytes in vivo, causing apoptosis (Kousteni et al. 2001; Tomkinson et al. 1997) and reductions in cell density (Mullender et al. 1996). These effects suggest that oestrogen deficiency may alter the mechanosensitivity of bone tissue during osteoporosis (Tatsumi et al. 2006).

In addition to effects on bone mechanosensitivity, osteoporosis is known to change the mechanical environment of osteocytes. Indeed, oestrogen deficiency in post-menopausal osteoporosis results in rapid bone loss, as well as changes in the bone strength and mineral density (Brennan et al. 2011a; Brennan et al. 2011b; Brennan et al. 2012; Compston et al. 1989; Lane et al. 1998; McNamara et al. 2006; Parfitt 1987). This can also manifest as distorted organisation of the osteocyte cell network in humans (Knothe Tate et al. 2004), and increased surface roughness of the surrounding lacunae and canaliculi (see Figure 2.19) (Sharma et al. 2012). This may indicate the occurrence of osteocyte osteolysis, wherein osteocytes are proposed to directly alter their surrounding mineralised matrix (Teti and Zallone 2009). This may also be degradation of the lamina limitans, an organic layer which surrounds the cells
(Scherft 1972), plays an important role in both physicochemical fluid interactions and fluid flow in the pericellular space (Knapp et al. 2002; Reilly et al. 2001), and is thought to be absent during bone formation (Takagi et al. 1991). Both of these processes may occur as a mechanobiological response by the cell to osteoporotic changes.

**Figure 2.19:** Sample TEM images of the surface of the lacuna in (a) a control (b) an ovariectomised rat model of osteoporosis (Sharma et al. 2012). Histology of the osteocyte network in health and disease (c) (Knothe Tate et al. 2004)

While never before observed, these microstructural changes in composition and microarchitecture likely alter the mechanical environment of cells embedded in osteoporotic bone. This might provoke a mechanobiological response, particularly in osteocytes, which have been shown to modify their mechanosensing focal adhesions, their material properties and their mechanosensitivity in response to changes in their mechanical environment (Bacabac et al. 2008; Vatsa et al. 2008). Moreover, a mechanobiological response to compensate for bone loss could explain the increases in stiffness and yield strength which have been observed to occur in osteoporosis despite deterioration in macroscopic bone properties. This thesis sets out to investigate this proposed phenomenon in detail, with the aim of elucidating the role of osteocyte mechanobiology in the pathogenesis of osteoporosis.
2.7 Summary

This chapter has presented a detailed review of bone physiology and anatomy, as well as the mechanobiology of its constituent cells, in both health and disease. In summary, bone is an active material which, through the process of remodelling, is capable of adapting itself in response to mechanical loading. This adaptive nature is orchestrated by osteocytes, with the coupled activities of bone-forming osteoblasts and bone-resorbing osteoclasts replacing old and damaged bone. While the mechanobiology of these cells is crucial to understanding this process, their complex closed mechanical environments are challenging to investigate experimentally. Therefore the precise mechanical stimuli that bone cells sense in vivo remain unknown and, as such, computational modelling techniques can provide greater insight into this environment. Furthermore, during osteoporosis oestrogen deficiency results in deteriorated macroscopic bone properties, bone architecture, reduced bone mass and changes in tissue composition. These changes likely alter the mechanical environment of bone cells, possibly inducing a mechanobiological response, although this remains to be confirmed.

In Chapters 4 and 5 a detailed computational investigation of the geometry, solid mechanics and fluid dynamics of the multi-physics osteocyte environment is performed, as it is the most mechanosensitive and influential cell in osteogenesis. Then, in Chapter 6, a novel experimental loading method is devised to study the mechanical environments of bone cells directly, and to elucidate the effect of osteoporosis on the mechanical stimuli they experience. Finally, a study is conducted in Chapter 7, which applies the models developed in Chapters 4 and 5 and alongside the data obtained in Chapter 6, in order to explore the effects of osteoporosis on various aspects of the osteocyte’s mechanical environment.
Chapter 3

Theory

3.1 Introduction
The theoretical basis for the computational models developed in Chapters 4, 5 and 7, as well as the image analysis in Chapter 6, are outlined in this chapter. Firstly, an overview of continuum mechanics is provided, alongside the theoretical framework and governing equations for the finite element theory, in Section 3.3. Secondly, fluid dynamics theory and the requisite governing equations are considered in Section 3.4. In Section 3.5, the theoretical basis and coupling of these techniques using fluid-structure interaction modelling is also expounded. The digital image correlation theory applied in Chapter 6 is presented in Section 3.6, and finally the elastostatics theory, employed to validate the experimental approach, is described in Section 3.7.

3.2 Notation
Here an introduction to the notation used in the current chapter is provided. Tensors and matrices are given capital letters while vectors are given lowercase letters. Bold type face font is applied to vectors, tensors and matrices, and their component parts are shown in italics. Index notation is used to illustrate manipulation of these quantities. For index notation a repeated index implies a summation. The coordinate system uses the axes \( x_1, x_2 \) and \( x_3 \), or \( x_i \) where \( i = 1, 2, 3 \) (summation over values of the indices from 1 to 3). Index notation summation convention is shown below using the dot product (also known as the scalar or inner product) of two vectors in three dimensions \( (u, v) \), which is the summation of the component parts.
Second order tensors are represented by nine components in 3D. Each component is denoted by two subscripts that define the location in the tensor. For example, the second order tensor $A$ in component form is $A_{ij}$, where the subscripts define the location within the tensor at row $i$ and column $j$. A fourth order tensor is defined as the linear tensor function of a second order tensor. For example, the linear elastic constitutive law relates the second order stress tensor, $\sigma$, and strain tensor, $\varepsilon$, through the fourth order elastic modulus tensor, $C$, as follows:

$$\sigma_{ij} = C_{ijkl}\varepsilon_{kl}$$  \hspace{1cm} (3.2)

### 3.3 Finite element theory and formulations

#### 3.3.1 Continuum Mechanics

The computer simulations in Chapter 4 and Chapter 7 are based in a finite deformation kinematic framework, which is outlined below. Similarly, all models in this thesis are assumed to exhibit linear elastic, isotropic behaviour, and thus this material model is described here.

#### 3.3.1.1 Fundamental Principles and Theoretical Formulations

Large deformation kinematics describes the deformation of a body by following its movement from an initial state to a deformed state, which are commonly referred to as the ‘reference configuration’ and ‘current configuration’ of that body respectively (Fagan 1992). This is represented in Figure 3.1 with position vectors, $\mathbf{x}$ and $\mathbf{y}$ used to define the location of a material point in the reference (position vector $\mathbf{x}$) and current configurations (position vector $\mathbf{y}$).
Figure 3.1: Schematic diagram of finite deformation kinematics, where $V_0$ is a reference volume that changes to a current volume $V$, and $u$ is a displacement vector of a material point defined by vectors $x$ and $y$ (Fagan 1992). P and Q are points which are mapped to p and q in the current volume, allowing characterisation of deformation of the body.

The displacement vector, $u$, of the material point is represented by:

$$u(x, t) = y(x, t) - x$$  \hspace{1cm} (3.3)

The velocity vector, $v$, is the time derivative of $u$ with respect to time, $t$:

$$du = v(x, t) = \frac{du}{dt}$$  \hspace{1cm} (3.4)

The deformation gradient tensor, $F$, relates the distance between two neighbouring material points to that in the reference configuration.

$$dy = F \cdot dx$$  \hspace{1cm} (3.5)
The Jacobian, $J$, the total volume change, is used to quantify volume changes in the deformation of the body, expressed as the determinant of the deformation gradient $F$ as in equation (3.7).

$$J = \det(F)$$

(3.7)

From the deformation gradient (equation (3.6)), the spatial velocity gradient, $L$ can be defined as:

$$L = \dot{F} \cdot F^{-1}$$

(3.8)

The spatial velocity gradient, $L$, can be decomposed into a symmetric rate of deformation tensor, $D$, and an asymmetric spin tensor, $W$. The spin tensor, $W$, is a measure of the average angular velocity associated with the deformed configuration. The rate of deformation tensor, $D$, is a measure of the strain rate associated with the deformed configuration and is from the spatial velocity gradient, $L$, as:

$$D = \text{sym}(L) = \frac{1}{2} (L + L^T)$$

(3.9)

$$W = \text{sym}(W) = (L - L^T)$$

(3.10)

The deformation tensor, $D$, can be integrated with respect to time to give the logarithmic strain tensor, $\varepsilon$, a measure of finite strain, calculated as:

$$\varepsilon(t) = \int_0^t D \, dt$$

(3.11)

In the present work the Cauchy stress, or true stress, $\sigma$, is the primary measure of stress and is defined as the force per unit area on the current (deformed) configuration.
3.3.1.2 Material Constitutive Theory

A continuum constitutive elasticity formulation is required to predict elastic
deformation under complex loading and boundary conditions. The strain tensor, $\varepsilon$, in equation 3.11 is symmetric whereby $\varepsilon_{ij} = \varepsilon_{ji}$ and therefore only six independent strain components are needed to represent a strain state. This can be written as:

$$\varepsilon_{ij} = \begin{bmatrix} \varepsilon_{11} & \varepsilon_{12} & \varepsilon_{13} \\ \varepsilon_{21} & \varepsilon_{22} & \varepsilon_{23} \\ \varepsilon_{31} & \varepsilon_{32} & \varepsilon_{33} \end{bmatrix}$$  \hspace{1cm} (3.12)

The primary measure of stress used in ABAQUS and in this thesis is the Cauchy, or “true” stress, $\sigma$. The three dimensional stress state of a material can be represented by the Cauchy stress tensor, $\sigma$ (i.e. $\sigma_{ij}$) as follows:

$$\sigma_{ij} = \begin{bmatrix} \sigma_{11} & \sigma_{12} & \sigma_{13} \\ \sigma_{21} & \sigma_{22} & \sigma_{23} \\ \sigma_{31} & \sigma_{32} & \sigma_{33} \end{bmatrix}$$  \hspace{1cm} (3.13)

The stress tensor is also symmetric, whereby $\sigma_{ij} = \sigma_{ji}$, therefore only six independent stress components needed to represent a stress state.

3.3.2 Finite element method

The finite element method is applied in software such as the ABAQUS package used in this thesis, to solve the constitutive equations of the material under various boundary conditions (ABAQUS 2011). The first step in FE analyses is the division of a structure into an assembly of subdivisions, known as elements. These elements are interconnected at nodes. A given input variable is assumed to act over each element in a predefined manner, with the number (i.e. mesh density) and type of elements chosen such that the variable distribution throughout the whole body is adequately approximated. The distribution across each element can be predefined by a polynomial (e.g. linear or quadratic) or a trigonometric function.
After the problem has been discretised, the governing equations for each element are calculated and assembled to give the system equations (Fagan 1992). These individual element equations generally take the form

$$ [k] \{U\} = \{F\} $$  \hspace{1cm} (3.14)

where $[k]$ is a square matrix, known as a stiffness matrix; $\{U\}$ is the vector of (unknown) nodal displacements; and $\{F\}$ is the vector of applied nodal forces. The requisite element and system equations can be derived for a four-noded tetrahedral element, with displacements $\{u\}$ calculated as:

$$ \{u\} = [N]\{U\} $$  \hspace{1cm} (3.15)

where $[N]$ is a shape function matrix. The general form of the shape functions is given by:

$$ N_\beta = \left(a_\beta + b_\beta x + c_\beta y + d_\beta z\right)/6V \hspace{1cm} \beta = i, j, k, l $$  \hspace{1cm} (3.16)

where $V$ is the total volume of the element and the constants $a, b, c$ and $d$ are functions of the nodal coordinates. The $[B]$ matrix relates the strains experienced in an element to the nodal displacements, and for a three-dimensional analysis all six strain components are required (Fagan 1992). Considering strain $\varepsilon_x$ in the $x$ direction, the variation in displacement is given by:

$$ u = N_i u_i + N_j u_j + N_k u_k + N_l u_l $$  \hspace{1cm} (3.17)

Therefore the strain $\varepsilon_x$ is:

$$ \varepsilon_x = \frac{\partial u}{\partial x} = \frac{\partial}{\partial x} \left(N_i u_i + N_j u_j + N_k u_k + N_l u_l \right) $$

$$ = \left(b_i u_i + b_j u_j + b_k u_k + b_l u_l \right)/6V $$  \hspace{1cm} (3.18)

Similar derivations can be performed for the other two direct strains and the shear strains, which can be summarised as follows:
The material property matrix \([D]\) for an isotropic material is:

\[
\left[\begin{array}{cccccc}
\varepsilon_x & 0 & 0 & b_j & 0 & 0 \\
0 & c_i & 0 & 0 & c_j & 0 \\
0 & 0 & d_i & 0 & d_j & 0 \\
\end{array}\right] = \frac{1}{6V} \left[\begin{array}{cccccc}
\delta & b_i & 0 & c_j & 0 & d_j \\
0 & c_i & b_i & 0 & c_k & 0 \\
d_i & 0 & b_i & d_k & 0 & d_k \\
\end{array}\right] \left[\begin{array}{c}
u_i \\
v_i \\
v_i \\
\end{array}\right] = [B][U] 
\] (3.19)

Thus, the stiffness matrix for the element can be calculated from:

\[
[D] = \frac{E}{(1+\nu)(1-2\nu)} \left[\begin{array}{cccc}
1-\nu & \nu & \nu & 0 \\
\nu & 1-\nu & \nu & 0 \\
\nu & \nu & 1-\nu & 0 \\
0 & 0 & 0 & 1-2\nu \\
0 & 0 & 0 & \frac{1-2\nu}{2} \\
0 & 0 & 0 & \frac{1-2\nu}{2} \\
\end{array}\right] 
\] (3.20)

This theory is applied using both ABAQUS in Chapter 4 and ANSYS in Chapter 5, as part of fluid-structure interaction (FSI) simulations, in order to model the osteocyte mechanical environment.

### 3.4 Computational fluid dynamics theory and formulations

The computer simulations in Chapter 5 and Chapter 7 are based on a two-way coupled structural and fluid dynamics framework. The following section elaborates on the theoretical background of CFD as a basis for the FSI analyses described in the next section.


3.4.1 Mass conservation principle and continuity equation

Fluid flow and transport phenomena are governed by basic conservation principles such as conservation of mass, momentum and energy. All these conservation principles are solved according to the fluid mechanics theory, which gives a set of partial differential equations, called the governing equations of the fluid.

The mass conservation principle states that the rate of increase of mass in a fluid element is equal to the net rate of flow of mass into a fluid element. Applying this physical principle to a fluid model results in a differential equation called continuity equation (Malalasekra 2007). This is derived by first defining the rate of increase of mass in the fluid element:

\[
\frac{\partial}{\partial t} (\rho \delta x \delta y \delta z) = \frac{\partial \rho}{\partial t} \delta x \delta y \delta z
\]  
(3.22)

where \( \rho \) denotes density and \( t \) is time. The mass flow rate across a face of an element is defined as the product of area and the velocity component normal to the face (see Figure 3.2). Fluids entering the element are given a positive sign as they increase the mass of the system, while those leaving are given a negative sign.

Figure 3.2: Mass flows in and out of a discrete fluid element (Malalasekra 2007)

Thus the net rate of flow of mass across the boundaries of an element is given by:
\[
(pu - \frac{\partial (pu)}{\partial x} \frac{1}{2} \delta x) \delta y \delta z - (pu + \frac{\partial (pu)}{\partial x} \frac{1}{2} \delta x) \delta y \delta z +

(pv - \frac{\partial (pv)}{\partial y} \frac{1}{2} \delta y) \delta x \delta z - (pv - \frac{\partial (pv)}{\partial y} \frac{1}{2} \delta y) \delta x \delta z +

(pw - \frac{\partial (pw)}{\partial z} \frac{1}{2} \delta z) \delta x \delta y - (pw - \frac{\partial (pw)}{\partial z} \frac{1}{2} \delta z) \delta x \delta y
\] (3.23)

where \(dx/dt = u, dy/dt = v, \) and \(dz/dt = w.\) The rate of increase of mass (equation 3.22) is now equated to the net rate of flow of mass into the element across its faces (equation 3.23). If the expression is divided by the volume of the element, \(\delta x \delta y \delta z,\) the following expression is obtained:

\[
\frac{\partial \rho}{\partial t} + \frac{\partial (\rho u)}{\partial x} + \frac{\partial (\rho v)}{\partial y} + \frac{\partial (\rho w)}{\partial z} = 0
\] (3.24)

which can be expressed as:

\[
\frac{\partial \rho}{\partial t} + div(\rho u) = 0
\] (3.25)

This is the continuity equation for a compressible fluid, where the first term of the equation is the rate of change of density with respect to time and the next term is net flow of mass out of the element boundaries. The analyses performed in this thesis involved liquids, which are modelled as incompressible fluids, and allow simplification of equation 3.25 as follows:

\[
div(u) = 0
\] (3.26)

or more clearly:

\[
\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0
\] (3.27)
3.4.2 Newton’s second law and momentum equation

Newton’s second law states that the rate of change of momentum of a fluid particle is equal to the sum of the forces acting on a particle. Using equation 3.25, the rate of increase of $x$-, $y$-, and $z$-momentum of a fluid particle can be expanded as:

\[
\rho \frac{Dx}{Dt} = \frac{\partial (\rho u)}{\partial t} + \text{div} (\rho u u) \tag{3.28}
\]

\[
\rho \frac{Dy}{Dt} = \frac{\partial (\rho v)}{\partial t} + \text{div} (\rho v u) \tag{3.29}
\]

\[
\rho \frac{Dz}{Dt} = \frac{\partial (\rho w)}{\partial t} + \text{div} (\rho w u) \tag{3.30}
\]

where $D$ is the total or substantive derivative, also known as the Lagrangian derivative. The forces acting on a body are a combination of both surface and body forces. The state of stress of a fluid element is defined in terms of the pressure, a normal stress denoted by $p$, and the nine shear stress components, denoted by $\tau$, shown in Figure 3.3. The usual suffix notation $\tau_{ij}$ is applied to indicate the direction of the shear stresses. The suffices $i$ and $j$ in $\tau_{ij}$ indicate that the stress component acts in the $j$-direction on a surface normal to the $i$-direction.

![Figure 3.3: (A) Stress components on three faces of a fluid element and (B) stress components in the $x$-direction (Malalasekra 2007)](image-url)
Considering the \( x \)-components of the forces due to pressure \( p \) and stress components \( \tau_{xx}, \tau_{yx} \) and \( \tau_{zx} \), (see Figure 3.3), the net force in the \( x \)-direction is the sum of the force components acting in that direction on the fluid element. To provide the total force per unit volume due to surface stresses, these forces are summed and divided by the volume \( \delta x \delta y \delta z \):

\[
\frac{\partial (-p+\tau_{xx})}{\partial x} + \frac{\partial \tau_{yx}}{\partial y} + \frac{\partial \tau_{zx}}{\partial z}
\]

(3.31)

The overall effect of body forces, such as gravity, can be included by defining a source \( S_{Mx} \) of \( x \)-momentum per unit volume per unit time. The \( x \)-component of the momentum equation is found by setting the rate of change of \( x \)-momentum of the fluid particle (3.28) equal to the total force in the \( x \)-direction on the element due to surface stresses (3.31), plus the rate of increase of \( x \)-momentum due to sources:

\[
\rho \frac{Du}{Dt} = \frac{\partial (-p+\tau_{xx})}{\partial x} + \frac{\partial \tau_{yx}}{\partial y} + \frac{\partial \tau_{zx}}{\partial z} + S_{Mx}
\]

(3.32)

Similarly for the \( y \)- and \( z \)-directions:

\[
\rho \frac{Dv}{Dt} = \frac{\partial \tau_{xy}}{\partial x} + \frac{\partial (-p+\tau_{yy})}{\partial y} + \frac{\partial \tau_{zy}}{\partial z} + S_{My}
\]

(3.33)

\[
\rho \frac{DW}{Dt} = \frac{\partial \tau_{xz}}{\partial x} + \frac{\partial \tau_{yz}}{\partial y} + \frac{\partial (-p+\tau_{zz})}{\partial z} + S_{Mz}
\]

(3.34)

When this law is applied for Newtonian fluid (shear stress is proportional to the rates of deformation) the resulting equations are called Navier-Stokes equations. The three-dimensional form of Newton’s law of viscosity for compressible flows involves two constants of proportionality: the first (dynamic) viscosity, \( \mu \), to relate stresses to linear deformations, and the second viscosity, \( \lambda \), to relate stresses to the volumetric deformation. The nine viscous stress components, of which six are independent, are

\[
\tau_{xx} = 2\mu \frac{\partial u}{\partial x} + \lambda \text{div}(\mathbf{u})
\]

(3.35)

\[
\tau_{yy} = 2\mu \frac{\partial v}{\partial y} + \lambda \text{div}(\mathbf{u})
\]

(3.36)
\[ \tau_{zz} = 2\mu \frac{\partial w}{\partial z} + \lambda \text{div}(\mathbf{u}) \]  
\hfill (3.37)

\[ \tau_{xy} = \tau_{yx} = \mu \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial x} \right) \]  
\hfill (3.38)

\[ \tau_{xz} = \tau_{zx} = \mu \left( \frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} \right) \]  
\hfill (3.39)

\[ \tau_{yz} = \tau_{zy} = \mu \left( \frac{\partial v}{\partial z} + \frac{\partial w}{\partial y} \right) \]  
\hfill (3.40)

If the above equations for viscous stress components (equation 3.35-3.40) are substituted into equations 3.32-3.34 the Navier-Stokes equations are derived as:

\[ \rho \frac{Du}{Dt} = -\frac{\partial p}{\partial x} + \frac{\partial}{\partial x} \left[ 2\mu \frac{\partial u}{\partial x} + \lambda \text{div}(\mathbf{u}) \right] + \frac{\partial}{\partial y} \left[ \mu \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial x} \right) \right] + \frac{\partial}{\partial z} \left[ \mu \left( \frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} \right) \right] + S_{Mx} \]  
\hfill (3.41)

\[ \rho \frac{Dv}{Dt} = -\frac{\partial p}{\partial x} + \frac{\partial}{\partial x} \left[ \mu \left( \frac{\partial u}{\partial y} + \frac{\partial v}{\partial x} \right) \right] + \frac{\partial}{\partial y} \left[ 2\mu \frac{\partial v}{\partial y} + \lambda \text{div}(\mathbf{u}) \right] + \frac{\partial}{\partial z} \left[ \mu \left( \frac{\partial v}{\partial z} + \frac{\partial w}{\partial y} \right) \right] + S_{My} \]  
\hfill (3.42)

\[ \rho \frac{Dw}{Dt} = -\frac{\partial p}{\partial x} + \frac{\partial}{\partial x} \left[ \mu \left( \frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} \right) \right] + \frac{\partial}{\partial y} \left[ \mu \left( \frac{\partial v}{\partial z} + \frac{\partial w}{\partial y} \right) \right] + \frac{\partial}{\partial z} \left[ 2\mu \frac{\partial w}{\partial z} + \lambda \text{div}(\mathbf{u}) \right] + S_{Mz} \]  
\hfill (3.43)

As the fluid is incompressible, these equations can be simplified and rearranged into the most useful form for implementation in the finite volume method:

\[ \rho \frac{Du}{Dt} = -\frac{\partial p}{\partial x} + \text{div}(\mu \text{grad } u) + S_{Mx} \]  
\hfill (4.44)

\[ \rho \frac{Dv}{Dt} = -\frac{\partial p}{\partial y} + \text{div}(\mu \text{grad } v) + S_{My} \]  
\hfill (4.45)

\[ \rho \frac{Dw}{Dt} = -\frac{\partial p}{\partial z} + \text{div}(\mu \text{grad } w) + S_{Mz} \]  
\hfill (4.46)
where \( \text{grad} \) is defined as a vector having coordinate components that are the partial derivatives of a function with respect to its variables. Combining these equations with 3.28-3.30 gives us equations which fully explain the momentum conservation principle, and govern the flow of a compressible Newtonian fluid (Malalasekra 2007):

\[
\frac{\partial (\rho u)}{\partial t} + \text{div}(\rho uu) = -\frac{\partial p}{\partial x} + \text{div}(\mu \text{grad} u) + S_{Mx} \tag{3.47}
\]

\[
\frac{\partial (\rho v)}{\partial t} + \text{div}(\rho vu) = -\frac{\partial p}{\partial y} + \text{div}(\mu \text{grad} v) + S_{My} \tag{3.48}
\]

\[
\frac{\partial (\rho w)}{\partial t} + \text{div}(\rho wu) = -\frac{\partial p}{\partial z} + \text{div}(\mu \text{grad} w) + S_{Mz} \tag{3.49}
\]

where \( \rho \) represents the density, \( \mathbf{u} \) represents velocity vector, \( u, v, w \) are the velocity components in Cartesian coordinate system, \( \mu \) is the dynamic viscosity and \( S_m \) represents the momentum source term. Since the studies performed in this thesis do not involve heat transfer, the energy equation is not considered here.

