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INTEGRATING MICRO-CT AND COMPUTATIONAL BONE MECHANICS TO EVALUATE BONE TREATMENT OUTCOMES

Dharshini Sreenivasan

Supervised by: Dr Justin Fernandez and Prof Jillian Cornish

A thesis submitted in fulfilment of the requirements for the degree of
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ABSTRACT

In this thesis, a computational pipeline is presented which is used to assess bone failure strength in response to anabolic treatments. The pipeline was validated on a 3D printed bone sample, which underwent an Instron compression test. This pipeline was evaluated as part of three clinical studies. The first, a large scale human clinical trial on the effects of low-dose fluoride therapy in postmenopausal women. Second, a whey protein diet in a murine model which also integrated material properties from a number of different sources at different scales. Third, a computational assessment of commonly used murine controls: OVX and Sham mice. Finally, the pipeline was applied to an external collaboration which looked at the influence of Vitamin D depletion in a murine model. In all studies, our modelling pipeline was able to predict the same trends as standard clinical measures, using a double blinded design. The reported failure from the Instron test formed the basis of the yield criteria for the computational work and was also able to isolate architecture from material properties for both trabecular and cortical bone. The overall outcome from this thesis was the development, refinement and validation of a computational pipeline, which was efficient, integrates clinical work and was tested in a real clinical scenario to provide confidence in the use of computational models.
ACKNOWLEDGEMENTS

Sometimes people come into your life for a moment, a day or a lifetime. It matters not the time they spent with you but how they impacted your life in that time. - Unknown

The past few years have been extremely challenging and yet the most academically rewarding. It would have, however, been far more daunting without the help of several key people.

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LIST OF ABBREVIATIONS

ABS acrylonitrile butadiene styrene
B.Ar mean total cross sectional bone area
B.Ar/T.Ar mean cortical area fraction
bLF bovine lactoferrin
BMD bone mineral density
BMUs bone multicellular units
BV/TV percentage bone volume
Cs.Th mean cross sectional thickness
DXA dual energy x-ray absorptiometry
DXF drawing interchange/exchange format
FE finite element
FDA food and drug association
LPR1 low density lipoprotein receptor-related proteins 1
LPR2 low density lipoprotein receptor-related proteins 2
Ma.Ar mean medullary area
Micro-CT micro-computed tomography
NaF sodium fluoride
OVX ovariectomised
PBS phosphate buffered saline
PTH parathyroid hormone
rhPTH recombinant human parathyroid hormone
RMS root mean square
ROI region of interest
Sham sham operated
SPM scanning probe microscopy
SRNaF slow release sodium fluoride
STL Stereolithograph
T.Ar mean total cross sectional tissue area
TbN trabecular number
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<td>TbSp</td>
<td>trabecular separation</td>
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### CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
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<tbody>
<tr>
<td>Maureen Watson</td>
<td>Micro-CT processing</td>
</tr>
<tr>
<td>Michael Dray</td>
<td>Histomorphometric processing</td>
</tr>
<tr>
<td>Jillian Cornish</td>
<td>co-supervision of work and paper reviewing</td>
</tr>
</tbody>
</table>

### Certification by Co-Authors

The undersigned hereby certify that:

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<td></td>
<td>22/04/2015</td>
</tr>
<tr>
<td>Jillian Cornish</td>
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<td>Fracture mechanics analysis</td>
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<tr>
<td>Andrew Grey</td>
<td>Principal investigator of clinical study and paper reviewing</td>
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Original article: D Sreenivasan, P.T. Tu, M Dickinson, M Watson, A Blaise, R Das, J Cornish and J Fernandez. Computer modelling integrated with micro-CT and material testing provides additional insight to evaluate bone treatments: Application to a beta-glycan derived whey protein mice model. 68 (2016) 9-20.

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<td>M Watson</td>
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Background and Introduction

Bone fabric is woven at submicroscopic, microscopic and macroscopic levels into an architectural masterpiece of biomechanical engineering.

- Seeman and Delmas (2006)

This chapter begins with the motivations behind this thesis. Following this, the anatomy of bone is covered by looking at its structure macroscopically (the whole organ structure of the bone) and microscopically (composition and orientation of bone struts). Following from this, bone remodelling, the process of breaking down and rebuilding bone, is discussed followed by pathological conditions that occur when there is an imbalance in the remodelling process. One pathological condition, osteoporosis, is discussed from its diagnosis to available therapies. Various anabolic treatments are reviewed including fluoride and a whey protein diet of which are evaluated as part of this thesis. Finally, previous computational approaches are discussed.
1.1 MOTIVATIONS

Osteoporosis is a worldwide problem affecting, on average, one in five men and one in three women over the age of 50. Anabolic treatments are typically evaluated using large and expensive clinical trials, consisting of many effect and control groups with large sample sizes for statistical significance. Computational modelling, on the other hand, is relatively inexpensive, fast to solve and can be used to test large sample sizes by running simulations in parallel. Further, they can be used to provide insight into bone properties that are hard to evaluate experimentally, such as the influence of bone architecture on strength, independent of material properties. While computer models are not at the level of maturity to fully replace clinical trials they can play a complimentary role, provide further insight and allow clinical trials to proceed with reduced numbers after pre-clinical computational evaluations. The major theme of this thesis is the development of a computational pipeline to compliment clinical bone treatments using a double blinded design. The aim: to see if it can reproduce similar trends and secondly to provide further insight by using 3D virtual strength tests.

In order to do this we took part in three clinical studies, (i) fluoride treatment on postmenopausal women, (ii) a