### 3.4.3 Finite volume method

The finite volume method is applied in CFD codes, such as the ANSYS software package used in this thesis, to solve the governing equations of the fluid under various boundary conditions (ANSYS 2011). The basic and foremost step of CFD is to mesh the region of interest by dividing it into smaller regions called control volumes, with the calculated scalar values stored at the centre of these volumes. The governing equations can be described with the general transport equation as follows (Malalasekra 2007):

\[
\frac{\partial (\rho \phi)}{\partial t} + \text{div}(\rho \mathbf{u} \phi) = \text{div}(\Gamma \text{grad} \phi) + S_{\phi} \tag{3.50}
\]
where the variable $\phi$ can be replaced by any scalar quantity and $\Gamma$ is the diffusion coefficient. The left hand side of the equation contains the rate of change term and convective term, whereas the diffusive term and source term lie on the right hand side of the equation. This is then integrated over the control volumes and Gauss’ divergence theorem is applied to give (Malalasekra 2007):

$$\frac{\partial}{\partial t} \int_{C_\nu} \rho \phi dV + \int_A n (\rho \phi u) dA = \int_A n (\Gamma \text{ grad } \phi) dA + \int_{C_\nu} S_\phi dV \quad (3.51)$$

This results in the conversion of the general transport equation into a system of algebraic equations, which are solved by iteration to converge upon a solution (Malalasekra 2007).

### 3.5 Fluid-structure interaction modelling

The computational modelling of biological cells and tissue using solid mechanics and finite element approaches, either neglect the presence of biological fluids or represent extracellular fluids using poroelastic assumptions. For specific fluid dynamics modelling of such problems, biological tissues are assumed to be rigid for the purposes of understanding fluid flow and shear stresses. In reality, biological cells and tissues are composed of elastic solids (e.g. cell membranes, collagen) that deform in response to the external fluid flow imposed by mechanical loading. To fully simulate this behaviour represents a challenging multi-physics problem, which recent advances in computational resources and software can now model. Fluid-structure interaction (FSI) tools can simulate the interaction between deformable structures and adjacent fluid flows. The computer simulations in Chapter 5 and Chapter 7 are based in a combined fluid–solid interaction framework, which is outlined further below.
3.5.1 Theoretical background
As multi-physics problems are challenging to solve by analytical methods, they must be solved either by using numerical simulations or experiments. Two different approaches exist for solving FSI problems using commercial software packages: the monolithic approach and the partitioned approach.

3.5.1.1 Monolithic approach
This approach involves the formulation of both sub-problems (fluid and structure) into one combined problem. The system of algebraic equations resulting from both systems of governing equations, described in Sections 3.3 and 3.4, are solved as a whole (Hou et al. 2012; Richter 2010). The interaction of fluid and structure at the interface is treated synchronously. This allows the conservation of properties at the interface, increasing the stability of the solution. The monolithic approach is described in the flow process diagram in Figure 3.4, where $S^f$ and $S^s$ denote the solutions of the fluid and solid domains respectively, at times $t_n$ and $t_{n+1}$.

![Flow process diagram of the monolithic approach](image)

**Figure 3.4:** Flow process diagram of the monolithic approach, where $S^f$ and $S^s$ denote the solutions of the fluid and solid domains respectively, at times $t_n$ and $t_{n+1}$.

While this approach is considered to be more robust than the partitioned approach, it is computationally expensive and cannot take the advantage of software modularity as the partitioned method does.

3.5.1.2 Partitioned approach
The other choice for solving FSI problems is the partitioned method. Here, both sub problems are solved separately i.e. the fluid flow does not change while the structural solution is calculated. The equations governing the flow and the
displacement of the structure are solved in time, alternating between two distinct solvers. The intermediate fluid solution is prescribed as a boundary condition for the structure and vice versa, and the iteration continues until the convergence criterion is satisfied. At the interface (boundary between fluid and solid), the exchange of information occurs according to the type of coupling technique applied (Hou et al. 2012; Richter 2010). The process of the partitioned method is described in Figure 3.5, using the same notation as detailed in the previous approach.

As mentioned in the above paragraph, the information is exchanged at the interface between two solvers. This process is defined as the coupling, and can be of two types: one-way coupling and two-way coupling.

### 3.5.2 One-way coupling

One-way coupling is applied if the motion of a fluid flow influences a solid structure but the reaction of a solid upon a fluid is negligible (Benra et al. 2011), or vice versa. The one-way coupling method is explained in Figure 3.6.

Initially, the fluid flow calculation is performed until convergence is reached. Then the resulting forces at the interface from fluid calculation are interpolated to the structural mesh. Next, the structural dynamic calculations are performed until the
convergence criterion is met. As a consequence the mesh is deformed according to the response of structure. This is repeated until the end time is reached.

![Flow process diagram of the one-way coupling procedure](image)

**Figure 3.6:** Flow process diagram of the one-way coupling procedure (Benra et al. 2011)

### 3.5.3 Two-way coupling

This type of coupling is applied to problems where the motion of a fluid influences a solid structure and, at the same time, the flow of fluid is influenced by the reaction of the solid.

The process of the strongly coupled two-way algorithm is shown in Figure 3.7. During the first time step converged solutions of the fluid calculations (equations 3.35-3.40) provide the forces acting on the solid body. Then the forces are interpolated to the structural mesh, similar to the one-way coupling, and the solution from the structural solver is obtained with these fluid forces as boundary conditions. As a consequence the mesh is deformed according to the response of the structure. These displacement values are then interpolated back to the fluid mesh, which results in deformation of the fluid domain. This is modelled using a mesh diffusion scheme and, through displacement of the fluid-solid interface, maintains separation of the
Eulerian fluid domain from the Lagrangian solid domain. The fluid solver then begins, and this process is repeated until both force and displacement values at the fluid solid interface are converged below a pre-determined limit (Benra et al. 2011), set to 1 per cent in the simulations in this thesis (ANSYS 2011). This allows a fully implicit solution of both solid and fluid domains within each staggered iteration step.

![Diagram](image)

**Figure 3.7:** Flow process diagram of the two-way coupling procedure (Benra et al. 2011)

Both one-way and two-way coupling are applied in the fluid-structure interaction modelling performed in this thesis, with the modelling methodology described in detail in Chapters 5 and 7.

### 3.6 Digital image correlation theory

In Chapter 6, an image-based method is used to experimentally quantify strains experienced by individual bone cells in situ under mechanical loading, using previously developed software package (MOIRE) (Luu et al. 2011; Pan et al. 2010a;
Pan et al. 2010b). This image analysis is performed by digital image correlation (DIC), an optical technique that combines image registration and tracking methods for accurate 2D measurements of changes in images. Correlation theories for the measurement of alterations in data were first applied to digital images in 1975 (Keating et al. 1975). These theories have been optimised in recent years to apply to numerous applications (Pan et al. 2009b), including confocal microscopy (Berfield et al. 2006). DIC is based upon the calculation of a correlation coefficient that is determined from pixel intensity array subsets on multiple corresponding images and extracting the deformation mapping function that relates the images.

Two-dimensional DIC involves the tracking of the same points (or pixels) in two different images, before and after deformation (see Figure 3.8). The displacements of a point \( P \) are calculated by creating a square reference subset of \((2M + 1) \times (2M +1)\) centred at point \( P(x_0, y_0) \) on the “undeformed image”, which is then used to track its corresponding location in the “deformed image”. The degree of similarity between the reference subset and the deformed subset is calculated by determining a cross-correlation coefficient. The peak value of the correlation is obtained, and thus the position of the deformed subset is resolved. The differences in the positions of these two points yield the in-plane displacement vector at point \( P \) (see Figure 3.8).

![Diagram of DIC](image)

**Figure 3.8**: Diagram of (A) a reference square subset before deformation and (B) a deformed subset after deformation (Pan et al. 2009b)

In order to separate out the deformation from the displacement of the “deformed image”, a point \( Q(x_i, y_j) \) around the centre point \( P(x_0, y_0) \) of the reference subset is chosen. This point can be mapped to point \( Q'(x'_i, y'_j) \) of the deformed subset
according to the shape function (also known as a displacement mapping function) (Schreier and Sutton 2002):

\[ \begin{align*}
\xi'_i &= x_i + \xi(x_i, y_j) \\
y'_j &= y_j + \eta(x_i, y_j)
\end{align*} \quad (i, j = -M; M) \tag{3.52} \]

Assumption of rigid body translation, where displacement of all points is the same, allows determination of the displacements using a zero-order shape function as follows:

\[ \begin{align*}
\xi_0(x_i, y_j) &= u \\
\eta_0(x_i, y_j) &= v
\end{align*} \tag{3.53} \]

In order to capture the shape change of the deformed subset, a first-order shape function that allows translation, rotation, shear, normal strains and a combination of these can be applied:

\[ \begin{align*}
\xi_1(x_i, y_j) &= u + u_x \Delta x + u_y \Delta y \\
\eta_1(x_i, y_j) &= v + v_x \Delta x + v_y \Delta y
\end{align*} \tag{3.54} \tag{3.55} \]

Finally, second-order shape functions can be employed to characterise more complex deformation of the deformed subset (Lu and Cary 2000):

\[ \begin{align*}
\xi_2(x_i, y_j) &= u + u_x \Delta x + u_y \Delta y + \frac{1}{2} u_{xx} \Delta x^2 + \frac{1}{2} u_{yy} \Delta y^2 + u_{xy} \Delta x \Delta y \\
\eta_2(x_i, y_j) &= v + v_x \Delta x + v_y \Delta y + \frac{1}{2} v_{xx} \Delta x^2 + \frac{1}{2} v_{yy} \Delta y^2 + v_{xy} \Delta x \Delta y
\end{align*} \tag{3.56} \tag{3.57} \]
For equations 3.54 - 3.57, \( \Delta x = x_i - x_0 \), \( \Delta y = y_j - y_0 \), \( u \) and \( v \) are the \( x \)- and \( y \)-directional displacement components of the reference point \( P(x_0, y_0) \). \( u_x, u_y, v_x, v_y \) are the first order displacement gradients of the reference subset and \( u_{xx}, u_{xy}, v_{xx}, v_{yy}, v_{xy} \) are the second-order displacement gradients of the reference subset.

As mentioned above, a cross-correlation (CC) coefficient is defined in advance of a DIC analysis in order to evaluate the degree of similarity between a reference image and a deformed image (Giachetti 2000; Tong 2005). This can be defined by the following equation:

\[
C_{CC} = \sum_{i=-M}^{M} \sum_{j=-M}^{M} [f(x_i, y_j)g(x'_i, y'_j)]
\]  

(3.58)

However, the CC criterion has been shown to be sensitive to lighting fluctuations, including offset and linear scale in illumination lighting (Pan et al. 2007). Therefore a normalised cross-correlation (NCC) has been developed, which is insensitive to linear scale issues and is described as:

\[
C_{NCC} = \sum_{i=-M}^{M} \sum_{j=-M}^{M} \frac{f(x_i, y_j)g(x'_i, y'_j)}{\bar{f} \bar{g}}
\]  

(3.59)

where:

\[
\bar{f} = \sqrt{\sum_{i=-M}^{M} \sum_{j=-M}^{M} [f(x_i, y_j)]^2}
\]  

(3.60)

\[
\bar{g} = \sqrt{\sum_{i=-M}^{M} \sum_{j=-M}^{M} [g(x'_i, y'_j)]^2}
\]  

(3.61)

Finally, a further refined criterion known as the zero-mean normalised cross-correlation (ZNCC) coefficient has been developed in order to deal with sensitivity to offset of lighting. As such the ZNCC is insensitive to all fluctuations in lighting and offers the most robust and noise-proof method for performing DIC (Pan et al. 2009b; Pan et al. 2007). The relationship can be summarised as follows:
where:

\[ f_m = \frac{1}{(2M+1)^2} \sum_{i=-M}^{M} \sum_{j=-M}^{M} f(x_i, y_j) \]  
(3.63)

\[ g_m = \frac{1}{(2M+1)^2} \sum_{i=-M}^{M} \sum_{j=-M}^{M} g(x'_i, y'_j) \]  
(3.64)

\[ \Delta f = \sqrt{\sum_{i=-M}^{M} \sum_{j=-M}^{M} [f(x_i, y_j) - f_m]^2} \]  
(3.65)

\[ \Delta g = \sqrt{\sum_{i=-M}^{M} \sum_{j=-M}^{M} [g(x'_i, y'_j) - g_m]^2} \]  
(3.66)

Lastly, in order to calculate the strain distribution a local least-squares fitting technique, the most practical and efficient method for strain estimation (Pan et al. 2009a; Pan et al. 2007; Wattrisse et al. 2001), is applied to a square window of \((2m + 1) \times (2m + 1)\) points. Therefore:

\[ u(i, j) = a_0 + a_1 x + a_2 y \]  
(3.67)

\[ v(i, j) = b_0 + b_1 x + b_2 y \]  
(3.68)

where \(i, j = -m: m\) are the local coordinates within the strain calculation window, \(u(i, j)\) and \(v(i, j)\) are the original displacements obtained by DIC, and \(a_{i=0,1,2}, b_{j=0,1,2}\) are unknown polynomial coefficients which need to be determined. Rewriting equations 3.67 and 3.68 in matrix form:
Similarly for \( b_0, b_1 \) and \( b_2 \). Thus, the least-squares method provides the unknown polynomial coefficients \( a_i=0,1,2 \) and \( b_j=0,1,2 \), allowing calculation of the Cauchy strains or Green strains resulting in characterisation of the strain field in the deformed image being analysed (Pan et al. 2009b; Pan et al. 2007):

\[
\begin{bmatrix}
1 & -m & -m \\
1 & -m+1 & -m \\
\vdots & \vdots & \vdots \\
1 & 0 & 0 \\
1 & m-1 & m \\
1 & m & m
\end{bmatrix}
\begin{bmatrix}
a_0 \\
a_1 \\
a_2
\end{bmatrix}
=
\begin{bmatrix}
u(-m,-m) \\
u(-m+1,-m) \\
\vdots \\
0 \\
u(m-1,m) \\
u(m,m)
\end{bmatrix}
\tag{3.69}
\]

In a 2D analysis these values can be applied to give the maximum principal strain as follows:

\[
\varepsilon_x = \frac{\partial u}{\partial x} = a_1
\tag{3.70}
\]

\[
\varepsilon_y = \frac{\partial v}{\partial y} = b_2
\tag{3.71}
\]

\[
\gamma_{xy} = \left( \frac{\partial u}{\partial y} \right) + \left( \frac{\partial v}{\partial x} \right) = a_2 + b_1
\tag{3.72}
\]

\[
\varepsilon = \frac{\varepsilon_x + \varepsilon_y}{2} + \sqrt{\left( \frac{\varepsilon_x - \varepsilon_y}{2} \right)^2 + \left( \frac{\gamma_{xy}}{2} \right)^2}
\tag{3.73}
\]

This DIC theory is applied in Chapter 6 to investigate the strains experienced by bone cells in situ using a previously developed software package (MOIRE) (Luu et al. 2011; Pan et al. 2010a; Pan et al. 2010b).
3.7 Theory of elastostatics of a spherical inclusion in homogenous biological media

In order to validate the strain observed in bone cells experimentally, a validation study is performed in Chapter 6 to investigate strains within a spherical object inside a homogenous medium. Fluorescent microspheres are embedded in a solid resin, which is then imaged under loading and analysed using DIC. These results are then compared to an analytical model of a spherical inclusion in homogenous biological media, the theory of which is described hereafter.

Previously, analytical models have been created of incompressible, homogenous tissues under two-dimensional plane-strain (Kallel and Bertrand 1996; Skovoroda et al. 1994) or plane-stress assumptions (Sumi et al. 1995). In a study by Bilgen and Insana, these tissue models are developed into a three-dimensional problem to describe a soft or stiff spherical inclusion embedded in a uniform matrix (Bilgen and Insana 1998). An analytical model relates the elasticity of the inclusion, the boundary conditions set by the external compression and the tissue dimensions to the strain and stress distributions throughout the media.

The spherical inclusion is assumed to be bonded to the surrounding matrix (see geometry in Figure 3.9A) i.e. the displacements and stresses are uniform across the boundary between the inclusion and the surrounding matrix. Figure 3.9 displays a cylindrical volume of height $2Z$ and diameter $2W$, with a spherical inclusion of radius $a$. A static compressive force $T$ is applied through a radius $R$ at both the top and bottom of the cylinder. The matrix and inclusion are assumed to be homogenous and isotropic elastic in nature, with elastic shear moduli described by $\mu$ and Poisson’s ratios by $\nu$. The parameters relating to the matrix and the inclusion are denoted by subscripts $b$ and $t$, respectively. Finally, small levels of deformation are assumed to occur such that a linear elastostatic analyses can be performed.
This provides an analytical solution for the component strains and component stresses along the $y$ and $z$ axes for an applied compression $T$. The solutions within the spherical inclusion are spatially constant and are as follows:

\[ \varepsilon_{yy}^t = \frac{T(1-v_b)}{2(1+v_b)} \left( -\frac{5(1-v_b)}{\mu_b(7-5v_b)+\mu_t(8-10v_b)} + \frac{(1-2v_t)}{\mu_b(2-4v_t)+\mu_t(1+v_t)} \right) \]  
(3.74)

\[ \varepsilon_{zz}^t = \frac{T(1-v_b)}{2(1+v_b)} \left( -\frac{10(1-v_b)}{\mu_b(7-5v_b)+\mu_t(8-10v_b)} + \frac{(1-2v_t)}{\mu_b(2-4v_t)+\mu_t(1+v_t)} \right) \]  
(3.75)

\[ \sigma_{yy}^t = 2\mu_t \left\{ \left(\frac{1+v_t}{1-2v_t}\right)H - 2F \right\} \]  
(3.76)

\[ \sigma_{zz}^t = 2\mu_t \left\{ \left(\frac{1+v_t}{1-2v_t}\right)H + 4F \right\} \]  
(3.77)

The corresponding solutions for the surrounding matrix also depend on the coordinate variables $y$ and $z$, and are as follows:
The constants $A$, $B$, $C$, $H$ and $F$ are determined by applying the above solutions to an infinite medium, i.e. $Z = W = \infty$, that is loaded uniformly i.e. $R = \infty$. By employing a classical approach, in which symmetrical nature of the displacements about the loading axis is applied to derive axially symmetric solutions (Goodier 1933), these constants are calculated as follows:

\begin{align*}
A &= \frac{T a^3 [(1-\nu_b+2\nu_c)\mu_b + (1-2\nu_b+\nu_c)\mu_t + 2\nu_b\nu_c(\mu_b-\mu_t)]}{6\mu_b(1+\nu_b)(2-4\nu_c)\mu_b + (1+\nu_c)\mu_t} \\
B &= \frac{T a^5(\mu_b-\mu_t)}{8\mu_b[(7-5\nu_b)\mu_b + (8-10\nu_b)\mu_t]} \\
C &= \frac{5Ta^3(\mu_b-\mu_t)(1-2\nu_b)}{8\mu_b[(7-5\nu_b)\mu_b + (8-10\nu_b)\mu_t]} \\
H &= \frac{T(1-\nu_b)(1-2\nu_b)}{2(1+\nu_b)(2-4\nu_c)\mu_b + (1+\nu_c)\mu_t} \\
F &= \frac{5T(1-\nu_b)}{4[(7-5\nu_b)\mu_b + (8-10\nu_b)\mu_t]} \\
\end{align*}

For a finite but relatively large height $Z$, an equivalent uniform displacement $U$ on the compressor can be written in terms of $T$ as follows:

\begin{equation}
U = \frac{TZ}{E} 
\end{equation}
where \( E \) denotes Young’s modulus, which can be related to the shear modulus \( \mu \) via:

\[
E = 2(1 + \nu)\mu \tag{3.88}
\]

Therefore \( T \) can be expressed as follows:

\[
T = \frac{\mu}{Z} \{ 2(1 + \nu)\mu \} \tag{3.89}
\]

Inserting this into equation 3.74 gives a relationship between the applied displacement and strain in the \( x \) and \( y \) directions within the spherical inclusion:

\[
\epsilon^t = \left( \frac{\mu}{Z} \right) \left( \frac{2\mu_b(1+\nu_b)(1-\nu_b)}{2(1+\nu_b)} \right) - \frac{5(1-\nu_b)}{\mu_b(7-5\nu_b) + \mu_t(8-10\nu_t)} + \frac{(1-2\nu_t)}{\mu_t(2-4\nu_t) + \mu_b(1+\nu_t)} \tag{3.90}
\]

This theory will be employed in Chapter 6 to validate the strains measured by the DIC method.

### 3.8 Summary

The theory outlined Sections 3.3-3.5 in this chapter formed the basis of computational simulations of the mechanical environment of the osteocyte, using an FE approach in Chapter 4 and an FSI approach in Chapter 5. The models developed using both techniques were applied in Chapter 7 to investigate the effects of osteoporosis on mechanical stimulation of osteocytes. Similarly, the theory developed in Sections 3.6 and 3.7 was employed in Chapter 6 to experimentally investigate the strain stimulation experienced by osteocytes, in both health and osteoporosis.
Chapter 4

Strain Amplification in Bone
Mechanobiology: A Computational
Investigation of the In Vivo Mechanics of Osteocytes

4.1 Introduction

As described in Chapter 2, osteocytes have long been regarded to be the primary mechanosensors of bone, due to their ubiquitous spatial distribution throughout bone and their extensive communication network, facilitated by cell processes that extend to other osteocytes and osteoblasts. In this chapter, an exploration of the mechanical environment surrounding the osteocyte in vivo was carried out, with the aim of elucidating the mechanical stimuli sensed by osteocytes in an accurate representation of in vivo lacunar-canalicular architecture. In particular, an attempt was made to resolve the apparent paradox that osteoblastic cells do not generate an appreciable anabolic response below approximately 10,000 με (You et al. 2000; Burger and Veldhuijzen 1993) despite the yield strain of bone observed at approximately 3,500-4,000 με (Carter et al. 1987; Mosley 2000). However, as osteocytes are embedded in a mineralised matrix, direct experimental studies of these cells within their native environment are challenging. At present high resolution imaging of the osteocyte environment under mechanical loading has been limited to 2D imaging of sections through the lacunae on an exposed optical microscopy plane (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001; Schneider et al. 2010).
Researchers have sought to overcome experimental challenges by developing idealised theoretical models of osteocyte canaliculi and predicted the approximate levels of shear stress (0.8-3Pa) at the cell membrane under load-induced fluid flow (Han et al. 2004; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994). In particular, these studies have shown that loading induced fluid drag on the pericellular matrix (PCM), and projections of the extracellular matrix (ECM) (McNamara et al. 2009), might amplify the strain experienced at the cell membrane to levels that initiate a biochemical response (Han et al. 2004; Wang et al. 2007; You et al. 2001b). Computational modelling has also been used as a tool to characterise the osteocyte mechanical environment, with idealised models of a whole osteocyte lacuna predicting a strain amplification factor of 1.26 to 1.52 for an applied global strain of 2,000 µε, increasing to 3 with the inclusion of idealised canaliculi in the simulations (Rath Bonivtch et al. 2007). However, both the analytical studies and this computational approach employed idealised geometries, which do not accurately represent osteocytes in vivo. Computational fluid dynamics models of individual canaliculi were developed using 3D axisymmetric models generated from 2D transmission electron microscopy images of osteocyte canaliculi, predicting that the canalicular geometry has a profound effect on shear stress experienced by the cell process (Anderson and Knothe Tate 2008). However, the lacunar and cell body geometry were not included in these models and, while the role of fluid flow was considered, direct mechanical stimulation of the osteocyte by the surrounding matrix components was not investigated. Therefore, detailed geometrically accurate models are required to fully understand the role of the complex 3D geometry of the lacunar-canalicular network on the mechanical stimulation experienced by osteocytes under physiological loading.

Therefore, the hypothesis that “Mechanical loading to the osteocyte is amplified by the native geometry of the osteocyte environment” was tested in this chapter. The objectives of the study in this chapter were (1) to develop solid models of osteocytes that closely represent their geometry in vivo and (2) to use computational methods to predict the loading conditions experienced by osteocytes during normal physiological activities. Confocal image-derived models were developed of an osteocyte and its immediate surroundings. These studies were compared to idealised models of osteocytes to examine the effect of confocal image-derived geometries on
strain predicted in the cell. The role of the pericellular matrix and extracellular matrix projections as strain amplifiers in osteocyte mechanobiology were also investigated. This chapter presents an adapted version of work previously published in the Journal of the Royal Society Interface (Verbruggen et al. 2012).

4.2 Materials and Methods

4.2.1 Confocal Imaging of osteocytes

To visualise osteocytes within their native environment fluorescent staining of the lacunar-canicular network and confocal imaging was performed. Thick transverse sections (400 µm) of the tibia of a male, 6-8 month old Sprague-Dawley rat were cut using a diamond blade saw (Isomet, Buehler) and then fixed in 4% paraformaldehyde for 24 hours at room temperature. Sections were then dehydrated in ascending graded ethanol (75%, 95%, and 100%, 5 minutes each). These sections were stained with FITC (Fluorescein Isothiocyanate isomer I, Sigma-Aldrich) to visualise the pericellular space, similar to the methods of Ciani et al. (2009). The samples were placed in a rotator (Agar Scientific) with FITC solution at 1% in ethanol and mixed for 4 hours at room temperature. The sections were then rinsed in 100% ethanol for 30 minutes, air dried and mounted on coverslips using DPX Mounting Media (Sigma-Aldrich). Samples and containers were covered in aluminium foil at all times to prevent photobleaching.

Confocal scans were taken using a confocal microscope (Zeiss LSM 51) with a 40x oil immersion lens, 1.25 numerical aperture, laser wavelength excitation of 488 nm and the pinhole set at 1 Airy unit. All scans were taken at a resolution of 2048 x 2048 pixels giving a field of view of 255 µm. An example of these images can be seen in Figure 4.1a, 4.1b, where lacunae can clearly be seen oriented concentrically around a Haversian canal. A z-stack was obtained at increments of 0.41 µm using laser scanning mode through the depth of the section.
Figure 4.1: (a) Confocal microscopy scan of the lacunar–canalicular network with 
(b) an individual osteocyte, (c) a finite element volume mesh of the osteocyte

4.2.2 Solid model/Mesh generation

Finite element models were constructed of confocal image-derived and idealised 
cells which included the cell membrane, pericellular matrix and the extracellular 
matrix.

4.2.2.1 Confocal image-derived models

Materialise MIMICS image processing software was employed to generate a three-
dimensional solid model. Confocal image stacks of four osteocyte lacunae were 
imported into MIMICS and thresholded to between -884 and -769 Hounsfield units. 
Thresholding allowed segmentation of the lacunar–canalicular space from the 
surrounding matrix. The lacunar geometries generated were similar to a slightly 
flattened ellipsoid with major axes ranging between 14-16 µm and minor axes of 
8.5-9.5 µm. The canaliculi generated had an average diameter of 0.5-0.7 µm. These 
models were then meshed using Materialise 3-Matic voxel-meshing software. A 
Boolean subtraction was performed to construct the calcified extracellular matrix 
(ECM). In addition, the mesh was offset by 0.08 µm to create a pericellular space 
and a pericellular matrix (PCM) of the same thickness was constructed by Boolean
subtraction. A representative geometry of the ECM, PCM and osteocyte in a confocal image-derived model is shown in Figure 4.2a. These geometries were then meshed using 4-noded C3D4 tetrahedral elements, see Figure 4.1c, and exported to ABAQUS finite element software.

In simulations where the PCM was removed, the ECM volume was increased to fill the space and contact the cell. These geometries were then meshed using 4-noded C3D4 tetrahedral elements.

**Figure 4.2:** Models of (a) confocal image-derived and (b) idealised osteocytes depicting extracellular matrix (blue), pericellular matrix (orange), and osteocyte cell body (green), with ECM projections visible longitudinally in (c) and in cross-section in (d)

### 4.2.2.2 Idealised model

A solid model of an idealised osteocyte lacuna was developed for comparison using ABAQUS finite element software. The lacuna was modelled as an ellipsoid with minor and major axes equal to 9 and 15 µm respectively, which were taken as the median dimensions from the confocal scans (Lin and Xu 2011). The canaliculi were modelled as channels in the ECM with a diameter of 0.6 µm. The osteocyte was also modelled as an ellipsoid with minor and major axes of 7.5 and 13.5 µm respectively, allowing for a surrounding PCM with a thickness of 0.75 µm (McNamara et al.)
2009; You et al. 2004). The cell processes were created by offsetting from the canaliculi by 0.08 µm (Wang et al. 2005). The PCM represented the entire space between the ECM and the osteocyte, and in simulations where the PCM is removed to examine the effect of its absence the ECM is enlarged to fill this space and contact the osteocyte. The geometries of the ECM, PCM and osteocyte are displayed in Figure 4.2b. These geometries were then meshed using 4-noded C3D4 tetrahedral elements.

4.2.2.3 Idealised model with ECM projections

In order to investigate the effect of ECM projections on strain transfer into the osteocyte, an idealised model was developed that included conical ECM protrusions, of height 0.08 µm and base radius 0.1 µm, which projected into the PCM space (Wang et al. 2005). The projections were organised in groups of four about the axis of the canaliculi, spaced 1 µm apart along its length (see Figure 4.2c, 4.2d). While the distance between adjacent projections has been observed to be approximately 0.1 µm (McNamara et al. 2009), spacing of 1 µm was selected in order to reduce the computing power required and to better observe the localised effects of individual projections.

4.2.3 Finite Element Analysis:

4.2.3.1 Material Properties and Loading

All structures were modelled as linearly elastic, isotropic materials. The viscoelastic nature of the cell was neglected for simplicity as physiological loading of bones occurs at a frequency of approximately 1 Hz, well below the relaxation time of 41.5 s measured in bone cells (Appelman et al. 2011; Darling et al. 2008). An elastic modulus of 16 GPa and Poisson’s ratio of 0.38 were attributed to the ECM (Deligianni and Apostolopoulos 2008). At present there is no experimental data available to define the material properties of the PCM surrounding the osteocyte. Therefore the properties of chondrocyte PCM were assumed, with an elastic modulus of 40 kPa and Poisson’s ratio of 0.4 (Alexopoulos et al. 2003; Alexopoulos et al. 2005). A modulus of 4.47 kPa and Poisson’s ratio of 0.3 was attributed to the osteocyte cell body and processes (Sugawara et al. 2008). Tie constraints were
applied to attach the PCM to the ECM and osteocyte where direct contact (i.e. no space between the components) occurred at their respective surfaces. Similarly, when ECM projections were included in the idealised model they were also tied directly to the osteocyte cell membrane.

Finite element simulations were conducted to investigate the strain experienced at the cellular level under global loading conditions representing under-loading (500 µε), homeostatic loading (1,500 µε) and loading during physical exertion (3,000 µε) (Burr et al. 1996). A displacement boundary condition was applied to model faces to generate compressive loads of 500, 1,500 and 3,000 µε globally. Uniaxial ramped static loading was applied symmetrically, with opposing faces constrained symmetrically to prevent rigid body motion. Periodic boundary conditions were not applied in these models as the realistic geometries are representative of individual cells, rather than repeating representative volume elements (RVE). Thus periodic boundary conditions were not appropriate, and were also not applied to the idealised models to maintain identical loading conditions. A strain amplification factor was calculated for each osteocyte as the maximum principal strain in the cell divided by the applied global strain.

4.3 Results

The finite element contour plots of the confocal image-derived and idealised cell models in Figure 4.3 and 4.5 show areas of each cell, which experience maximum and minimum principal strains within specific intervals of microstrain respectively, under global applied loading of 3,000 µε. Qualitatively, it can be seen from these images that the both the maximum and minimum principal strains are greater in the realistic models (a-d) than in the idealised models (e, f).

For an applied load of 500 µε, representing low levels of physiological loading, more than 99% of confocal image-derived and idealised osteocyte volumes experienced strains lower than 1,000 µε. Maximum principal strains of 3,800-3,900 µε (3,858 ± 49 µε) were experienced in the confocal image-derived cells, while 1,100 µε was the maximum strain predicted in the idealised model.
Figure 4.3: Maximum principal strain distribution in four confocal image-derived models of osteocytes (a-d) and idealised osteocytes without ECM projections (e), and with ECM projections included (f) under global loading of 3,000 με.

Figure 4.4: Image of confocal image-derived osteocytes (a) and (b) loaded at 3,000 με, with indentations circled showing presence of ECM projections and resulting maximum principal strain concentration.
Figure 4.5: Minimum principal strain distribution in four confocal image-derived models of osteocytes (a-d) and idealised osteocytes without ECM projections (e), and with ECM projections included (f) under global loading of 3,000 $\mu$e.

Figure 4.6: Image of confocal image-derived osteocytes (a) and (b) loaded at 3,000 $\mu$e, with indentations circled showing presence of ECM projections and resulting minimum principal strain concentration.
Homeostatic physiological loading of 1,500 µε resulted in 2-13% of the volumes of confocal image-derived osteocytes stimulated above 2,000 µε, while this figure was just 1% for the idealised model. Maximum principal strains predicted in confocal image-derived osteocytes were 11,000-13,000 µε (11,933 ± 612 µε), with 3,300 µε being the maximum strain experienced by the osteocyte in the idealised model. While maximum strains in confocal image-derived models were above 10,000 µε, these were predicted in a negligible proportion (less than 0.001%) of the models.

Figure 4.7: Maximum principal strain distribution in confocal image-derived (a-d, as in Figure 4.3) and idealised osteocytes (e-f in Figure 4.3) subjected to 3,000 µε compression. Results show idealised models both with and without ECM projections.

The strain distribution as a percentage volume of the osteocyte cell body is shown in Figure 4.7, with element volumes and strain values at element centroids organised into bands to clearly show the proportions of the cells that are well in excess of 3,000 µε. For an applied load of 3,000 µε, representing vigorous physiological activity, the confocal image-derived models predicted strain values above 3,500 µε throughout 7-28% of the cell body, whereas only 0.1% of the volume of the idealised osteocytes
experienced strain above this level, see Figure 4.7. Maximum principal strains predicted in the confocal image-derived osteocytes were 23,000-26,000 µε (24,233 ± 1,033 µε), compared to a maximum strain of 6,600 µε in the idealised model. The strains observed in the models are used to calculate a strain amplification factor of 7.7-8.9 for the confocal image-derived models. The strain amplification factor for the idealised model without the ECM projections was 2.2. All four confocal image-derived osteocyte models experienced strains greater than 10,000 µε in approximately 1% of the cell volume when 3,000 µε global loading was applied. However, the idealised model without the projections did not see these strains. In addition a slight decrease in the volume of the PCM, of 0.0836-0.1191% (0.0986 ± 0.0144%), was also observed during loading in all models.

**Figure 4.8:** Maximum principal strain distribution in confocal image-derived (a-d, as in Figure 4.3) and idealised osteocytes subjected to 3,000 µε compressive loading, without a pericellular matrix
Figure 4.9: Minimum principal strain distribution in confocal image-derived (a-d, as in Figure 4.5) and idealised osteocytes (e-f in Figure 4.5) subjected to 3,000 με compression. Results show idealised models both with and without ECM projections.

Figure 4.10: Minimum principal strain distribution in confocal image-derived (a-d, as in Figure 4.5) and idealised osteocytes subjected to 3,000 με compressive loading, without a pericellular matrix.
Similar results were observed for minimum principal strains, shown in Figure 4.9, with much greater strain stimulation of realistic osteocytes above 3,500 µε compared to idealised osteocytes, for an applied global load of 3,000 µε. Minimum principal strains predicted in the confocal image-derived osteocytes were 20,000-26,000 µε, compared to a minimum principal strain of 5,800 µε in the idealised model. Similar to the results for maximum principal strains, all four confocal image-derived osteocyte models experienced strains greater than 10,000 µε in approximately 1% of the cell volume when 3,000 µε global loading was applied, while this was not observed in the idealised model without the ECM projections.

Evidence of the presence of ECM projections was detected in the confocal image-derived models constructed from confocal images, as depicted in Figure 4.4 and 4.6, (a) and (b). Indentations indicating the presence of ECM projections are highlighted, with strain concentrated in these areas on the cell process. Maximum strain levels predicted around these points ranged from 4,500 µε to 5,000 µε, resulting in amplification of the applied loading by 50-67%. This generated a strain amplification factor of 1.5-1.67. The idealised model that includes the ECM projections (Figure 4.2f) shows more concentrated strain in the cell processes than the idealised model without these (Figure 4.2e). It can be seen from the graph in Figure 4.7 that in the idealised loading case of 3,000 µε, the inclusion of ECM projections resulted in a 0.25% increase in the proportion of the osteocyte experiencing high strain levels (>3,500 µε). Maximum strains localised around the ECM projections in the idealised model were 6,600-12,600 µε, which is 220-420% higher than the applied global loading. This resulted in a strain amplification factor for the model of 4.2. Simulations of the confocal image-derived and idealised models without a pericellular matrix were performed. The results of the 3,000 µε loading case are shown in Figure 4.8 and 4.10. It can be seen that 1-17% of the confocal image-derived models experience strain levels above 3,500 µε, while the idealised model senses this strain in less than 0.1% of its volume. Maximum principal strains in the confocal image-derived models without a PCM were found to be 29,000-69,000 µε (57,925 ± 16,630 µε), while a maximum strain of 7,000 µε was predicted in the idealised model without a PCM. The strain amplification factors for these models were calculated as 9.8-23.3 for the confocal image-derived models and 2.3 for the idealised model. Similar results were observed for minimum principal strains (see
Figure 4.10), with values of 33,000-60,000 µε and 8,000 µε for realistic and idealised models respectively, in the absence of a PCM.

4.4 Discussion

In this study geometrically accurate three-dimensional computational models of osteocytes in their native environment were developed to study in detail the effects of physiological loading on the mechanical stimuli experienced by the osteocyte. The results show for the first time that even under strains of 3,000 µε, 7-28% of the volume of the osteocyte experiences strains in excess of 3,500 µε, and indeed 1% of an osteocyte volume experiences strains in excess of 10,000 µε with maximum principal strains of 23,000-26,000 µε. Minimum principal strains were also investigated due to the applied compressive loading, with similar results observed in both realistic and idealised geometries. In contrast elevated strains (> 3500 µε) were predicted in only 1% of the idealised model under similar loading conditions, with a maximum principal strain of 6,600 µε. The strain amplification factor was shown to be 3.5-4 times higher in confocal image-derived models compared to an idealised model. Most notably, while all osteocyte models experienced strains above the 5,000 µε level, which generates some biological response in vitro, only the confocal image-derived models predicted maximum strains well in excess of 10,000 µε, which is the approximate strain stimulus necessary to elicit a significant osteogenic response in osteocytes (Burger and Veldhuijzen 1993). These findings corroborate those of Anderson et al. (Anderson and Knothe Tate 2008), demonstrating the importance of accurate 3D geometrical modelling when simulating the osteocyte in vivo mechanical environment, and the strain amplification effect of the lacunar-canalicular architecture. The results of this study also served to reinforce two theories, (1) the PCM plays an important role in osteocyte mechanical stimulation (Burra et al. 2010; Burra et al. 2011; Reilly et al. 2003a) and (2) ECM projections act to amplify matrix strains at the cell membrane (Wang et al. 2007).

It must be noted that a number of assumptions were made in the development of these models. Firstly the ECM, PCM and cell materials were assumed to be linear elastic isotropic materials, and material properties were assumed based on values available for cultured osteocytes and chondrocytes (Alexopoulos et al. 2003;
Alexopoulos et al. 2005; Deligianni and Apostolopoulos 2008; Sugawara et al. 2008). For the purposes of this study it was impractical to include an advanced material model due to the highly non-linear nature of the geometry, which resulted in 4.5 million elements and an average computational solving time of 12 hours. The mechanical behaviour of osteocytes in vivo have not been characterised at this scale as direct analysis of such a closed biological system has proven unfeasible. Furthermore, the ECM is composed of collagen fibrils and mineral crystals that are intricately organised within this matrix and have been shown to affect stress distribution about the lacuna (Currey 2003; Ascenzi et al. 2008; Hofmann et al. 2006). It would therefore be more appropriate to model the ECM as an anisotropic or transversely isotropic material (Neil Dong and Edward Guo 2004; O'Mahony et al. 2001). It should also be noted that the distance between the osteocyte and the ECM was assumed to be constant at 0.08 µm in the confocal imaged-derived models. This is due to the fact that confocal microscopy is limited by the wavelength of light (~488 nm), which is much greater than this gap size (~80 nm). Therefore, as this gap cannot be imaged using confocal resolution, data from much higher resolution TEM studies are used. However, it is not expected that in vivo the pericellular space is a uniform thickness as tracer studies and TEM imaging have shown average values 0.078 µm ± 0.038 µm (McNamara et al. 2009; Wang et al. 2005; You et al. 2004). Due to the experimental challenges of obtaining the precise size of the gap at all locations surrounding the cell body, it was necessary to make this assumption. However, it is important to note that an average gap size was assumed whereas in reality certain regions would be in closer proximity to the ECM. At such locations strain transfer to the osteocytes would be increased, as is clearly demonstrated by the results of the idealised ECM projections study.

Furthermore, the cell body and membrane are highly non-linear materials (Lim et al. 2006), and the internal actin cytoskeleton in particular plays an integral role in cell mechanics by actively adapting in response to applied mechanical forces (Ofek et al. 2010; McGarry et al. 2009; McGarry 2009). An analysis of transversely isotropic cells was also performed in order to investigate the effect of anisotropy of the cell on the strain stimulus to its membrane. The results of this analysis show that there is a 3.7-12.2% increase in maximum principal strains experienced by a transversely isotropic osteocyte compared to an isotropic model. Therefore this analysis further
supports the conclusion that the inclusion of an actin cytoskeleton to accurately reflect the anisotropy in vivo would enhance the strain amplification which occurs due to the geometry of the lacunar-canalicular network.

A final limitation of these models is the absence of an active actin cytoskeleton. Future studies should incorporate this behaviour in order to provide more realistic simulations of osteocyte mechanobiology in vivo (Han et al. 2004). However, the overall focus was on the role of geometric changes and based on these studies it is expected that the strain amplification effect observed here would be more pronounced if an active actin cytoskeleton and a fluid-saturated PCM were included.

While previous computational research has examined idealised models of the osteocyte mechanical environment in 3D (McCreadie and Hollister 1997; Rath Bonivtch et al. 2007), this study represents the first development of accurate 3D finite element geometries of the osteocyte, with its canaliculi and surrounding matrix, to predict osteocyte mechanobiology. It is reported here that the maximum strains predicted in confocal image-derived models are approximately 17,000-20,000 µε higher than strains predicted through previous idealised computational approaches (McCreadie and Hollister 1997; Rath Bonivtch et al. 2007), but are lower than the maximum strains of approximately 35,000 µε reported from experimental imaging of lacunae on a 2D exposed plane (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001). It must be noted that those experimental studies characterised strain in the lacunae, as opposed to strain measured in the cell (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001), and also that the authors proposed that microdamage artefacts might be exacerbating local tissue deformation (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001). Therefore, it is unlikely that such high strain levels would indeed be experienced by the osteocyte in vivo, and the results of this chapter correlate better with average strains between 7,500 µε and 20,000 µε reported in experimental studies (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001). The results demonstrate that the in vivo geometrical characteristics of the surrounding ECM and PCM lead to a high degree of strain transfer into the cell body and must be considered for understanding mechanotransduction in osteocytes in vivo.
The current study provides direct evidence that the pericellular matrix does indeed act to amplify the strains experienced by the cell membrane, which was previously shown using two-dimensional idealised analytical models of a transverse section of a canaliculus (Han et al. 2004; Wang et al. 2007; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994). It is interesting to note that in this study it was observed that the presence of a pericellular matrix reduces the maximum strain in confocal image-derived osteocytes by approximately 80%, but that the total volume of the cells stimulated above 3,500 µε increases by 4-10%. This implies a strain shielding effect, whereby the PCM absorbs very high strains while also increasing the overall strain transfer to the cell. A similar effect has been predicted in recent computational studies of chondrocytes, which found a wider overall stress distribution despite a decrease of 20-52% in maximum stress values in the cell when a PCM was included (Alexopoulos et al. 2005; Appelman et al. 2011). In the idealised simulation maximum strains actually increased by 37% with the inclusion of a PCM, alongside a slight increase in the volume experiencing strain above 3,500 µε. This may be explained by the simplified geometry of the idealised model resulting in poor strain amplification in the absence of a PCM. The PCM has a low elastic modulus and deforms significantly under compression compared to the calcified extracellular matrix, resulting in high strain transfer to the osteocyte cell membrane. These results emphasise the important role that the PCM plays in osteocyte strain sensing and mechanotransduction.

It should be noted that in vivo the pericellular matrix consists of jelly-like glycocalyx and the interstitial fluid within this matrix is capable of flowing through the lacunar-canalicular network under mechanical loading of the ECM (Wang et al. 2004). Analytical models developed by Weinbaum et al., which assume a PCM which tethers the cell process to the canalicular wall using matrix fibres through which interstitial fluid is free to flow under mechanical loading (Han et al. 2004; Wang et al. 2007; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994), suggest that the stimulus arising from loading-induced fluid flow plays an important role in osteocyte mechanobiology. Computational fluid dynamics has also shown that fluid flow characteristics within osteocyte canaliculi have a profound effect on shear stress experienced by cell process (Anderson and Knothe Tate 2008). While fluid dynamics were not incorporated into the models in the current study, a decrease in the volume
of the PCM was predicted (~0.1%) and thus it is proposed that, due to such volumetric changes under mechanical loading, fluid flow would indeed occur and might provide an additional stimulus to the cell. Further studies of fluid flow within this matrix arising from this observed volume change reported here are performed in Chapter 5 using fluid-structure interaction (FSI) modelling techniques, providing a unique insight into mechanical stimulation of osteocytes under matrix strain and loading-induced fluid flow in vivo.

It is interesting that evidence of projections of the ECM into the pericellular space can be seen in the confocal image-derived geometries, which have been identified previously using TEM and AFM techniques (Knapp et al. 2002; McNamara et al. 2009; Reilly et al. 2001). Localised strain amplification appears to occur around these ECM projections, magnifying the applied strain by 50-67%. When these projections were incorporated into the idealised model, local strain amplification of 220-420% of global strain was predicted, increasing the strain amplification factor of the overall model by 190%. These findings highlight the ability of the ECM projections to act as powerful strain amplification mechanisms in osteocyte mechanobiology, particularly due to their location in the most mechanosensitive region of the cell (Adachi et al. 2009b). Furthermore, the spacing between adjacent projections was increased from 0.1 µm to 1 µm to reduce the large computational power required for the simulations. Employing a spacing of 0.1 µm would result in the presence of many more projections, which could in turn impart even greater strain stimulus to the osteocyte, and this is investigated further in Chapter 7 of this thesis.

Of particular interest is the loading case representing vigorous physiological activity (3,000 µε), as it is under this level of global mechanical loading that appreciable strain above 10,000 µε is observed in the osteocyte. This is significant as previous in vitro studies have shown that strain levels above approximately 10,000 µε result in elevated levels of intracellular cytosolic calcium mobilisation (Ca^{2+}) and alkaline phosphatase (ALP) in cultured osteocyte-like cells (Smalt et al. 1997; You et al. 2000). As Ca^{2+} mobilisation is an early intracellular signalling event involved in bone mechanotransduction and ALP is a marker for bone mineralisation and ECM deposition, these are good indicators of the anabolic effect of mechanical stimulus. Past studies applying large strains (>10,000 µε) have also observed increased nitric
oxide (NO) and prostaglandin (PG) production, both essential for an osteogenic response, at these strain levels (Pitsillides et al. 1995; Yeh and Rodan 1984). In contrast more recent studies have suggested that production of NO and PGs, as well as mRNA expression of the important bone remodelling protein osteopontin, may be more closely linked with loading-induced fluid flow than direct substrate stimulation (Owan et al. 1997; Smalt et al. 1997; You et al. 2000). However, these studies did not investigate direct mechanical loading above 5,000 µε. Therefore, it is possible that the elevated strain levels (>10,000 µε) predicted in the current models and in previous experimental studies (Nicolella et al. 2005; Nicolella et al. 2006; Nicolella et al. 2001) may also stimulate these biochemical osteogenic responses, particularly due to the strain amplification effects of the PCM and ECM projections.

4.5 Conclusions
In this chapter it was reported confocal image-derived models subjected to physiologically active loading levels (3,000 µε) predict 350-400% greater strain amplification experienced by osteocytes compared to an idealised cell. Furthermore, it was predicted that the PCM increases the cell volume stimulated above 3,500 µε by 4-10% and that ECM projections amplify strain to the cell by approximately 50-420%. In addition, decreases in the volume of this matrix were also predicted, suggesting the occurrence of mechanically-driven interstitial fluid flow. These are the first confocal image-derived computational models of osteocytes in vivo and the results confirm the hypothesis that “Mechanical loading to the osteocyte is amplified by the native geometry of the osteocyte environment,” providing a greater understanding of osteocyte mechanobiology. However, these models did not incorporate the role of the fluid occupying the pericellular space, which is believed to act as a stimulus for osteocytes in vitro. In Chapter 5, a fluid-structure interaction approach is applied to investigate the role of fluid flow in mechanical stimulation of osteocytes. The strains within the environment of the osteocyte will be investigated experimentally in Chapter 6, and compared with those predicted here. Finally, in Chapter 7 the models developed here are applied alongside the experimental results to determine the effect of changes in bone tissue properties on osteocyte stimulation during osteoporosis.
Chapter 5

Fluid Flow in the Osteocyte Mechanical Environment: A Fluid-Structure Interaction Approach

5.1 Introduction

The precise mechanical stimuli that osteocytes, the cells that orchestrate bone remodelling, experience in vivo have been much debated. Experimental studies comparing substrate strain with fluid flow in vitro point to loading-induced fluid flow around the osteocyte as the primary stimulus for bone growth (Owan et al. 1997; Smalt et al. 1997; You et al. 2000). The fluid within the pericellular space is driven to flow under normal mechanical loading and thereby generates a shear stress on the osteocyte cell membrane, which is thought to act as a stimulus for biochemical signalling (Knothe Tate and Knothe 2000; Knothe Tate et al. 1998a; Knothe Tate et al. 1998b; Knothe Tate et al. 2000; Wang et al. 2000). This theory has been substantiated by experimental observations of bone cells responding more to flow derived shear stress than to streaming potentials or chemotransport (Bakker et al. 2001), although chemotransport may modulate the response to fluid flow (Donahue et al. 2003). In vitro cell culture studies have observed osteogenic responses in osteocyte-like cells exposed to fluid shear stress levels between 0.4 and 1.2 Pa within in vitro flow experiments (Bacabac et al. 2004; Bakker et al. 2001), while a similar osteogenic threshold of 10,000 με has been observed for direct strain loading (Burger and Veldhuijzen 1993; You et al. 2000). However, due to the location of osteocytes embedded within a mineralised extracellular matrix, direct
experimental studies to elucidate their in vivo mechanical environment are challenging.

Thus analytical models of idealised osteocyte canaliculi under load-induced fluid flow have been developed and applied to predict the in vivo range for shear stress (0.8-3 Pa) and deformation of osteocyte cell membranes (Han et al. 2004; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994). Computational finite element modelling techniques have also been applied to idealised models of the lacunar-canalicular system and have predicted abrupt changes in the drag forces within the canaliculi arising from changes in geometry or proximity to bone microporosity and the Haversian canals (Mak et al. 1997). One study investigated the fluid environment of an idealised osteocyte and predicted high shear stresses within the canaliculi whereas the osteocyte cell body is primarily exposed to hydrodynamic pressure (Anderson et al. 2005). Furthermore, theoretical and computational studies have suggested that projections of the ECM amplify the shear stress or strain stimulus to the osteocyte (Han et al. 2004; Verbruggen et al. 2012; Anderson and Knothe Tate 2008). Ultra high voltage electron microscopes (UHVEM) have also been used to develop highly detailed computational models of 80 nm long sections of osteocyte canaliculi, and these have been applied to predict that the geometry of the pericellular space greatly affects the velocity of fluid flow around the osteocyte cell processes (Kamioka et al. 2012).

In all previous computational models bone cells and tissue were modelled using either solid mechanics approaches (where extracellular fluids were modelled using poroelastic assumptions) or fluid dynamics modelling, wherein the biological tissues were assumed to be rigid for the purposes of understanding fluid flow and shear stresses. In reality, osteocytes are composed of an elastic cell membrane that deforms in response to the external fluid flow imposed by mechanical loading. The assumption that osteocytes are static rigid bodies precludes the elucidation of cellular strains arising from fluid flow in the lacunar canalicular network (Anderson et al. 2005; Anderson and Knothe Tate 2008; Kamioka et al. 2012). To fully simulate this behaviour represents a most challenging multi-physics problem, which is too complex to solve analytically and requires a fluid-structure interaction (FSI) approach. Furthermore, previous studies have used idealised geometries (Anderson et al. 2005; Rath Bonivtch et al. 2007) or have modelled limited portions of the cell
(Anderson and Knothe Tate 2008), but computational models have recently shown that the use of idealised geometries predicts stress and strain values which are significantly lower than those predicted by geometries developed from physiological imaging (Anderson and Knothe Tate 2008; Verbruggen et al. 2012). In vitro experimental studies have shown that the geometry of the bone cell affects its response to strain (Bacabac et al. 2008). Therefore, an approach which examines the effect of the complex architecture of the lacunar-canalicular network on fluid shear stress and cellular strains in osteocytes is required.

Therefore, it was hypothesised that “Loading-induced interstitial fluid flow significantly contributes to the mechanical stimulation of osteocytes in vivo.” The objective of the research in this chapter was to use computational methods to predict the mechanical environment of osteocytes in vivo under physiological loading. Using representative models of the osteocyte and its environment with fluid structure interaction (FSI) techniques, both shear stresses on the cell membrane and resulting strain within the osteocyte arising from mechanically-driven interstitial fluid flow in vivo were examined. This chapter is modified from previously published work (Verbruggen et al. 2013).

5.2 Materials and Methods

5.2.1 Model generation

Fluorescent staining of thick transverse sections of the mid-diaphysis of the tibia of a male 6-8 month old Sprague-Dawley rat was performed, using FITC (Fluorescein Isothiocyanate isomer I, Sigma-Aldrich) solution at 1 per cent in ethanol to visualize the pericellular space (Verbruggen et al. 2012). While ethanol will damage any remaining cellular tissue, it will not affect the visualisation of the lacunar-canalicular architecture. Confocal scanning of osteocytes was performed using a confocal microscope (Zeiss LSM 51) with a 40x oil immersion lens at laser wavelength excitation of 488 nm, allowing visualisation of their native environment.

Confocal microscopy allowed acquisition of a z-stack of scans through the depth of the sample, taken at 2048 x 2048 x 47 pixels with a field of view of 255 µm, resulting in a resolution of 0.1 µm in x-y plane and 0.6 µm in the z axis, that were
then used to develop geometrically accurate models of osteocytes as previously described (Verbruggen et al. 2012). Briefly, MIMICS (Materialise) image processing software was used to generate 3D reconstructions of the images, with thresholding to between -884 and -769 Hounsfield units to allow segmentation of the lacunar-canalicular space from the surrounding matrix (Verbruggen et al. 2012). These models were then meshed using 3-Matic (Materialise) voxel-meshing software to create four solid osteocyte models with anatomically accurate geometries (Verbruggen et al. 2012). Boolean operations were applied to generate a surrounding extracellular matrix (ECM) and pericellular fluid space (PCS), offset from the osteocyte model by 0.08 µm, based on experimental measurements of the geometry of the osteocyte environment (McNamara et al. 2009; Wang et al. 2005; You et al. 2004). These models were meshed with ANSYS SOLID72 tetrahedral elements and exported to ANSYS. Models of an idealised osteocyte and an idealised osteocyte with ECM projections were generated to compare with the representative geometries (Verbruggen et al. 2012), similar to the methods described above. The four representative geometries and the idealised geometry are shown in Figure 5.1 (A-D) and Figure 5.1 (E) respectively.
Figure 5.1: Composite images of the three components of the representative models (A-D) and the idealised model (E). The face on which loading and inlet pressure conditions are applied is also shown (F). In each model the solid extracellular bone matrix (grey) is cut back to reveal the fluid-filled pericellular space (blue), which is in turn cut back to reveal the solid osteocyte beneath (green).

5.2.2 Fluid-structure interaction analyses:

5.2.2.1 Solid Material and Fluid Properties

All solid structures were modelled as linearly elastic, isotropic materials. An elastic modulus of 16 GPa and Poisson’s ratio of 0.38 was attributed to the ECM (Deligianni and Apostolopoulos 2008). A modulus of 4.47 kPa and Poisson’s ratio of 0.3 was attributed to the osteocyte cell body and processes (Sugawara et al. 2008).

The properties of the interstitial fluid were assumed to be similar to salt water, with a density of 997 kgm$^{-3}$ and a dynamic viscosity of 0.000855 kgm$^{-1}$s$^{-1}$ (Anderson et al. 2005). Flow within the lacunar-canalicular system was assumed to be laminar in nature.
5.2.2.2 Boundary conditions and loading
Two-way fluid-structure interaction simulations were conducted using coupled ANSYS Multi-physics software to investigate the behaviour of interstitial fluid under physiological conditions. Two levels of fluid-solid interactions are performed to simulate interaction between the ECM and the interstitial fluid and also between this fluid and the osteocyte cell membrane.

The initial two-way FSI simulation is conducted through a bi-directional coupling of the ANSYS CFX solver to the ANSYS Structural finite element (FE) solver. A displacement boundary condition was applied to model faces to generate a compressive load of 3,000 µε, representative of vigorous physiological loading (Burr et al. 1996). Due to the fact that mechanical loading in bone occurs on the whole organ level, with compression and tension occurring in different regions driving fluid across the bone network, a pressure gradient is applied across the models to represent this (Knothe Tate 2003; Manfredini et al. 1999; Steck et al. 2003; Knothe Tate and Niederer 1998). Therefore, in order to simulate in vivo loading conditions, an inlet pressure of 300 Pa was assigned to the inlets on one face and the remaining inlets were defined as outlets at a relative pressure of 0 Pa (Manfredini et al. 1999; Steck et al. 2003; Knothe Tate and Niederer 1998), similar to the pressure gradient applied by Anderson et al. (Anderson et al. 2005).

While in vivo loading of bone is dynamic and cyclic (Fritton et al. 2000), the materials in these models were assigned linear elastic material properties, and it was thus assumed fluid flow would not differ during unloading as has been determined mathematically (Wang et al. 2001). This allowed simplification of boundary conditions to ramped static loading. Loading was applied uni-axially and symmetrically, to represent longitudinal compression of long bones in vivo (Taylor et al. 1996), with opposing faces constrained symmetrically to prevent rigid body motion. Using a staggered iteration approach inherent in ANSYS coupling software, the deformations at the interface between the ECM and the PCS resulting from the applied loading are mapped onto the fluid domain. The resulting fluid equations are solved and forces are relayed back to the solid ECM domain as new boundary conditions, allowing gradual mesh motion and a strongly coupled solution through further iterations. This staggered iteration approach is performed repeatedly within each step until convergence of the field equations and a fully implicit solution is
achieved. Upon solution of this FSI analysis, the loading-induced fluid flow can be analysed. The pressure load on the cell membrane arising from the flow is then exported to ANSYS Structural and interpolated onto the surface of the solid osteocyte domain. This FSI analysis facilitates investigation of the deformation and strain in the cell generated by the interstitial fluid boundary conditions imposed by global matrix loading.

5.3 Results

The complex multi-physics nature of this study allows analysis of multiple aspects of the osteocyte mechanical environment, which are examined in sequence here.

5.3.1 Interstitial fluid velocity

The streamline plots in Figure 5.2B and Figure 5.3 show the velocity distribution on the surface of representative osteocytes. Qualitatively it can be seen from these images that regions of highest velocities are located within osteocyte canaliculi, with maximum velocities of 238.1-325.7 µm/s (298.48 ± 35.84 µm/s) in representative models and 219.2 µm/s in the idealised model. In contrast fluid velocities were much lower surrounding the osteocyte cell body, with magnitudes of approximately 38-79 µm/s (60.58 ± 16.5 µm/s) predicted in the representative models and approximately 27 µm/s observed in the idealised models (see Figure 5.7A). It can also be seen that the fluid velocity within canaliculi is affected by variations in surface roughness of the canalicular wall. This is noticeable in the effect of ECM projections on fluid flow in the osteocyte canaliculi as shown in Figure 5.6. Fluid velocity is seen to increase in these areas in both models (Figure 5.6A and 5.6C).
Figure 5.2: (A) Maximum principal strain distribution in the bone matrix around a representative osteocyte, (B) streamlines of the fluid flow within the pericellular space around the osteocyte, (C) shear stress imparted on the cell membrane by the fluid flow and (D) the resulting strain within the osteocyte. A composite image of these contour plots is also shown (E)
Figure 5.3: Images of velocity streamlines in the space surrounding representative osteocytes

Figure 5.4: Contour plots of shear stress on representative osteocyte cell membranes
5.3.2 Shear stress on the cell membrane

The shear stress distribution on the surface of the representative osteocytes resulting from the interstitial fluid flow is shown in Figure 5.2C and Figure 5.4. Visually the highest shear stresses are predicted within the osteocyte canaliculi, resulting in stress concentrations on the cell processes (see Figure 5.6B). A similar effect is seen in idealised models when ECM projections are included (see Figure 5.6D). Maximum shear stresses are shown in Figure 5.7B, and are observed to increase to 6.9 Pa in the idealised models with the inclusion of ECM projections in the osteocyte canaliculi. The representative models experience even higher levels of shear stress stimulus, with maximum shear stresses of approximately 9.5-15.2 Pa (11.6 ± 2.5 Pa) located in the canaliculi. All osteocyte models experienced shear stress above the level of shear stress required for bone growth (0.8 Pa) (Han et al. 2004; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994), with the amount of the cell membrane stimulated to this level shown in Figure 5.8A.

Figure 5.5: Contour plots of the maximum principal strain distributions in representative osteocyte cell membranes
5.3.3 Strain within the osteocyte

The strain experienced in the representative osteocyte cell membranes is visible in the contour plots in Figure 5.2D and Figure 5.5. The strain distribution in the representative and idealised osteocytes is shown in Figure 5.8B. While cellular strains greater than 10,000 µε were predicted in discrete areas of the models most of the cell membranes, approximately 81-98% (90.1 ± 6.1%) were strained by less than 1,000 µε.

Figure 5.6: Contour plots showing the stimulus amplification effect within the canaliculi, with the effect of the ECM projections clearly visible on (A) velocity and (B) shear stress in a representative osteocyte, and similarly (C, D) in an idealised model.
Figure 5.7: Average interstitial fluid velocities within the pericellular space in osteocyte models (A) and the resulting maximum shear stresses on the osteocyte cell membranes (B).

Figure 5.8: The percentage of the osteocyte models stimulated above 0.8 Pa (A) and the strain distribution in the osteocytes arising from the mechanically-driven fluid flow (B).

5.4 Discussion

The study in this chapter develops the first fluid-structure interaction models to investigate the complex interaction between the solid and fluid phases within the osteocyte environment in vivo. These models simulate interstitial fluid flow arising from mechanical deformation of the bone matrix and pressure gradients under loading conditions representative of vigorous activity. This study allows the investigation of the velocities of the interstitial fluid, the shear stress imparted onto the surface of the cell and the resulting strain in the osteocyte cell processes and...
body. Such findings predict the mechanical stimuli to osteocytes under loading conditions known to stimulate an osteogenic response.

The modelling of a complex mechanical environment such as the lacunar-canalicular network necessitates a number of assumptions. The resolution of the images used to generate the models (0.1 µm), is amongst the highest obtainable on commercially available confocal microscopes. As the diameter of the canaliculus is ~100 nm, the most highly fluorescent canaliculi are more easily discernible and less fluorescent canaliculi were omitted during thresholding operations, resulting in fewer processes than exist in vivo. Although transmission electron microscopy (TEM) resolutions could overcome this issue, obtaining the full 3D information of an osteocyte through serial TEM is unfeasible due to the small size of ultrathin slices required (~9 nm) relative to the size of an osteocyte environment (~50 µm). It must be noted that, as the greatest stimulation in the models occurs in the canaliculi, it is expected that inclusion of more canaliculi, as are present in vivo, would present even more sites for amplification of stimulation at these levels of loading.

For the purposes of this study the ECM and osteocyte materials were assumed to be linear elastic isotropic materials, with cell properties assigned from values observed in cultured bone cells (Sugawara et al. 2008), and the mesh-like pericellular matrix was not included. The viscoelastic nature of the cell was neglected as physiological loading of bones occurs at a frequency of approximately 1 Hz, well below the relaxation time of 41.5 s measured in bone cells (Appelman et al. 2011; Darling et al. 2008). Furthermore, the inherent anisotropy of the cell was neglected in this study in order to focus on the multi-physics effects of loading-induced fluid flow. However, the effect of transverse isotropy was investigated previously in Chapter 4 through a finite element approach and reported 3.7-12.2% greater strain transfer to the osteocyte when compared to isotropic cells (Verbruggen et al. 2012). Thus, the stimulation predicted in the current study would be enhanced with the inclusion of an active actin cytoskeleton, which has been shown to result in higher reaction forces and increased tension inside the cells (Dowling et al. 2012; McGarry 2009; McGarry et al. 2009; Ofek et al. 2010; Ronan et al. 2012). As the fluid dynamics of the interstitial fluid are not well understood, laminar uni-directional flow was assumed based on studies of the nano-scale dimensions of the canalicular channels (Anderson et al. 2005; Cheng and Giordano 2002). Realistically dynamic flow and cyclic loading conditions occur in vivo, and experimental studies suggest that shear strain
rate on the cell membrane is an important mechanical factor in bone mechanobiology (Bacabac et al. 2004; Fritton et al. 2000; Goldstein et al. 1991; Lanyon and Rubin 1984). The incorporation of dynamic physiological boundary conditions in future studies, along with an active actin cytoskeleton, would further amplify the stimulus observed in this study and provide more realistic simulations of osteocyte mechanobiology in vivo.

Mechanical loading in bone occurs on the whole organ level, with compression and tension occurring on opposite regions in the bone. This global loading generates a much higher pressure gradient than across the region of a single cell, driving fluid from one side of the organ to the other and resulting in gradients as large as the 300 Pa gradient applied in this study (Manfredini et al. 1999; Steck et al. 2003). A recent computational study has predicted pressure gradients within individual canaliculi as high as 1 Pa/nm, the equivalent of an approximately 800 Pa pressure gradient along the length of a single canaliculus (Kamioka et al. 2012). While this global loading was represented as a localised pressure gradient in this study it is anticipated that an anatomically accurate lacunar-canalicular network, employing a multi-scale modelling approach to derive the boundary conditions across multiple scales (Vaughan et al. 2012), may elucidate the pressure gradient induced across individual osteocytes.

While previous researchers have employed CFD techniques to predict fluid flow in the lacunar-canalicular system (Anderson et al. 2005; Anderson and Knothe Tate 2008; Kamioka et al. 2012), this study provides the first investigation of the effect of bone matrix deformation on flow of interstitial fluid, and subsequent deformation of the osteocyte cell membranes in a fully three-dimensional anatomically-accurate osteocyte environment. FSI modelling has recently been applied to model individual cells under in vitro conditions (Vaughan et al. 2013a) and to investigate the mechanics of the bone marrow cavity (Birmingham et al. 2012a), but these techniques have not yet been applied to the in vivo environment of individual osteocytes. It was observed here that average velocities of approximately 60.5 µm/s occur within the pericellular space, which are similar to those observed in experimental fluorescein tracer studies (~60 µm/s) (Price et al. 2011).

Examination of the strain distribution in the osteocyte resulting from loading-induced fluid flow shows that strain levels are much lower than 10,000 µε, which in
in vitro substrate strain studies have shown is required to stimulate osteoblast and osteocyte activity (Burger and Veldhuijzen 1993; You et al. 2000). Indeed, most of the cell volumes (81-98%) experience strains below 1,000 µε, an order of magnitude lower than that required for an osteogenic response. This implies that mechanically-driven fluid flow alone in the canalicular system is not sufficient to generate a strain-related osteogenic response. In a previous study, presented in Chapter 4, it was predicted strain stimuli of 23-26,000 µε by a solid mechanics finite element approach using these representative models, assuming that the PCM was a solid continuum (Verbruggen et al. 2012). This glycocalyx matrix has been shown to reduce permeability by an order of magnitude (Anderson et al. 2008), resisting flow and inducing strain in the osteocyte cell membrane (Goulet et al. 2009; Gururaja et al. 2005; Sansalone et al. 2012). Thus the inclusion of such a matrix would likely reduce the magnitudes of fluid velocity and the resulting shear stresses. However, the presence of tethering elements in the PCM would also likely result in an increase in strain transfer to the osteocyte predicted here, as has been demonstrated analytically (Han et al. 2004; Wang et al. 2007; You et al. 2001b; Zeng et al. 1994). The models in the current study do not include tethering elements as these are too small (~0.5 µm long) to be accurately captured at confocal resolutions, while serial TEM imaging of an entire osteocyte with tethering elements would prove extremely challenging due to the small size (~9 nm) of ultrathin sections required relative to the size on an osteocyte environment (~50 µm). Thus, it is proposed that the absence of these tethers of the peri-cellular matrix accounts for the low strain values predicted along the osteocyte membrane. The effects of these tethering elements are included in an idealised form in Chapter 7 in order to elucidate the effects of osteoporosis on the local osteocyte mechanical environment.

There has been much debate within the osteocyte community as to whether the stimulus to the osteocyte is amplified through tethering elements (Han et al. 2004) or through ECM projections that disturb the flow or attach directly to the cell processes (Anderson and Knothe Tate 2008; Verbruggen et al. 2012; Wang et al. 2007). While tethering elements have not been included in this study, the results do show that variations in the canalicular geometry, particularly the ECM projections, act as shear stress stimulus amplifiers in the osteocyte geometry, similar to previous computational fluid dynamics (CFD) studies (Anderson and Knothe Tate 2008).
These projections have been shown to contact the cell process (Kamioka et al. 2012; McNamara et al. 2009) and also co-localize with areas of β3 integrin staining (McNamara et al. 2009). β3 integrin is a key protein in αvβ3 focal adhesions which have been implicated in matrix invasive and attachment processes (Chatterjee and Chatterjee 2001; Huang et al. 2000) between bone surfaces and osteoclasts (Clover et al. 1992; Engleman et al. 1997; Horton et al. 1991). Additionally, osteopontin is a β3 ligand (Horton et al. 1991; Ross et al. 1993), which is present in abundance along the canalicular wall (Devoll et al. 1997b; McKee and Nanci 1996a; Noda et al. 2003; Sodek and McKee 2000). This evidence strongly suggests that these punctate represent a form of focal adhesion between the osteocyte and the canalicular wall. These attachments would result in both increased strain transfer from the ECM to the osteocyte (Verbruggen et al. 2012; Wang et al. 2007), and increased shear stress stimulus to the osteocyte through disrupted fluid flow as seen previously (Anderson and Knothe Tate 2008) and predicted in the current study.

The shear stimuli predicted here fall within the range of 0.1-2.2 Pa, which has been shown in cell culture studies of osteoblastic cells to result in increased nitric oxide (NO), prostaglandin (PGE2) and osteopontin production, biochemicals known to play a key role in the osteogenic (bone forming) response of osteoblasts (Bacabac et al. 2004; Bakker et al. 2001; Owan et al. 1997; Smalt et al. 1997). Furthermore, in vitro studies have also shown increases in intracellular calcium (Ca2+), an important bone mechanotransduction signalling factor, at shear stress levels of 2 Pa (You et al. 2000). Therefore, these findings suggest that under global loading conditions representative of vigorous activity (3000 µε), the individual osteocyte is sufficiently stimulated to produce biochemical signals for bone formation.

Interestingly, the results of the current study predict shear stresses on the cell processes in all models that are within the range of 0.8-3 Pa predicted analytically to occur in vivo (Han et al. 2004; Weinbaum et al. 1994; You et al. 2001b; Zeng et al. 1994), and are similar to values (~5 Pa) suggested by tracer studies (Price et al. 2011). Highly variable fluid velocities and shear stresses are predicted within the canaliculi, with inhomogeneous flow patterns occurring similar to those recently observed using detailed fluid mechanics models of individual canaliculi (Kamioka et al. 2012). However, in the current study both the maximum velocities and highest shear stress levels were predicted to occur within the canaliculi, and such stimuli
were not predicted surrounding the cell body. Furthermore, it was found that the inclusion of ECM projections along the wall of the canaliculi in idealised models resulted in an increase of 152% in shear stress stimulus to the cell. These stimuli were further amplified around these localised projections in representative osteocytes, with increases of 203-300% experienced. This is in agreement with previous finite element and fluid mechanics studies which found that the geometry of the canaliculi can greatly affect the stimulus experienced by the osteocyte cell process (Anderson et al. 2005; Anderson and Knothe Tate 2008; Kamioka et al. 2012; Verbruggen et al. 2012). The current study corroborates this evidence, showing that under physiological loading conditions, representative of the in vivo multi-physics environment, interstitial fluid velocities and shear stresses that are both significantly greater in the canaliculi than around the osteocyte cell body. Furthermore, this is the first computational study to explore the effects of both the complex interplay between fluid and solid mechanics and the intricate architecture of the lacunar-canalicular network on osteocyte mechanobiology.

The mechanical stimulation within canaliculi is particularly interesting as focal adhesion complexes are localised in osteocytic processes (Kamioka et al. 2006; McNamara et al. 2009; Vatsa et al. 2008) and have been predicted to cause strain concentrations on osteocyte processes by analytical and fluid mechanics modelling approaches (Verbruggen et al. 2012; Wang et al. 2007). Previous cell culture studies have shown that the cell process contains more concentrated and highly organised actin filaments (Tanaka-Kamioka et al. 1998), and for these reasons the cell process is believed to be the most mechanosensitive area of the osteocyte (Adachi et al. 2009b; Burra et al. 2010; Klein-Nulend et al. 1995b; Wu et al. 2011). The findings of this chapter show that the canaliculus is a more mechanically active region of the lacunar-canalicular network than the lacuna, with the cell processes exposed to the greatest velocities and shear stimuli. Therefore, these findings support the hypothesis that osteocyte cell processes in the canaliculi play an important role in osteocyte mechanobiology (Adachi et al. 2009b).
5.5 Conclusions

In this chapter it was reported that fluid-structure interaction models of the osteocyte mechanical environment under global loading conditions representative of vigorous activity predict maximum shear stress (~11 Pa) stimulus and average fluid velocities (~60.5 µm/s) at the level of the individual osteocyte. Interestingly, these values were found to be within the range known to stimulate bone cells in vitro, and thus validate the hypothesis that “Loading-induced interstitial fluid flow significantly contributes to the mechanical stimulation of osteocytes in vivo.” Furthermore, it was observed that the highest stimuli occur in the canaliculi about the osteocytic process, reinforcing the theory that this is the most mechanically active and mechanosensitive region of the osteocyte. These are the first computational models to simulate the complex multi-physics mechanical environment of the osteocyte in vivo and to incorporate the complex 3D lacunar-canalicular architecture, providing a deeper understanding of osteocyte mechanobiology. Although this research demonstrates the stimulation of osteocytes by loading-induced fluid flow, the absence of a pericellular matrix will affect the mechanical stimulation of the osteocyte. This matrix is included in an idealised form in Chapter 7, as part of an investigation of osteocyte stimulation during the disease of osteoporosis.
Chapter 6

Temporal Changes in the Micromechanical Environment of Osteoblasts and Osteocytes in an Animal Model of Osteoporosis

6.1 Introduction

Osteoporosis is a debilitating bone disease, which is characterised by an imbalance in normal bone cell remodelling (Braidman et al. 2001b), and results in severe bone loss (Compston et al. 1989; Lane et al. 1998), significantly reduced strength (Brennan et al. 2011b; Brennan et al. 2012; Parfitt 1987), micro-damage (Burr 2003; Dai et al. 2004; Mashiba et al. 2001; Schaffler 2003) and altered tissue porosity (Knothe Tate et al. 2004; KnotheTate et al. 2002; Sharma et al. 2012). Previous studies have shown altered mechanical properties of trabecular bone in ovariectomised rats compared to sham-operated controls (McNamara et al. 2006; McNamara and Prendergast 2005). Furthermore, tissue-level mineral distribution is altered in both trabecular and cortical bone in a sheep model of osteoporosis (Brennan et al. 2011a; Kennedy et al. 2009), and changes in mineralized crystal maturity, mineral-to-matrix ratio, and collagen cross-linking also occur (Brennan et al. 2012). Such changes might occur as a compensatory mechanism triggered by bone loss during osteoporosis. Alternatively, it may be that oestrogen deficiency itself leads directly to changes in tissue composition, which consequently alters the mechanical environment of osteoblasts and osteocytes. This change in mechanical stimuli sensed by these cells may then initiate a mechanoregulatory response.
resulting in bone loss. Computational simulations of bone adaptation have predicted osteoporotic-like trabecular architecture (Van Der Linden et al. 2001), as well as altered bone resorption rates and osteocyte strain levels (Mulvihill et al. 2008) in response to changes in tissue stiffness. However, it remains that the mechanical stimulation experienced by osteoblasts and osteocytes within osteoporotic bone in vivo has never been characterised, and as such these theories remain conjecture.

As osteocytes are embedded in a mineralized matrix, direct experimental investigation of their mechanical environment is challenging. High resolution microscopy of exposed two-dimensional bone sections under mechanical loading (Nicolella et al. 2001) have predicted perilacunar strains in the range of 7,500-35,000 µε (Nicolella et al. 2005; Nicolella et al. 2006). AFM techniques have measured osteoblast strains as high as 40,000 µε under an applied load of 20 nN in vitro (Charras and Horton 2002; Charras et al. 2001). However, the experimental approaches of Nicolella et al. involved milled sections of bone tissue and surface polishing to expose embedded osteocytes and can introduce microcracks (Nicolella et al. 2005; Nicolella et al. 2006), and such methods might alter the mechanical environment of the cell (Reilly 2000; Zioupos and Currey 1994). Furthermore, point loading through AFM techniques is not representative of the substrate strain that osteoblasts on bone surfaces are exposed to in vivo. In Chapters 4 and 5 computational modelling of the in vivo mechanical environment of individual osteocytes predicted maximum strains and shear stresses of 23-26,000 µε and 11 Pa respectively (Verbruggen et al. 2013; Verbruggen et al. 2012), whereas osteoblasts were shown to experience maximum strains of approximately 1,270 µε for applied loading of 1,000 µε (Charras and Horton 2002; Charras et al. 2001). While these models provide an insight into bone cell mechanical behaviour, an experimental approach that does not necessitate destruction of or interference with the local mechanical environment is required to investigate the in situ strain environment of cells in healthy and osteoporotic bone.

Therefore, in this chapter two hypotheses were proposed: (a) “Rapid bone loss at the onset of osteoporosis increases mechanical stimulation of osteoblasts and osteocytes” and (b) “A compensatory mechanobiological response by osteocytes to increased loading results in subsequent alterations in local tissue mineralisation and stiffness in late-stage osteoporosis.” The objective of this research was to develop a combined micromechanical loading and confocal microscopy technique to
characterise the local mechanical environment of osteocytes and osteoblasts, from healthy and osteoporotic bone, in a rat model of osteoporosis. A purpose-built loading rig was designed to characterise the mechanical environment of osteoblasts and osteocytes in situ under physiological loading conditions. Thus, the effects of both short- and long-term osteoporosis on the local mechanical environments of osteocytes and osteoblasts were investigated.

6.2 Materials and Methods

6.2.1 Custom-designed loading device

In order to visualise the local mechanical environment of the cells, a custom loading device was designed that is compatible with a confocal microscope (Zeiss LSM 51) and comprised of a specialised loading stage and sample grips to ensure that samples could be held flush with the microscope objective (Figure 6.1). A high-torque stepper motor (ST2818L1006, Nanotec) and gearing provided transmission to a precision bi-directional ball power screw (SD0401, ABSSAC), and thus applied microscale displacements to cortical bone samples (of length 10 mm) during imaging. The applied loading is displacement-controlled, with displacements applied to the whole bone in specified increments, with speeds and magnitudes controlled using commercial software (NanoPro 1.6, Nanotec). The device is capable of applying bi-directional uniaxial tensile or compressive loading at increments as small as 50 με, allows controlled application of loading up to 3,000 με, representative of vigorous physiological loading (Burr et al. 1996).
Figure 6.1: Diagram of custom-designed micro-loading device in position under the confocal microscope (A) and close-up (B). Relationship between bone sample, loading platens and microscope objective shown in (C) and (D)

6.2.2 Validation of loading device and digital image correlation (DIC) analysis

The custom-built loading device was validated for the application of bi-directional, uniaxial compression loading on a poly(methyl methacrylate) (PMMA) sample (length 12 mm and radius 3.2 mm) with embedded fluorescent microspheres. Briefly, a PMMA resin (8510, Akasel) was combined with a curing agent (8562, Akasel) and fluorescent microspheres (10 µm diameter) at a dilution of 1 µL/mL (Fluoresbrite 18140-2, Polysciences Inc.). A sonicator (2510E-MT, Branson Ultrasonics) and rotator (SB3, Stuart) were used to ensure dispersion of the microspheres throughout the sample. The samples were formed by filling 12 mm lengths of 3.2 mm diameter silicone tubing (HV-96410-16, Masterflex) with the PMMA resin and allowing it to set overnight. The samples were then inserted into the grips for the experimental loading.
A compressive displacement load equivalent to 3,000 µε was applied to PMMA/microsphere construct and a series of confocal images were captured for DIC analysis (see Figure 6.2A). The maximum principal strain distribution within each sphere was determined from a series of images of each loaded sphere using Digital Image Correlation (DIC) analysis with a previously developed software package (MOIRE) (Luu et al. 2011; Pan et al. 2010a; Pan et al. 2010b), which is capable of tracking displacements of pixels in the images (see Figure 6.2B). A correlation coefficient is calculated for each pixel by comparing the deformed image with the reference image. A zero-mean normalised cross-correlation (ZNCC) coefficient is then determined for each image pixel, both at the edges and within the microsphere. Once the correlation coefficient extremes (maximum and minimum) have been detected, the full-field deformation can be determined as described in equations 3.72-3.75 in Chapter 3. The loading and DIC analysis was repeated for ten different microspheres and was compared to the results of an analytical solution for a homogenous material with spherical inclusions under loading (Bilgen and Insana 1998).

Briefly, the analytical solution allows for calculation of the strain within a spherical object embedded in a homogenous material of different material properties (see Figure 6.2C). The inclusion of the microspheres will likely reduce the effective stiffness of the PMMA, and the relationship between the material properties, geometry and displacement is summarised in the following equation:

\[
\varepsilon^t = \left(\frac{U}{Z}\right) \left(\frac{2\mu_b(1 + v_b)(1 - v_b)}{2(1 + v_b)}\right) \left(\frac{5(1 - v_b)}{[\mu_b(7 - 5v_b) + \mu_t(8 - 10v_b)]} \right) \\
+ \left(\frac{1 - 2v_t}{\mu_b(2 - 4v_t) + \mu_t(1 + v_t)}\right)
\]

where the shear modulus and Poisson’s ratio are denoted by \(\mu_b, v_b\) and \(\mu_t, v_t\) for the matrix and microsphere respectively, displacement is denoted by \(U\) and specimen length by \(2Z\) (Bilgen and Insana 1998). PMMA was assumed to have a shear modulus of 1.7 GPa and Poisson’s ratio of 0.3. The polystyrene microspheres remain spherical after embedding and are radially isotropic, with a shear modulus of 2.1 MPa and a Poisson’s ratio of 0.3.
Figure 6.2: Confocal image of PMMA-embedded fluorescent microsphere (A), with the contour plot of strain within it under 3,000 µε loading (B). Diagram of analytical solution for spherical inclusion in an homogenous material (C), adapted from (Bilgen and Insana 1998). Comparison of experimental and analytical results over a range of applied loads is shown in (D).

Analysis of the experimental results was compared to the analytical solution at load steps of 500 µε, 1,000 µε, 1,500 µε, 2,000 µε and 2,500 µε and 3,000 µε. The strain observed experimentally displayed close correlation to the analytical solution over multiple applied loads, see Figure 6.2D. The percentage error at each of the 500 µε, 1,000 µε, 1,500 µε, 2,000 µε, 2,500 µε and 3,000 µε load steps was 9.41%, 3.37%, 4.13%, 1.14%, 6.81% and 1.88% respectively. At 3000 µε an average strain within the microspheres of 486 µε (486 ± 32.1 µε) was observed by the DIC technique, while the analytical solution predicts a value of 477 µε.
6.2.3 Animal model and sample preparation

Ovariectomised rat bone is employed in this study as it has been deemed an appropriate model for post-menopausal osteoporosis in humans (World-Health-Organization 1998), with many shared characteristics with human diseased bone (Kalu 1991). Four groups of 8-month old female Wistar (Charles River) rats were used in this study; (1) a group in which rats were ovariectomised five weeks prior to the experiment (n=2) to induce oestrogen deficiency, (2) a control sham-operated group (n=2), and a 34-week postoperative (3) ovariectomised (n=2) and (4) control sham group (n=2). Animals were anaesthetised using isoflurane gas and then sacrificed by CO₂ inhalation. Upon sacrifice of the animals, checks were performed to confirm the presence or absence of the ovaries for SHAM and OVX animals respectively. Immediately prior to sacrifice, rats were injected with FITC (Fluorescein Isothiocyanate Isomer 1, 30 µL at 10 mg/mL, Sigma-Aldrich F7250) to stain the lacunar-canicular network, similar to previous methods (Ciani et al. 2009). All procedures were carried out following institutional ethical approval and under an animal license granted by the Irish Department of Health B100/4424.

One femur from each animal was extracted and placed in α-minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all Sigma-Aldrich) at 37 °C, in order to maintain cell viability within the samples. Additionally, the current study was performed in less than five hours to minimise post-extraction time.

Femora extracted from rats were cut proximally and distally using a diamond blade saw (Isomet, Buehler) to produce 10 mm femoral shaft specimens. These were further cut to produce longitudinal, semi-cylindrical samples that could be loaded and imaged simultaneously in the custom-built device (see Figure 6.3(A-C)). The live samples were kept in media during cutting and preparation, and rinsed with phosphate-buffer solution (PBS) immediately prior to loading to prevent autofluorescence of the media. After cutting and prior to loading, samples were incubated for 30 minutes in FITC to enhance staining and a plasma membrane stain (CellMask Orange Plasma Membrane, 20 µL at 5 mg/mL, Invitrogen C10045) in order to visualise the osteoblast and osteocyte cell membranes. All preparation and loading occurred within 3 hours after extraction, with samples covered in aluminium foil to
prevent photobleaching. Phosphate buffered saline (PBS) was regularly applied to samples during loading and imaging to prevent dehydration. Custom designed epoxy resin grips were made for each sample using a mould to prevent bone fracture and edge effects during loading.

6.2.4 Confocal imaging and mechanical loading conditions

Using the custom-built loading device, bi-directional, uniaxial compression loading of 3,000 µε, representative of vigorous physiological activity (Burr et al. 1996), was applied longitudinally to the bone samples. Cells were imaged in the mid-diaphysis of the femur in order to avoid characterising cells that might experience large displacements occurring at the proximal and distal surfaces near the grips. Confocal scans (Zeiss LSM 51) were taken with a 63x oil immersion lens, with 0.08 mm thick glass coverslips (CB00070RA1, Menzel-Glaser) separating the moisture in the sample from immersion oil and allowing imaging through the depth of the sample. Wavelength excitations of 488 nm and 543 nm were applied to scan the pericellular space and the cell membrane respectively (see Figure 6.3D), at a resolution of 1024 x 1024 pixels, pixel size of 0.1 µm and optical slice thickness of 0.6 µm. Multi-tracking was also performed to illuminate both the lacunar-canalicular network and the osteocyte cell membrane contained within (see Figure 6.3E and 6.3F respectively). Confocal scans of the osteocytes and osteoblasts can be seen in Figure 6.3F and 6.3G respectively. Photobleaching or leaching of the stains was not observed during the experiments. Imaging was performed at a depth of at least 50 µm, to prevent imaging of damaged regions from the cutting process. At this depth osteocytes and osteoblasts are easily discriminated in confocal microscopy due to the location of osteocytes within the bone matrix compared with the presence of osteoblasts on the bone surfaces.
Figure 6.3: Diagram of removal of proximal and distal ends of femur, followed by longitudinal sectioning of the sample (A-C). Imaging was performed at the mid-diaphysis, approximately 50 µm below the cut surface, indicated by the dotted line in (A) and the box in (C). Confocal scans performed from cut face through depth of bone (D), allowing visualisation of the lacunar-canalicular network in green (E) and osteocytes (F) and the osteoblasts (G) in red.

Scans were taken of the cells every 3 seconds for each 250 µε loading step in order to build a series of images to represent cell deformation during loading, resulting in a strain rate of 83.3 µε/s. For each femur sample loaded, the number of cells images was limited to ten osteoblasts and ten osteocytes, due to the amount of cells present at the mid-diaphysis. This resulted in a total of 160 cells, with 80 of each cell type for both OVX and SHAM animals, at 5- and 34-weeks post-operation. Sample images of both an osteocyte and osteoblast are shown at 0 µε in Figure 6.5A and 6.5B respectively.
6.2.5 Cell Viability

In order to investigate whether cells imaged away from the cut surface were detrimentally affected by the cutting process, a cell viability study was performed. A femur was harvested from a 4-month old female Wistar rat, sectioned and processed as described above. Instead of a cell membrane stain, the sample was incubated in a Live/Dead Viability/Cytotoxicity assay (L-3224, Invitrogen) for three hours. Confocal scans of the sample were taken five hours post-extraction at 10x magnification with excitation wavelengths of 488 nm, at depths of 0 and 50 µm below the cut surface. The resulting images are shown in Figure 6.4, with green indicating viability and red indicating cytotoxicity. Additionally, the percentage of live and dead cells at each depth has been quantified using ImageJ: 34.7% live vs. 65.2% dead at 0 µm; 89% live vs. 11% dead at 50 µm. These results show that although cell death occurs at the cut surface, at a distance 50 µm from the surface (the location at which where the strain analyses were conducted) there is a substantial population of live cells. As damage to the surrounding matrix would likely have a detrimental effect on cell viability, it can be inferred that the local mechanical environment of the osteocyte is not damaged during cutting.
Figure 6.4: Confocal scans of the same location in a femur sample at (A) 0 µm and (D) 50 µm from the cut surface. Cell viability is indicated by green staining (B and E), while cytotoxicity is denoted by red (C and F) (scale bar: 100 µm)

6.2.6 Digital Image Correlation (DIC) analysis

The DIC methods described above were applied to analyse a series of images of the loaded osteoblasts and osteocytes. This allowed the maximum principal strain field in the cells to be calculated, providing contour plots of strain distributions within the cells, shown in Figure 6.5C and 6.5D. The percentage area of a cell stimulated within a specific range of strain is determined by dividing the number of pixels at strain values within this range by the total number of pixels that represent the cell. As this is a 2D DIC analysis, the contour plots provide details of a section through the cell and is therefore denoted as the percentage area of this section of the cell strained.

6.2.7 Statistical Analysis

Ten of each bone cell type were analysed from each bone sample, giving n=20 per group (SHAM, OVX, etc.). Data are expressed as a mean ± standard deviation. Statistical differences between groups were determined using an ANOVA-crossed factor model, defined using the general linear model ANOVA function. Tukey’s test method for comparison between groups was used to determine statistical significance defined as \( p < 0.05 \) (MINITAB v. 16).
6.3 Results

6.3.1 Mechanical environments of osteoblasts and osteocytes in healthy bone

The maximum principal strain distribution experienced by a sample osteoblast and osteocyte as a result of the applied organ-level loading is shown in Figure 6.5. These strains experienced by osteoblasts and osteocytes, as a proportion of the cell area, are shown in Figure 6.6 and Figure 6.7 respectively. Strains experienced by osteoblasts from healthy bone exceeded the osteogenic strain threshold (10,000 µε) in a larger proportion of the cell (14.99 ± 3.59%) than osteocytes (7.76 ± 4.59%). Maximum strains experienced by osteoblasts in healthy bone were 24,449 µε (24,449 ± 2,631 µε), with healthy osteocytes experiencing strains of 37,252 µε (37,252 ± 5,629 µε).

![Figure 6.5](image)

**Figure 6.5:** Confocal images of (A) a sample osteocyte and (B) osteoblast at 0 µε. Digital image correlation (DIC) is applied to characterise the maximum principal strain in the (C) osteocyte and (D) osteoblast at 3,000 µε (scale bar: 10 µm)
A significant drop in the proportion of the cell exceeding the osteogenic strain threshold (10,000 µε) occurs in osteoblasts (2.16 ± 4.39% vs. 14.99 ± 3.59%, \( p \leq 0.039 \)) at 34 weeks after the SHAM operation. However, there was no significant change in this value for osteocytes (5.77 ± 2.60% vs. 7.76 ± 4.59%, \( p = 1.000 \)).

*Figure 6.6:* Average strain distributions observed after 5 and 34 weeks in osteoporotic (OVX) and healthy (SHAM) osteoblasts as a percentage of cell area. \( n = 20 \) samples per group of 2 animals, \(^a p < 0.05\) versus SHAM-5 at corresponding strain level, \(^b p < 0.05\) versus OVX-5 at corresponding strain level.

Contour plots showed greater variability in strain at the cell membranes than within the cell body for all cell types. This effect was exacerbated in the osteocyte environment, with a more heterogeneous distribution and the highest and lowest strains occurring within the cell processes.
Figure 6.7: Average strain distributions observed after 5 and 34 weeks in osteoporotic (OVX) and healthy (SHAM) osteocytes as a percentage of cell area. 
n=20 samples per group of 2 animals, \(a \ p<0.05\) versus SHAM-5 at corresponding strain level, \(b \ p<0.05\) versus OVX-5 at corresponding strain level

6.3.2 Mechanical environment of osteoblasts and osteocytes during osteoporosis

The effect of osteoporosis on strain within osteoblasts and osteocytes was examined, with the strain distribution for OVX and SHAM samples compared in Figure 6.6 for osteoblasts and Figure 6.7 for osteocytes. Strains exceeding the osteogenic strain threshold (10,000 \(\mu\varepsilon\)) in osteoblasts in bones exposed to 5 weeks of oestrogen deficiency occur in a similar proportion of the cell to healthy bone at 5 weeks (16.77 ± 4.78% vs. 14.99 ± 3.59%). However, strains exceeding the osteogenic strain threshold occur in a significantly larger proportion of osteocytes at 5 weeks of oestrogen deficiency compared to healthy osteocytes (17.77 ± 3.33% vs. 7.76 ± 4.59%, \(p \leq 0.027\)). In addition, osteoporotic osteoblasts exhibited maximum strains of 32,082 \(\mu\varepsilon\) (32,082 ± 4,014 \(\mu\varepsilon\)) while osteocytes experienced 46,881 \(\mu\varepsilon\) (46,881 ± 7,081 \(\mu\varepsilon\)).
After 34 weeks of oestrogen deficiency, the proportion of osteoblast and osteocyte cell areas experiencing strains above the osteogenic threshold is significantly lower than osteoblasts (2.71 ± 8.36% vs. 16.77 ± 4.78%, \( p \leq 0.010 \)) and osteocytes (1.83 ± 2.24% vs. 17.77 ± 3.33%, \( p \leq 0.001 \)) at 5 weeks oestrogen deficiency. Furthermore, there was no significant difference between osteoporotic cells and healthy cells after 34 weeks oestrogen deficiency for either osteoblasts (2.71 ± 8.36% vs. 2.16 ± 4.39%, \( p = 1.000 \)) or osteocytes (2.71 ± 8.36% vs. 5.77 ± 2.60%, \( p = 0.998 \)).

Similar to the contour plots of the healthy cells, the highest and lowest strains occurred at the cell membranes of both cell types, particularly in the cell processes of osteocytes. This strain behaviour within osteoblasts and osteocytes was not found to vary between osteoporotic or healthy bone cells.

### 6.4 Discussion

The study in this chapter represents the first direct experimental investigation of the effect of local mechanical environment on osteocytes and osteoblasts in situ, and the changes which occur during oestrogen deficiency. With the development of a custom-designed loading device for simultaneous confocal imaging and mechanical loading, and the application of digital image correlation (DIC) techniques, the strain distribution in the cells in both healthy and osteoporotic bone tissue was characterised in a rat model of osteoporosis. It is reported here for the first time that osteoblasts and osteocytes in both healthy and osteoporotic bone experience strains that are sufficient to stimulate osteogenic responses in vitro (>10,000 με) under applied global loading conditions representative of vigorous physical activity. However, while osteocytes in osteoporotic bone initially (5 weeks) experience osteogenic strains (>10,000 με) in a greater area of the cell (11%) than those in healthy bone, there is no significant difference for osteoblasts. In contrast, in long-term oestrogen deficiency (34 weeks) there is a significant decrease in the proportion of both osteoblasts and osteocytes exceeding the osteogenic strain threshold compared to the respective cells at 5 weeks oestrogen deficiency, such that there is no longer a significant difference between either osteoblasts or osteocytes in 34-week osteoporotic and healthy bone. This demonstrates that for an equivalent global mechanical stimulus (3,000 με) applied at the cellular scale, the local stimuli
experienced by osteoblasts and osteocytes are highly dependent on disease state. Moreover, these results suggest that oestrogen deficiency alters the mechanical environment of osteoblasts and osteocytes initially, but that a mechanobiological response occurs during long-term oestrogen deficiency to restore the mechanical environment.

A possible limitation of this study may arise due to the fact that, although confocal imaging allows the visualisation of the intact osteocyte environment under loading, DIC techniques can only be performed on a chosen 2D plane through the cell body. Thus, the assumption is made that the percentage area of the cell strained is representative of the strain experienced by the whole cell body in 3D. Furthermore, the movement of fluid flow, or movement of the cell out of the plane of the scan, cannot be visualised directly using DIC techniques. However, despite this limitation, this method represents the first approach to elucidate cellular strains in their local mechanical environment without destructive interference. Indeed, as the fluid is still present, the observed strains will be also be representative of fluid-induced cellular strains, and in fact the balance of direct strain versus fluid-induced strain may be a critical modulator of mechanotransduction in bone. It should also be noted that immunohistochemistry was not performed to identify the phenotype of the cells and thus it is possible that the cells on the bone surface are quiescent bone lining cells rather than active osteoblasts. However, these cells are present in the periosteum on the surface of femur, and therefore reside in the same local mechanical environment that osteoblasts and bone lining cells experience when on the bone surface. It should also be noted that a low number of animals were used in this study (n=2 per group). Nonetheless, significant differences were observed in stimulation of cells between animal groups. Further studies are needed to conclusively determine the mechanobiological link between these changes in stimulation of the cells and changes in the bone tissue properties caused by oestrogen deficiency, and these studies should include more animals per group to provide a greater degree of statistical confidence.

Previous experimental studies of bone cell mechanobiology have largely involved in vitro cell culture techniques (Charras and Horton 2002; Charras et al. 2001; Owan et al. 1997; Smalt et al. 1997; You et al. 2000). This study provides the first exploration of the strain experienced by osteocytes and osteoblasts in their local environments.
Maximum strains in healthy osteocytes reported here were approximately 37,000 µε, which are far in excess of the applied loading of 3,000 µε. Strain amplification was previously observed in the bone matrix of sectioned lacunae under 2,000 µε loading (35,000 µε) (Nicolella et al. 2005; Nicolella et al. 2006), but these methods altered the mechanical behaviour of the osteocyte environment and did not measure strains within the embedded cells directly. Previous theoretical and computational studies have proposed that strain amplification in osteocytes is governed by a glyocalyx or integrin attachments to the extracellular matrix (Han et al. 2004; Verbruggen et al. 2012; Wang et al. 2007). Such amplifications might explain how bone surface strains of 1,000 µε and 2,000 µε (Fritton et al. 2000; Burr et al. 1996) are amplified above the 10,000 µε threshold required to induce observed anabolic biochemical responses by bone cells in vitro (Pitsillides et al. 1995; Smalt et al. 1997; You et al. 2000). The results of the current study verify these predictions by providing experimental evidence that strain amplification, sufficient to elicit an osteogenic response, occurs in vivo. Whether such amplification is due to the presence of a glyocalyx or integrin attachments, which are present and remain intact in the live loaded samples, is not clear from the results and further studies are required to definitively address this question. It should also be noted that some of these high strain levels may be traumatic for the cells and may induce cell death (Knothe Tate and Niederer 1998). However, it has also been postulated that this death will trigger resorption and reconstruction processes, which may explain the decrease in bone cell stimulation for both healthy and osteoporotic bone over time observed here (Currey 1964).

This results of this chapter show that, osteoblasts are stimulated to a greater extent than osteocytes (based on proportion of the cell above 10,000 µε). Osteoblasts reside on the surface of bones and are exposed to the natural surface bending of bone during loading (Raab-Cullen et al. 1994; Fritton et al. 2000) and shear stress from the marrow (Birmingham et al. 2013; Coughlin and Niebur 2012a), but are also connected to the bone surface and other cells by discrete attachments (Grigoriou et al. 2005; Shapiro 2008). These factors would serve to amplify mechanical stimulation to these cells in vivo and likely account for the high strains observed in the current study. It is interesting to note that in vitro studies have shown that osteoblasts are more responsive to mechanical strain stimuli than fluid shear (Klein-Nulend et al. 1995b; Mullender et al. 2004a), whereas osteocytes are far more
responsive to fluid shear stimuli (Ajubi et al. 1996; Klein-Nulend et al. 1995b; Pitsillides et al. 1995; Westbroek et al. 2000), and as such their exposure to a high strain environment might play an important role in bone mechanobiology.

Despite the greater strain stimulation of osteoblasts observed here, the osteocyte likely experiences an additional stimulus resulting from loading-induced interstitial fluid flow, to which osteocytes are highly responsive (Ajubi et al. 1996; Klein-Nulend et al. 1995b; Westbroek et al. 2000). Furthermore, osteocytes were observed to experience greater maximum strain stimuli than osteoblasts, despite a smaller proportion of the osteocyte exceeding the osteogenic strain threshold. Interestingly, it was observed qualitatively that osteocytes experienced the greatest extremes of strain stimuli in the cell processes, which have been shown to be the most mechanosensitive area of the osteocyte (Adachi et al. 2009b; Burra et al. 2010; Klein-Nulend et al. 2013; Wu et al. 2011).

In this chapter cellular strains are compared to the applied strain to gain an insight into how the bone cell mechanical environment is altered in bone tissue from healthy and osteoporotic bone under the same boundary conditions. At the onset of osteoporosis micro-structural changes in bone strength (Brennan et al. 2011b; Brennan et al. 2012; Parfitt 1987), mass (Compston et al. 1989; Lane et al. 1998; Parfitt 1987), mineral density (Brennan et al. 2011a; Brennan et al. 2011b; Busse et al. 2009), trabecular architecture (Brennan et al. 2012; Busse et al. 2009; Compston et al. 1989; Lane et al. 1998), trabecular mineral and matrix composition (Brennan et al. 2011b; Brennan et al. 2012; Busse et al. 2009; McNamara et al. 2006) and micro-damage (Burr 2003; Dai et al. 2004; Mashiba et al. 2001; Schaffler 2003) occur. Previous studies have suggested that changes in osteocyte geometry occur during oestrogen-deficiency (Knothe Tate et al. 2004; KnotheTate et al. 2002; Sharma et al. 2012). Using ImageJ image analysis software the circularity of each cell body was calculated (i.e. the degree of roundness), and was found to be similar for SHAM (0.2779 ± 0.1236) and OVX osteocytes (0.2277 ± 0.1418). While a difference in the cell body during oestrogen deficiency was not observed, changes in geometry or the surface roughness of the canaliculi may occur during osteoporosis (Knothe Tate et al. 2002; Knothe Tate et al. 2004; Sharma et al. 2012). Indeed, these possible osteoporosis-related alterations to the canaliculi are investigated computationally in
Chapter 7. However, as the local bone cell mechanical environment is maintained intact after extraction, the experimental method in the current study captures the effects that the features described above would have on the strain sensed by the cell in vivo. Indeed, it is proposed here that it is in fact these complex changes in tissue structure and composition that dictate the cellular strains reported here. It is also important to note that oestrogen is thought to be necessary for osteocytes to respond to mechanical loading (Lanyon 1996), suggesting that oestrogen deficiency may alter the mechanosensitivity of bone tissue during osteoporosis (Tatsumi et al. 2006). Therefore while increased mechanical stimulation is observed here, further studies are required to investigate the subsequent osteocyte mechanotransduction.

The initial bone loss during oestrogen deficiency likely alters the micro-mechanical loading conditions of osteoblasts and osteocytes, but this has never before been demonstrated. The results of in this chapter show for the first time that both osteocytes and osteoblasts are initially (5 weeks) exposed to higher strains in osteoporotic bone than in healthy bone. However, in long-term oestrogen deficiency (34 weeks) the strains have returned to the levels of cells in healthy bone of the same age. Therefore, it is proposed that the elevated strains in the osteocytes, arising from the initial bone loss, acts as a stimulus to initiate an adaptive response in an effort to achieve homeostatic levels of strain. This response then leads to changes in bone tissue mineral content and modulus (Brennan et al. 2011a; Brennan et al. 2011b; Busse et al. 2009; McNamara et al. 2006) and these changes restore the strain environment to levels experienced by healthy cells. Increases in the thickness of individual trabeculae (Brennan et al. 2012; McNamara et al. 2006; Waarsing et al. 2004; Waarsing et al. 2006) occur in osteoporotic bone following the initial bone loss, which may also serve to compensate for bone loss and restore the strain environment of osteoblasts and osteocytes to homeostatic levels (Skerry 2008). It has recently been shown using both nanoindentation and quantitative backscattered electron imaging (qBEI) that increased fragility in cortical bone from osteoporotic humans is not accompanied by reduced stiffness or hardness in the material (Fratzl-Zelman et al. 2009). Furthermore, studies on cortical bone during osteoporosis in a sheep model observed a decrease in stiffness in short-term osteoporosis (12 months post-OVX) (Kennedy et al. 2009), similar to that observed in trabecular bone (Brennan et al. 2009; Brennan et al. 2011b). While long-term osteoporosis was also
studied (31 months post-OVX), the elastic modulus at this time point was not published (Healy et al. 2010). However, it was observed that despite increased porosity and turnover, the compressive strength of the bone did not change significantly over time (Healy et al. 2010; Kennedy et al. 2009). These studies suggest that temporal changes in cortical tissue properties occur in osteoporosis that are comparable to those observed in trabecular bone. Thus it has been proposed that a compensatory mechanobiological response to osteoporosis may also occur in cortical bone, to counter altered tissue mechanics due to oestrogen deficiency (Healy et al. 2010).

6.5 Conclusions

In this chapter, the first experimental evidence was shown that osteocytes in healthy bone tissue experience higher maximum strains ($37,252 \pm 5,629 \mu \varepsilon$) than osteoblasts ($24,449 \pm 2,631 \mu \varepsilon$), whereas osteoblasts experience elevated strains ($> 10,000 \mu \varepsilon$) throughout a greater proportion of their cell body than osteocytes. Most interestingly, it was observed that osteoporotic osteoblasts and osteocytes sense osteogenic strain magnitudes in a greater proportion of the cell (2-11%), with 25-31% greater maximum strains, than healthy cells. This validated the hypothesis that “Rapid bone loss at the onset of osteoporosis increases mechanical stimulation of osteoblasts and osteocytes.” Furthermore, it was seen that osteocytes are more stimulated than osteoblasts in osteoporotic bone, exhibiting 46% higher maximum strains. However, it was also seen that, in long-term osteoporosis, cellular strains decrease significantly compared to short-term osteoporosis, such that there is no significant difference between cells in healthy and osteoporotic bone. This suggests that a mechanobiological response has occurred to alter the mechanical environment, perhaps in an attempt to restore homeostasis, and suggests confirmation that “A compensatory mechanobiological response by osteocytes to increased loading results in subsequent alterations in local tissue mineralisation and stiffness in late-stage osteoporosis.” This study provides a greater understanding of the mechanobiology of osteoblasts and osteocytes during the disease of osteoporosis. The results from this chapter are employed in Chapter 7, using the models developed in Chapters 4 and 5 to investigate the effects of osteoporosis on the mechanical environment of the osteocyte.
Chapter 7

Mechanisms of Osteocyte Stimulation in Health and Osteoporosis

7.1 Introduction

The studies in Chapters 4 and 5 provided a novel insight into the mechanical strains and shear stresses experienced by osteocyte cells in their native environment in vivo using computational approaches. The experimental study in Chapter 6 examined the changes in the mechanical stimulation of the osteocyte in both health and osteoporosis. However, the reason for the changes in stimulation observed in these experiments remains to be established, and merits further investigation. In this chapter, the computational methods developed in Chapters 4 and 5 are applied, incorporating the experimental results obtained through the study described in Chapter 6, to further investigate potential explanations for alterations in the local mechanical stimulation of osteocytes during osteoporosis.

Osteoporosis is a disease that causes significant bone loss, architectural deterioration and degradation of macroscopic bone properties (Compston et al. 1989; Parfitt 1987). It has also been shown that complex changes in the tissue level mechanical properties occur following oestrogen deficiency in animal models (Brennan et al. 2011b; McNamara et al. 2006). The results from Chapter 6 are potentially explained by these changes in tissue properties, as they showed increased mechanical stimulation experienced by osteocytes embedded in bone tissue from an early oestrogen deficiency animal model compared to controls, an effect which was mitigated at the later stage of the animal model. The author proposed in Chapter 6 that a mechanobiological response may occur, as a result of the increased cell
stimulation experienced in early oestrogen deficiency, and that this response may manifest as an increase in bone tissue stiffness, which has been observed previously in trabecular bone (McGrotty et al. 2006; McNamara et al. 2006), serving to return mechanical stimuli on osteocytes to control levels. Furthermore, as tissue stiffness is highly correlated to mineral content (Currey 1988), the changes in mechanical stimulation observed in this thesis may result from a mechanobiology-driven change in mineralisation (McNamara 2010), though this link remains to be confirmed.

As well as the macroscopic and tissue level changes in bone tissue properties during osteoporosis, alterations in the local osteocyte environment have been observed. In particular it has been demonstrated that the lacunar-canalicular network in humans with osteoporosis is disorganised, with a more tortuous canalicular anatomy than healthy subjects (Knothe Tate et al. 2002; Knothe Tate et al. 2004). As demonstrated previously (Anderson and Knothe Tate 2008; Kamioka et al. 2012), including in Chapters 4 and 5 of this thesis (Verbruggen et al. 2013; Verbruggen et al. 2012), osteocyte stimulation is highly sensitive to the surrounding lacunar-canalicular architecture. Therefore, this increase in tortuosity during osteoporosis would likely affect the mechanical stimulation of the osteocyte in vivo. Similarly, the lacunar and canalicular walls have been observed to be rougher, with loose collagen fibrils and matrix debris, in an oestrogen deficient rat model of osteoporosis (Sharma et al. 2012). It has been postulated that this degradation could disrupt the ECM and PCM attachments connecting the cell processes to the canalicular wall (Sharma et al. 2012), which could in turn affect osteocyte mechanosensation and viability (Lanyon and Skerry 2001; Plotkin et al. 2005). Together these complex changes would be expected to alter the mechanical stimulation of the osteocyte, but the correlation between such changes and the mechanical environment of the osteocyte has not yet been established.

In order to investigate the role of changes in the lacunar-canalicular architecture on the mechanical environment of the osteocyte during osteoporosis, the attachments between the cell membrane and the extracellular matrix must be considered. Studies have previously investigated the role of proteoglycan pericellular matrix (PCM) elements, which tether the osteocyte to the surrounding matrix (You et al. 2004; Han et al. 2004; You et al. 2001b), and projections of the extracellular matrix (ECM) into the canaliculi, which disturb the flow or attach to the cell process via integrin
A particular challenge to understanding these mechanisms is the multi-physics nature of the osteocyte environment, which comprises solid cell membranes and a pericellular mesh of proteoglycans (You et al. 2004) that deform under fluid flow within the canalicular space. To date no computational approach has been capable of modelling this complex multi-physics behaviour, incorporating both mechanisms into a full scale model of the osteocyte and allowing elucidation of their strain amplification roles in health or osteoporosis.

In this chapter it was hypothesised that “The extracellular environment alters the mechanical stimulation of osteocytes during osteoporosis”. Therefore, the first objective of this study was to apply the experimental results from Chapter 6 alongside the previously developed finite element (FE) models from Chapter 4 to determine the link between changes in bone tissue properties and osteocyte mechanobiology during osteoporosis. Secondly, the computational fluid-structure interaction (FSI) methods from Chapter 5 were employed to explore the effects of osteoporosis on the local osteocyte mechanical environment by (a) including a more tortuous canalicular anatomy and (b) reducing the density of the PCM and ECM attachments to represent disrupted attachment to canalicular wall.

7.2 Materials and Methods

The models in this chapter use two different approaches, FE and FSI, in order to investigate whether changes in the extracellular environment alter the mechanical stimulation of osteocytes during osteoporosis. Firstly, the effects of changes in bone tissue properties on osteocyte stimulation were investigated. In order to elucidate the effects of tissue property changes during osteoporosis in isolation from loading-induced interstitial fluid flow, the FE models developed in Chapter 4 were employed. Secondly, localised changes to the lacunar-canalicular architecture have been observed in osteoporosis; a more tortuous canalicular anatomy (Knothe Tate et al. 2002; Knothe Tate et al. 2004) and rougher, degraded canalicular walls (Sharma et al. 2012). Idealised FSI models, in which these osteoporosis-related changes in lacunar-canalicular architecture can be simulated, facilitate investigation of the effects of these alterations on the mechanical environment of the osteocyte.
Chapter 7

7.2.1 Finite element model

The previously developed representative finite element models, described in Chapter 4 (Verbruggen et al. 2012), were employed to investigate the effects of alterations in material properties on the strain stimulation experienced by the osteocyte. These anatomically representative geometries were constant for each analysis. All materials were assumed to be isotropic and linear elastic with the following material properties: Elastic moduli of 16 GPa, 40 kPa and 4,471 Pa and Poisson’s ratios of 0.38, 0.4 and 0.3 were assigned to the ECM, the solid continuum PCM and the osteocyte respectively (Alexopoulos et al. 2003; Alexopoulos et al. 2005; Deligianni and Apostolopoulos 2008; Sugawara et al. 2008). However, in order to vary the tissue stiffness to determine its effect on osteocyte stimulation, as described in Section 7.2.3, a pressure boundary condition must be used in place of the displacement applied in Chapter 4. Therefore, a pressure was applied to an ECM surface to generate compressive loading equivalent to a global load of 3,000 µε.

7.2.2 Fluid-structure interaction model

An FSI model of an idealised osteocyte lacuna was developed, as described in detail in Chapter 5 (Verbruggen et al. 2013), which included ECM projections and PCM tethering elements. The ECM projections were modelled as conical protrusions, of height 0.08 µm and base radius 0.1 µm, which projected into the pericellular space in groups of four about the axis of the canaliculi (see Figure 7.1) (Wang et al. 2005). PCM tethering elements were included as cylinders of length 0.08 µm and radius 0.008 µm (Lemonnier et al. 2011; You et al. 2004), attaching the canalicular wall to the osteocyte cell process (Wang et al. 2005), and were organised in groups of eight about the axis of the canaliculi (see Figure 7.1). The ECM projections and PCM tethering elements were included at a spacing of 0.13 µm and 0.05 µm respectively, in order to closely represent their observed distribution in vivo (McNamara et al. 2009; You et al. 2004). Due to the small scale of PCM tethering elements and ECM projections relative to the size of the osteocyte, it was necessary to employ symmetry boundary conditions to reduce computational cost. Thus, a model representing an octant of the osteocyte environment, as shown in Figure 7.1, was generated that could characterise strains in these features at a high resolution.
The material properties of the ECM and osteocyte are the same as those described in Section 7.2.1. The flexural rigidity \((EI)\), defined as the product of the elastic modulus \((E)\) and the moment of inertia \((I)\), of PCM tethering elements has been determined previously as 700 pNm\(^2\) (Weinbaum et al. 2003). Assuming that a PCM tethering element is a solid cylinder, its moment of inertia can be calculated as follows:

\[
I = \frac{\pi r^4}{4}
\]

where \(r\) is the radius of the PCM tethering element. Taking the experimentally determined radius of 8 nm for these fibres (Lemonnier et al. 2011; You et al. 2004), and dividing the flexural rigidity by the resulting moment of inertia, an elastic modulus of 2.18 MPa can be calculated for the PCM tethering elements. Poisson’s ratio is assumed to be 0.4, similar to the experimentally-derived properties of the actin cytoskeleton (Gittes et al. 1993).

The properties of the interstitial fluid were assumed to be similar to water, with a density of 997 kgm\(^{-3}\) and a dynamic viscosity of 0.000855 kgm\(^{-1}\)s\(^{-1}\) (Anderson et al. 2005). Flow within the lacunar-canalicular system was assumed to be laminar in nature. Similar boundary conditions were applied to those described in Chapter 5. Briefly, a compressive uni-axial load of 3,000 µε is applied by means of a displacement boundary condition, while a pressure of 300 Pa is applied to the inlet on one face and the other openings are defined as outlets at a relative pressure of 0 Pa (Knothe Tate and Niederer 1998; Anderson et al. 2005; Manfredini et al. 1999; Steck et al. 2003). A staggered iteration FSI analysis is then conducted, as outlined in the author’s previous study in Chapter 5 (Verbruggen et al. 2013), with the results of this analysis interpolated onto the surface of the osteocyte to allow elucidation of the strain within the cell.
**Figure 7.1**: Diagram of osteocyte model showing the three components of the osteocyte environment (ECM, PCM, Osteocyte), modelled as an octant using symmetry. The location and arrangement of the PCM tethering elements and ECM projections is indicated.

### 7.2.3 Investigation of the effects of altered bone tissue properties on osteocyte mechanical stimulation

In order to determine whether the changes in strain observed in the experimental study in Chapter 6 can be explained by changes in tissue mineralisation, the elastic modulus of the ECM tissue surrounding the osteocyte was varied while applying a constant stress. Thus, the modulus was gradually reduced until the average volumes experiencing osteogenic strain across the four representative models matched the average volume experienced by cells from the experimental results of Chapter 6, for the SHAM and OVX at both 5 and 34 weeks post-operative.
To elucidate whether these changes in properties could be related to experimentally reported changes in tissue mineralisation (Brennan et al. 2009; Brennan et al. 2011b; Busse et al. 2009), the resulting elastic moduli were then converted to a corresponding calcium content using a previously developed power law equation (Currey 1988):

$$\log(E) = -9.16 + 4.30 \log(Ca)$$

where $E$ denotes the elastic modulus and $Ca$ denotes calcium content. These values for calcium content were then converted to weight percentage calcium (wt% $Ca$).

### 7.2.4 Investigation of the effects of a more tortuous canalicular anatomy on osteocyte mechanical stimulation

In order to investigate observed changes in the tortuosity of the lacunar-canalicular network during osteoporosis (Knothe Tate et al. 2002; Knothe Tate et al. 2004), an idealised osteocyte model was generated in which a more tortuous canalicular anatomy was included. As quantitative data on the degree of increased tortuosity in osteoporosis is not available, this was modelled by alternately adjusting the axis of one of the canaliculi by 45° every 2 µm along its length. In order to isolate the effect of this geometry from the amplifying effects of the PCM tethering elements and ECM projections, this increased tortuosity was applied in models both with and without these strain amplification mechanisms, which are described in detail in Section 7.2.2 above.

### 7.2.5 Investigation of the effects of altered strain amplification mechanisms on osteocyte mechanical stimulation

Exploration of the effects of disrupted attachment of the cell process to the canaliculus during osteoporosis, which has been proposed based on experimental observations (Sharma et al. 2012), required a separate parameter variation study in which the density of each type of strain amplification mechanism was reduced. Thus, the spacing between ECM projections was varied between 0.13µm, 0.5 µm, 0.75 µm and 1 µm, while the distance between adjacent PCM tethering elements varied between 0.05 µm, 0.1 µm and 1 µm.
7.3 Results

The results of this study are presented in detail below. Each section addresses a specific question related to the global hypothesis, which is “The extracellular environment alters the mechanical stimulation of osteocytes during osteoporosis”.

7.3.1 Are changes in osteocyte stimulation explained by altered bone tissue properties?

The temporal differences in the experimentally observed volume of osteocytes stimulated above the osteogenic strain threshold (>10,000 µε), between healthy and osteoporotic bone, are shown in Figure 7.2. The proportion of the osteocyte models exceeding this threshold at various degrees of ECM stiffness is shown alongside them. The elastic modulus of the ECM was varied incrementally between 2 and 4 GPa to match the proportion of osteogenic stimulation to the experimental results. The maximum and minimum values are shown for comparison in Figure 7.2, with the elastic moduli that closely match the experimental data from Chapter 6 also shown. These values are listed in Table 7.1 alongside their corresponding mineral content, expressed as calcium content, for control and oestrogen deficient data at 5 and 34 weeks post-operation. These results indicate that, at 5 weeks post-OVX, a weight percentage decrease of 0.66 wt% Ca, corresponding to a decrease in elastic modulus of 0.425 GPa, could explain the increased strain stimulation experienced by osteocytes during the early stages of osteoporosis in Chapter 6. Similarly, by 34 weeks the mineralisation could increase to 17.94 wt% Ca, representative of a 1.175 GPa increase in stiffness, thus explaining the decreased strain stimulation at 34 weeks post-OVX observed in Chapter 6.
Figure 7.2: Strain distribution in computational models at various degrees of stiffness (highlighted above their respective data), alongside the strain distributions observed in Chapter 6, at various stages of both health and oestrogen deficiency. Osteogenic strain stimulation is shown for the maximum and minimum values (2 and 4 GPa) of the range within which the modulus was varied.

<table>
<thead>
<tr>
<th></th>
<th>Elastic Modulus E (GPa)</th>
<th>Calcium Ca (mg/g)</th>
<th>Weight Percentage wt% Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM-5</td>
<td>2.75</td>
<td>170.76</td>
<td>17.08</td>
</tr>
<tr>
<td>OVX-5</td>
<td>2.325</td>
<td>164.23</td>
<td>16.42</td>
</tr>
<tr>
<td>SHAM-34</td>
<td>3.4</td>
<td>179.40</td>
<td>17.94</td>
</tr>
<tr>
<td>OVX-34</td>
<td>3.5</td>
<td>180.62</td>
<td>18.06</td>
</tr>
</tbody>
</table>

Table 7.1: Differences in elastic modulus and calcium content based on experimentally observed cell strains.
7.3.2 Are osteoporosis-related changes in osteocyte stimulation explained by altered canalicular tortuosity?

The velocity, shear stress and strain distributions experienced by osteocytes with a tortuous canalicular structure are compared to a non-tortuous anatomy in Figure 7.3. These results are graphed in Figures 7.4A, 7.4B and 7.5 for velocity, shear stress and strain respectively. This data demonstrates that a decrease in fluid velocity and shear stress stimulation occurs with a tortuous anatomy in the model without the PCM and ECM attachments. However, this effect appears negligible with the inclusion of the PCM and ECM attachments. In contrast, the strain stimulation experienced by the osteocyte can be seen to increase noticeably with a more tortuous geometry, both with and without the presence of PCM and ECM attachments. This strongly suggests that a more tortuous canalicular anatomy in osteoporosis increases the strain simulation of the osteocyte, and that this osteoporotic geometry effect is independent of PCM and ECM attachments. This change in osteogenic strain stimulation is compared in Figure 7.5 to the changes in osteogenic strains observed experimentally in Chapter 6. It can clearly be seen that, while a more tortuous anatomy in osteoporosis may contribute to increases in strain stimulation, it cannot completely account for the larger increase observed experimentally.
Figure 7.3: Changes in velocity, shear stress and maximum principal strain distribution in models with cellular attachments (A–C), and also with a more tortuous anatomy, representative of osteoporosis (Knothe Tate et al. 2002; Knothe Tate et al. 2004) (D–F)

Figure 7.4: Effects of a more tortuous canalicular anatomy on (A) velocity and (B) shear stress, both with and without ECM and PCM attachments
Figure 7.5: Effects of a more tortuous canaliculair anatomy on maximum principal strain distribution within an osteocyte, both with and without ECM and PCM attachments. The experimental data of Chapter 6 is also shown, for healthy and oestrogen deficient bone.

7.3.3 Are osteoporosis-related changes in osteocyte stimulation explained by altered strain amplification mechanisms?

The effects of ECM projections and PCM tethering elements on fluid flow, shear stress and strain experienced by the osteocyte are shown in Figure 7.36. Both ECM projections and PCM tethering elements appear to act to slow the overall fluid flow, resulting in correspondingly lower shear stresses (see Figures 7.7 and 7.8). However, the presence of these cellular attachments acts to increase strain experienced by the osteocyte (see Figure 7.9), thus suggesting an inverse relationship between velocity/shear stress and strain within the cell. It should also be noted that no uncoupling of the solid and fluid domains was observed during these simulations.
Figure 7.6: Streamlines showing velocity around the osteocyte and within canaliculi, shear stress imparted onto the cell membrane and resulting maximum principal strain distribution within the osteocytes for (A-C) the osteocyte model, (D-F) osteocyte model with PCM tethering elements and (G-I) osteocyte model with ECM projections respectively. The effect of these features on velocity streamlines is highlighted in the diagram.

When both PCM tethering elements and ECM projections are included at their observed normal in vivo spacing the lowest values for velocity (26.55 µm/s), shear stress (6.08 Pa) and volume strained above the osteogenic strain threshold (2.82%) are observed. When the density of each of these attachments is reduced, as is proposed to occur during oestrogen deficiency (Sharma et al. 2012), slight increases in velocity, shear stress and strain occur (see Figures 7.7, 7.8 and 7.9). However, these differences are so small as to be negligible, with values that are still sufficiently high to generate an osteogenic response (shear stress: 7.2-7.7 Pa; strain:
3.28-3.79%), demonstrating that reduced density of these attachments in disease does not result in decreased stimulation for bone growth.

Figure 7.7: Maximum interstitial fluid velocities within the pericellular space in osteocyte models with varying densities of PCM tethering elements (A) and ECM projections (B)

Figure 7.8: Maximum shear stresses on the osteocyte cell membrane with varying densities of PCM tethering elements (A) and ECM projections (B)

Figure 7.9: Changes in strain distribution with increasing density of (A) PCM tethering elements and (B) ECM Projections
7.4 Discussion

This study employs finite element and fluid-structure interaction computational techniques to elucidate the mechanisms by which osteocytes are stimulated in vivo, in both healthy bone tissue and bone from an animal model of osteoporosis. By simulating mechanical loading, it was observed that changes of 0.425-1.175 GPa in tissue stiffness, and corresponding changes in mineral content, could explain the changes in osteocyte stimulation during the progression of osteoporosis, which were observed in Chapter 6. Furthermore, an osteoporosis-related increase in canalicular tortuosity was shown to result in increased osteogenic strain stimulation, although this increase was not large enough to explain the experimental results in Chapter 6. However, a reduction in the presence and density of cellular attachments, disruption of which has been proposed to occur during osteoporosis (Sharma et al. 2012), does not significantly affect the levels of shear stress or strain stimulus, suggesting that it does not affect PCM permeability.

In order to replicate the intricate environment of the osteocyte, a number of assumptions were necessary. These assumptions concerning material properties, isotropy, laminar flow, etc. have been detailed previously in Chapters 4 and 5. Limitations particular to this study involve a lack of available quantitative data on both increased tortuosity and degradation of the canalicular walls. While increased tortuosity (Knothe Tate et al. 2002; Knothe Tate et al. 2004) and decreased attachments to the ECM (Sharma et al. 2012) have been indicated by experiments, the degree of changes that occur remain to be quantified. Therefore, the increased tortuosity and reduced density of attachments are modelled here based on assumed parameters, and further experimental studies to determine the quantitative changes in geometry that occur during osteoporosis are required. It should also be noted that, to date, no approach exists to link osteoporotic changes in the macroscopic stiffness of bone tissue with changes in loading-induced fluid flow in the local osteocyte environment. For this reason the elastic modulus variation study was performed using a finite element approach, as it is not known what effect osteoporosis would have on the applied 300 Pa pressure gradient representing macroscopic fluid flow. Incorporation of fluid flow could potentially add to the stimulation the effects of changes in tissue properties in a full FSI simulation, but further experimental studies...
are required to firmly establish the link between osteoporotic tissue changes and loading-induced fluid flow.

The previous study, outlined in Chapter 6, showed increased stimulation experienced in early oestrogen deficiency, but that in the later stage of osteoporosis this was mitigated. It was proposed that a cell-driven mechanobiological response may occur due to the increased stimulation experienced in early oestrogen deficiency, and that this response may manifest as increased bone tissue stiffness, which has been observed in trabecular bone (McGrotty et al. 2006; McNamara et al. 2006).

In this chapter, finite element models with representative osteocyte geometries were employed to determine whether these changes could be explained by alterations in bone tissue stiffness and mineralisation during oestrogen deficiency. By varying the elastic modulus of the ECM in the models to achieve similar strains as those observed in the experimental studies of Chapter 6, it was predicted that an initial decrease in tissue stiffness of 0.425 GPa relative to controls, followed by a later increase of 1.175 GPa over long-term osteoporosis, could explain the mechanical stimuli observed experimentally. Previous studies have also observed temporal changes in bone tissue stiffness of trabeculae from an ovine model of osteoporosis, and reported initial (12 months post-OVX) decreases in mineral content and stiffness (by 3.4 GPa) before increasing (by 0.7 GPa) to match control values in late-stage osteoporosis (31 months post-OVX) (Brennan et al. 2009; Brennan et al. 2011b). This suggests that a compensatory mechanobiological response occurs in response to initial increased loading, resulting in subsequent increases in stiffness to reduce this loading. Similarly, cortical bone in ovine studies has been observed to result in decreased stiffness (by 1.7 GPa) in early (12 months post-OVX) osteoporosis (Kennedy et al. 2009). These ovine studies also found that, as the animals progressed into long-term oestrogen deficiency, there was no significant difference in cortical compressive strength by 31 months post-OVX (Healy et al. 2010; Kennedy et al. 2009).

These changes in tissue properties in both trabecular and cortical bone corroborate the predictions of the models in the current study, but are not in the same range of values, possibly due to differences between the animal models or time points. Nonetheless, together with these experimental findings, the current study supports
the hypothesis that a compensatory mechanobiological response to changes in tissue properties occurs in osteoporotic bone. Furthermore, it has been proposed that these changes in stiffness during osteoporosis could be the result of increased mineralisation (McNamara 2010) or alteration in the mineralisation of the lamina limitans (Takagi et al. 1991). Conversion of the elastic moduli in the current study to mineral content demonstrates a corresponding initial significant decrease in mineralisation of 0.66 wt% Ca, followed by later increase of 1.4 wt% Ca such that there is no significant difference compared to controls. This same trend, with an initial decrease of 2.1 wt% Ca followed by a subsequent increase of 0.6 wt% Ca, was observed in an ovine model of temporal changes in mineralisation during osteoporosis (Brennan et al. 2009; Brennan et al. 2011b). Future studies could use similar techniques to further investigate the connection between mineralisation and in vivo osteocyte stimulation. The predictions of the models in the current study demonstrate that observed osteoporotic changes in tissue properties may be correlated to osteocyte stimulation, elucidating a possible mechanobiological link between the cellular environment and macroscopic bone properties in the development of osteoporosis.

One significant alteration to the osteocyte environment, which has been shown to occur in osteoporosis, is an increase in tortuosity of the canaliculi (Knothe Tate et al. 2002; Knothe Tate et al. 2004). By applying the computational modelling approaches developed in this thesis, it was predicted that such changes can lead to an increase in osteogenic strain stimulation of osteocytes, both with and without cellular attachments. These results reinforce the findings of Chapter 4 that osteocytes are highly sensitive to changes in canalicular geometry (Verbruggen et al. 2012), and suggests that increased canalicular tortuosity is a mechanism by which osteocytes may sense or react to osteoporotic changes. However, when compared to the increases in osteogenic strain stimulation observed in the experimental study in Chapter 6 it was seen that, while increased tortuosity may contribute, it cannot completely explain the difference in osteoporosis. Moreover, it is not known whether this increased tortuosity is a direct response to oestrogen deficiency, or whether the canalicular geometry is altered by the osteocytes themselves when the loading environment is altered during osteoporosis, in order to heighten sensitivity to strain (Harris et al. 2007). The observed increases in tortuosity may also be caused by a
“slackening” of the cell processes, with resulting alterations to the canalicular geometry, although this has not been directly observed. However, these theories remain conjecture, and therefore further studies which illuminate the temporal changes in canalicular anatomy during disease may provide a greater understanding of the development of this feature.

While previous theoretical studies have investigated both PCM tethering elements and ECM projections (Han et al. 2004; Wang et al. 2007; You et al. 2001b), this multi-physics computational study seeks to explore these mechanisms of stimulation in the context of loading-induced fluid flow in the normal and osteoporotic environments of a whole osteocyte cell. Studies have shown significant degradation and resulting increased roughness of the lacunar and canalicular walls in an ovariectomised model of osteoporosis (Sharma et al. 2012). It has been suggested that this degradation may disrupt cellular attachments to the canalicular wall (Lanyon and Skerry 2001; Plotkin et al. 2005; Sharma et al. 2012), modelled here as decreased density of ECM projections and PCM tethering elements. The results of the current study show that reduced density of either attachment type does not significantly affect the shear stress or strain stimulus to the osteocytes, indicating that it may not be the mechanism by which osteocytes sense changes in disease.

### 7.5 Conclusions

The results of this chapter determined the effect of bone tissue stiffness and mineralisation on osteocyte stimulation, thus elucidating a possible mechanobiological link in the temporal development of osteoporosis. Furthermore, osteoporosis-related canalicular tortuosity was shown to result in increased osteogenic strain stimulation, though to a lesser extent than that observed experimentally in Chapter 6. Finally, it was observed that a reduction in the presence and density of cellular attachments, disruption of which has been proposed to occur during osteoporosis, does not alter the levels of shear or strain stimulus to the osteocyte. This research indicated that the changes in the extracellular environment during osteoporosis, arising from altered mineralisation and lacunar-canalicular architecture, lead to an altered mechanical stimulation of osteoblasts and osteocytes. The findings of this chapter, along with those of Chapters 4 and 5, provide a novel
insight into the complex in vivo mechanical environment of osteocytes and confirm that some aspects of “The extracellular environment alter the mechanical stimulation of osteocytes during osteoporosis.” Moreover, the findings compliment the experimental approach in Chapter 6, providing a novel insight into the osteocyte environment in both health and osteoporosis.
Chapter 8

Discussion and Conclusions

8.1 Introduction
This chapter summarises the main findings of this thesis, drawing together the insight obtained from the different experimental and computational techniques performed to provide a greater understanding of bone mechanobiology, in particular relating to the temporal changes occurring during osteoporosis. Finally, recommendations for further work and future perspectives in the field are discussed.

8.2 Main Findings of Thesis
The research described in this thesis has focused on the mechanical environment of bone cells during physiological loading, in both healthy and osteoporotic bone. Computational modelling was performed using finite element and fluid-structure interaction techniques to investigate the different mechanical stimuli experienced by osteocytes. An experimental method was also developed to analyse the strain environment of osteoblasts and osteocytes in both healthy and oestrogen-deficient bone, allowing characterisation of the mechanobiological changes that occur in osteoporosis. The key contributions of each chapter are summarised as follows:

1. The first study of this thesis used finite element modelling to investigate the role of anatomically accurate geometry in dictating mechanical stimulation of osteocytes in vivo. The results of this study provided direct evidence that representative geometries predict 350-400% greater strain amplification experienced by osteocytes compared to an idealised cell, and also that the
pericellular and extracellular matrix components amplify strain stimulation to the cell by 10-420%. These anatomically representative models demonstrated the significance of geometry to strain amplification within the osteocyte mechanical environment and thus confirmed the hypothesis that “Mechanical loading to the osteocyte is amplified by the native geometry of the osteocyte environment.”

2. The second study employed fluid-structure interaction (FSI) modelling to investigate the complex multi-physics environment of osteocytes in vivo. Building upon the realistic models developed in the first study, fluid-structure interaction methods were used to simulate in vivo loading of osteocytes. The results of these studies predicted average interstitial fluid velocities (~60.5 µm/s) and average maximum shear stresses (~11 Pa) surrounding osteocytes in vivo. Interestingly, the greatest mechanical stimulation of the osteocyte was observed in the cell processes and these stimuli were within the range of stimuli known to stimulate osteogenic responses by osteoblastic cells in vitro, suggesting validation of the hypothesis that “Loading-induced interstitial fluid flow significantly contributes to the mechanical stimulation of osteocytes in vivo.” These computational FSI models provide a novel insight into the complex multi-physics mechanical environment of osteocytes in vivo.

3. The third study of this thesis aimed to characterise the strain environment of osteoblasts and osteocytes under physiological loading conditions, in healthy and osteoporotic bone, in a rat model of osteoporosis. A custom-designed loading device compatible with a confocal microscope was constructed to apply deformation to fluorescently stained femur samples from healthy and ovariectomised rats. It was shown that, although osteocytes in healthy bone tissue experience higher maximum strains than osteoblasts, a larger proportion of the osteoblast cell body exceeded the osteogenic strain threshold compared to that of the osteocyte. Most interestingly, osteoporotic osteoblasts and osteocytes initially (5 weeks post-OVX) experienced higher maximum strains than healthy cells and exceeded the osteogenic strain threshold in a larger proportion of the cell. This validated the hypothesis that “Rapid bone loss at the onset of osteoporosis increases mechanical stimulation of osteoblasts and osteocytes.” However, after prolonged
oestrogen deficiency (34 weeks) there was no significant difference between cells in healthy and osteoporotic bone, suggesting confirmation that “A compensatory mechanobiological response by osteocytes to increased loading results in subsequent alterations in local tissue mineralisation and stiffness in late-stage osteoporosis.” The results of this study show for the first time that the mechanical environment of osteoblasts and osteocytes is altered during early-stage osteoporosis. It was proposed that a mechanobiological response restores the homeostatic mechanical environment by late-stage osteoporosis.

4. The final study of the thesis combines the computational models developed in Chapters 4 and 5, with the experimental results of Chapter 6, to investigate whether changes in tissue properties, lacunar-canalicular architecture, or PCM and ECM attachments, during osteoporosis could explain the altered mechanical stimulation of osteocytes observed. A parameter variation study was used to determine the effect of bone tissue stiffness and mineralisation on osteocyte stimulation, and elucidated a possible mechanobiological link between increases in strain stimulation of the osteocyte and subsequent increases in mineralisation of bone tissue. Furthermore, osteoporosis-related canalicular tortuosity was shown to result in increased osteogenic strain stimulation. However, it was observed that a reduction in the presence and density of cellular attachments, disruption of which has been proposed to occur during osteoporosis, does not substantially alter the levels of shear or strain stimulus to the osteocyte. The findings of this chapter, along with those of Chapters 4 and 5, provide a greater understanding of the complex in vivo mechanical environment of osteocytes and confirm that some aspects of “The extracellular environment alter the mechanical stimulation of osteocytes during osteoporosis.” Moreover, the findings compliment the experimental approach in Chapter 6, providing a novel insight into osteocyte mechanobiology in both healthy bone and osteoporosis.

These findings are further considered in the context of current understanding of bone mechanobiology and osteoporosis below.
8.3 Insight into bone mechanobiology

The findings of this thesis provide a novel insight into the mechanobiology of osteocytes. Since the identification of the osteocyte as the most mechanosensitive bone cell (Ajubi et al. 1996; Klein-Nulend et al. 1995b; Westbroek et al. 2000), now thought to be the primary regulator of osteogenesis (Birmingham et al. 2012b), much research has focussed on the stimuli they sense in vivo. The challenges of directly observing these embedded cells in vivo have led researchers to model the environment numerically in an attempt to overcome these experimental obstacles. While previous theoretical models have investigated the effects of loading-induced fluid flow in an idealised canaliculus with various extracellular components, both the lacunar-canicular geometry and the osteocyte cell body were neglected (Han et al. 2004; Wang et al. 2007; You et al. 2001b). Finite element modelling has also been applied to determine the strains in the extracellular matrix of an idealised lacuna (Rath Bonivtch et al. 2007). Furthermore, computational fluid dynamics techniques were applied to an idealised pericellular space (Anderson et al. 2005) and later to a 3D representation of a realistic 2D geometry (Anderson et al. 2008).

All of these studies added new knowledge to the field, shedding further light on the in vivo mechanical environment of the mechanosensitive osteocyte. However, an approach that incorporated representative geometries, discrete cellular attachments and multi-physics boundary conditions was required to more closely mimic the in vivo environment of the osteocyte. The computational studies conducted during the course of this PhD built upon these previous models of the osteocyte mechanical environment (see Figure 8.1), exploring both the complex geometry and the multi-physics nature of this system. The results of these studies demonstrated the importance of the pericellular matrix, the significance of loading-induced fluid flow as a stimulus, and the dominant role of the canalicular geometry on the mechanical stimulus to the osteocytes. Development of these models also allowed exploration of various effects of osteoporosis on the local osteocyte environment, which are considered in the following section.
In addition to the previous modelling approaches described above, attempts have been made to experimentally investigate the local mechanical environment of osteocytes. A key study in this area employed digital stereoimaging techniques to characterise the strain distribution in the bone matrix surrounding osteocyte lacunae (Nicolella et al. 2006). This was performed by sectioning and polishing bone tissue to expose osteocyte lacunae on a microscopy plane, allowing visualisation of matrix deformation under loading (Nicolella et al. 2006). However, this experimental approach involves milling and polishing, both of which can introduce microcracks (Nicolella et al. 2005; Nicolella et al. 2006), and thus alter the mechanical environment of the cell (Reilly 2000; Zioupos and Currey 1994). Additionally, AFM techniques have previously been used to measure osteoblast strains under an applied loads in vitro (Charras and Horton 2002; Charras et al. 2001). However, point loading through AFM techniques is not representative of the substrate strain that osteoblasts on bone surfaces are exposed to in vivo.

Therefore, despite these important studies, a clear gap in knowledge of strains experienced by bone cells in vivo existed, and the experimental study in Chapter 6 sought to address this. Using a custom-designed loading device, which was compatible with a confocal microscope, it was possible to directly observe individual osteoblasts and osteocytes under applied loading in their native mechanical environments. Application of digital image correlation (DIC) techniques provided a
novel insight into the strain stimulation experienced by osteoblasts and osteocytes under loading conditions analogous to vigorous physiological activity, deepening current understanding of bone cell mechanobiology. This study also facilitated an investigation of the temporal effects of osteoporosis on bone cell stimulation, which is discussed further in the following section.

8.4 Insight into the mechanobiological effects and temporal development of osteoporosis

The mechanobiology of osteocytes, as explored in this thesis, is crucial to the adaptive nature of bone. Therefore it likely plays a key role in the disease of osteoporosis, particularly in the sequence of events that occur through the progression of the disease. As described in Chapter 2, osteoporosis is characterised by reduced bone mass and degradation of micro-architecture. This results in deterioration of bone strength and increasingly brittle material properties, ultimately leading to greater risk of fracture.

The disease of osteoporosis, at its core, arises from defective cellular behaviour that disrupts the natural bone remodelling cycle (McNamara 2011). While the most common form of the disease, post-menopausal osteoporosis, is driven by oestrogen deficiency, the sequence of events ultimately resulting in bone fracture has not yet been fully characterised. For a long period of time it was accepted that bone loss was the predominant change in osteoporotic bone, and that bone tissue properties were either decreased or unchanged (Bohic et al. 2000; Gadeleta et al. 2000; Loveridge et al. 2004; Rohanizadeh et al. 2000). However, this theory was first confounded by a study that showed a counterintuitive increase in stiffness and mineralisation in long-term osteoporosis (McNamara et al. 2006). This study was further corroborated by a study demonstrating increased calcium content within individual trabeculae during oestrogen deficiency (Busse et al. 2009). Oestrogen deficiency was also found to result in increased heterogeneity in mineral composition, both in individual trabeculae and across different anatomical sites (Brennan et al. 2011a; Busse et al. 2009). These unexpected findings led to further investigations of temporal changes in bone material properties over the course of osteoporosis, in an attempt to explain
discrepancies from different studies involving various animal models and experimental time points. Studies of trabeculae in an ovine model of osteoporosis observed an initial significant decrease (12 months post-OVX) in both mineral content and elastic modulus relative to controls (Brennan et al. 2009), followed by increases in both properties in long-term oestrogen deficiency (31 months post-OVX) such that there was no longer a significant difference compared to controls (Brennan et al. 2011b). Similarly, studies on cortical bone during osteoporosis in an ovine model observed a decrease in tissue stiffness in short-term osteoporosis (12 months post-OVX) (Kennedy et al. 2009). While long-term osteoporosis was also studied (31 months post-OVX), the elastic modulus at this time point was not published (Healy et al. 2010). However, it was observed that despite increased porosity and turnover, the compressive strength of the bone did not change significantly over time (Healy et al. 2010; Kennedy et al. 2009). These studies demonstrate that complex changes in both trabecular and cortical tissue properties occur during the temporal development of osteoporosis. Interestingly, the results of this thesis (Chapter 6), show that the mechanical stimulation of osteocytes is initially increased (5 weeks post-OVX), but returns to control levels by 34 weeks post-OVX. The results of Chapter 7 further explain this finding, and shed light on the various studies demonstrating contradictory changes in bone tissue properties and mineralisation during osteoporosis.

Two theories were previously proposed to describe the series of events which are set in motion by oestrogen deficiency, shown in Figure 8.2 (McNamara 2010). These theories were developed in an attempt to unite knowledge that has been captured by research in disparate fields, with a particular focus on understanding how increased tissue properties could arise in trabeculae of ovariectomised sheep, and are described in greater detail elsewhere (McNamara 2010).

The first theory describes the sequence of events initiating with increased osteoclast activity, which arises from upregulation or downregulation of various molecules during oestrogen deficiency (see Figure 8.2A) (McNamara 2010), and is commonly accepted as the primary event in the bone loss cascade. Osteoclast bone resorption results in reduced bone mass, and it was proposed that this bone loss results in elevated mechanical stimuli in osteocytes, embedded in the remaining bone and thereby subject to elevated loading. It was thus suggested that altered mechanical
stimulation may elicit a secondary adaptive mechanobiological response from the osteocytes and osteoblasts to alter the local mineral composition of the bone tissue to compensate for this bone loss (McNamara 2010). This would ultimately lead to more brittle bone, micro-damage and an associated greater risk of fracture, together with the depleted bone mass and architecture.

The alternative theory, outlined in Figure 8.2B, proposes initiation of the bone loss cascade through the effects of oestrogen deficiency on osteoblasts (McNamara 2010). Differential expressions of various genes would result in changes in collagen matrix production and tissue mineralisation, giving rise to increased stiffness of the local tissue matrix caused by osteoblasts. Thus it was proposed that bone resorption may occur as a secondary mechanobiological response to this increased stiffness, as the resulting lower loading would stimulate both osteocytes and osteoclasts to produce a resorption response. The resulting loss of bone mass would trigger increased micro-damage and ultimately cause osteoporotic fractures.
The findings of the experimental study in Chapter 6 determined that, at the onset of osteoporosis (by 5 weeks post-OVX), the proportion of osteogenic strain experienced by the osteocyte increases significantly compared to controls. A significant decrease in strain stimulation is then observed after 34 weeks of oestrogen deficiency, returning to strain levels experienced by controls. Moreover, there was no significant change in controls over this period, suggesting that these changes are entirely related to oestrogen deficiency. It was proposed that a mechanobiological response to initial decreased bone mass may be occurring, in an attempt to return the mechanical strain experienced by the osteocyte to homeostasis. This proposed mechanobiological link in the temporal development of osteoporosis was investigated further in the final study in Chapter 7. By processing the experimental data from Chapter 6 using the computational models developed in...
Chapter 4 the bone tissue stiffness, and consequently mineralisation, was derived in early- and late-stage osteoporosis. The results were found to demonstrate a similar pattern, an initial decrease in tissue stiffness of 0.425 GPa relative to controls, followed by a later increase of 1.175 GPa over long-term osteoporosis, to previous experimental studies of ovine bone. These ovine studies involving trabecular bone observed initial (12 months post-OVX) decreases in mineral content and stiffness (by 3.4 GPa) before increasing (by 0.7 GPa) to match control values in late-stage osteoporosis (31 months post-OVX) (Brennan et al. 2009; Brennan et al. 2011b). Similar initial decreases in stiffness (by 1.7 GPa) were also observed in ovine cortical bone in early-stage osteoporosis (Kennedy et al. 2009).

In order to better understand the sequence of events in the bone loss cascade, the research from Chapters 6 and 7 must be placed in the context of known temporal changes due to oestrogen deficiency in an ovariectomised rat model of osteoporosis. It has been observed that osteoclast activity increases rapidly during oestrogen deficiency in rats, with significant increases in osteoclast numbers observed 1 week post-OVX (Hughes et al. 1996). Furthermore, analysis of bones from ovariectomised rats have shown that decreases in bone volume fraction, trabecular number and connectivity density of trabeculae occur as early as 4 weeks post-OVX (Keiler et al. 2012), indicative of significant bone loss. The research from Chapter 6 has shown that, immediately after this time point (5 weeks post-OVX), there is an increase in strain stimulation of the cells embedded in the depleted trabecular bone tissue. It has also been shown that significant increases in trabecular stiffness occur at later time points in rats, at 12 and 34 weeks post-OVX (McNamara et al. 2006). The research from Chapter 6 suggests that these changes might restore the strain stimulation on osteocytes to homeostasis in late-stage osteoporosis (>34 weeks), a theory substantiated by the previously observed return of increased stiffness to control levels by 54 weeks (McNamara et al. 2006). The computational simulations of alterations in tissue properties in Chapter 7 corroborate this sequence, demonstrating that an initial decrease in mineralisation and stiffness followed by a later increase, can explain changes in strain stimulation. Taken together with the experimental results from previous studies, the results of this thesis provide further evidence that changes in bone mass, mineralisation and properties progress according to the sequence proposed in Figure 8.2A. Based on these findings an evolved sequence is
proposed and presented in Figure 8.3. As the temporal development of post-menopausal osteoporosis, outlined here based on previous experiments (Hughes et al. 1996; Keiler et al. 2012; McNamara et al. 2006) and corroborated by the studies performed in this thesis, does not match the second theory (Figure 8.2B) it can be refuted as an explanation of the bone loss cascade.

**Figure 8.3:** Flowchart depicting the research from Chapters 6 and 7 in the context of a proposed theory for the bone loss cascade (Adapted from (McNamara 2010)).

Time indicates weeks after ovariectomy in a rat model of osteoporosis
Finally, the FSI models developed in Chapter 5 were used to explore the implications of osteoporosis on osteocyte mechanobiology, investigating the effects of (a) a more tortuous canalicular geometry and (b) disrupted cellular attachment in the canaliculi, which are believed to occur in osteoporosis (Knothe Tate et al. 2002; Knothe Tate et al. 2004; Sharma et al. 2012). Increased canalicular tortuosity was found to result in greater strain stimulation, although to a lesser extent than observed experimentally in Chapter 6, while the velocity of interstitial fluid flow was decreased. This lower velocity is in agreement with previously proposed theory that this increased tortuosity could affect the permeability of the pericellular space (Sharma et al. 2012), which would disrupt normal interstitial fluid flow around the osteocyte and may inhibit biochemical signalling or nutrient supply during osteoporosis. Decreased density of strain amplification mechanisms (PCM tethering elements and ECM projections) as a result of disrupted cellular attachment, thought to occur in osteoporosis (Sharma et al. 2012), was not found to significantly affect the mechanical stimulation of the osteocyte. However, it is not known what effect osteoporosis has on the attachments of the pericellular matrix themselves, and any change in the properties of this important matrix could have consequences for mechanosensation of the osteocyte. Furthermore, it has been proposed that osteocytes may be capable of altering their canalicular environment in order to heighten sensitivity to mechanical loading (Harris et al. 2007). Thus, it is possible that the observed increased tortuosity (Knothe Tate et al. 2002; Knothe Tate et al. 2004) and surface degradation (Sharma et al. 2012) may form part of the mechanobiological response that occur in the temporal pathogenesis of osteoporosis. Therefore, the computational models developed in this thesis, in combination with future improvements in imaging of the osteocyte during osteoporosis, will provide a basis for further study of the development of osteoporosis.

8.5 Future work

The studies described in this thesis shed further light on the mechanobiology of bone cells, and demonstrate the potential importance of this field for study of osteoporosis. Based on the findings of this thesis, the following recommendations are made for future research;
8.5.1 TEM-resolution modelling of attachments in the extracellular osteocyte environment

Imaging methods, such as transmission electron microscopy (TEM), allow visualisation of the intricate components of the extracellular environment, and have been employed in the past to explore the osteocyte ultrastructure (You et al. 2004), as they operate at much higher resolutions than confocal microscopy. However, as TEM imaging requires ultra-thin slices, it is extremely challenging to perform serial sectioning and imaging, to develop the z-stack of images required for generation of 3D computational models of osteocytes. Developments in TEM imaging that combine serial TEM imaging of sections with rotation of the sample under TEM to develop a 3D reconstruction (Soto et al. 1994), have been used to investigate neurons with volumes of interest up to a few hundred µm² (Shoop et al. 2002). This method has provided the possibility of examining sections of osteocytes, a canaliculus for example, in extremely high resolution (Schneider et al. 2010). As this resolution could capture the PCM tethering elements and ECM projections in fine detail, this may facilitate the development of truly representative multi-physics models of the osteocyte mechanical environment. Furthermore, it is not known what effect oestrogen deficiency would have on the pericellular matrix and, as it has been shown that degradation of this matrix can affect mechanosensation of the osteocyte (Reilly et al. 2003b), any alteration that could occur in disease could also disrupt the strain experienced by the cell. Similarly, as predicted by the computational models in Chapters 4, 5 and 7, integrin attachments at ECM projections serve as important stimulus amplifiers in the lacunar-canalicular network. High resolution serial TEM imaging was not performed in this thesis as this technology remains to be tested on thick sections of mineralised bone tissue at volumes large enough to contain entire osteocytes, and also because this method would produce datasets too large for most current 3D reconstruction programs to process. However, future advancements of both automated TEM imaging techniques and computational power will likely facilitate development of these extremely detailed computational models, which could be used to compare the environments and attachments in both healthy and osteoporotic cells.
8.5.2 Experimental characterisation of the effect of disruption of attachments on the mechanical stimulation of osteocytes

It has been shown in vitro that degradation of the pericellular matrix surrounding osteocyte-like cells greatly affects their ability to respond to mechanical stimulation via some pathways (Reilly et al. 2003b). Similarly, blocking antibodies have been developed recently that can be used in vitro to interfere with integrin attachments such as $\alpha_V\beta_3$ (Lee et al. 2008), which have been shown to be abundant along the mechanosensitive cell process (McNamara et al. 2009). Therefore, these two methods could potentially be applied to disrupt the attachments of the osteocyte to its surroundings, which the computational models in this thesis have shown to be important. This interference could be applied to ex vivo samples, and then loaded using the techniques developed in Chapter 6, allowing comparative analysis of the effects of each on the strain experienced by the cell. While not performed in this thesis, this would deepen current understanding of the mechanisms by which the osteocyte is stimulated and could be repeated with osteoporotic tissue to determine their role in mechanosensation during disease.

8.5.3 Computational simulations incorporating complex material models of the osteocyte and its environment

In order to elucidate the effects of various elements of the mechanical environment in which the osteocyte resides, the material properties of all models were simplified to linear elastic isotropic materials. However, in reality the mineralised extracellular matrix, the glycocalyx pericellular matrix and the osteocyte display complex material behaviours, particularly at the microscopic scale of the cell (Han et al. 2004; O'Mahony et al. 2001; You et al. 2004). Indeed, the cell itself possesses its own actin cytoskeleton and can react to mechanical or biochemical cues to alter its morphology (Lim et al. 2006). Furthermore, the ECM is composed of collagen fibrils and mineral crystals that are intricately organised within this matrix and have been shown to affect stress distribution about the lacuna (Currey 2003; Ascenzi et al. 2008; Hofmann et al. 2006). Indeed, recent models of chondrocyte cells which include an ECM have demonstrated the importance of anisotropy resulting from collagen fibril alignment, with increased stress in the cell when the fibres are aligned with the
direction of maximum principal strain (Dowling et al. 2013). This study also demonstrated the importance of the PCM stiffness, which governs the deformation within the cell, particularly when it is significantly lower than the stiffness of the ECM (Dowling et al. 2013). Therefore, though experimental data on the material properties of the lacunar canalicular environment and internal cytoskeleton are scarce, future developments of osteocyte models should endeavour to incorporate more realistic models of material behaviour. Indeed, the inclusion of an active actin cytoskeleton model, similar to those that have recently been developed for cells in vitro (McGarry 2009; McGarry et al. 2009), would provide a novel insight into the interaction between the osteocyte and its environment and deepen the understanding of osteocyte mechanobiology. However, given the high computational cost of simulating the complex lacunar-canalicular architecture with simple linear elastic behaviour, the inclusion of such active material models will likely require further advancements in computational resources.

8.6 Conclusion

In conclusion, this thesis has presented computational and experimental studies performed throughout the course of the author’s PhD studies in the fields of bone mechanobiology and the disease of osteoporosis. A comprehensive literature review of the present state of research was conducted, followed by a rationale for the studies carried out. The methods and findings of each study were detailed in each chapter, along with a discussion of the implications for the fields of research.

Together, these studies provide a novel insight into the closed mechanical environment of the osteocyte, in both health and during osteoporosis. Computational methods were employed to investigate the multi-physics mechanical environment that osteocytes experience under physiological loading in vivo. An experimental method was developed concurrently that facilitated direct observation of osteoblasts and osteocytes under loading. Finally, by applying the computational models to simulate the strain environment that was observed experimentally in osteoporotic osteocytes, it was shown that changes in the mechanical strain experienced by osteocytes occur, which are explained by osteoporotic alterations in mineralisation that have been experimentally observed. Thus, this thesis provides a missing
mechanobiological link in the temporal development of post-menopausal osteoporosis, and the information elucidated from this body of work may inform future treatments for osteoporosis.


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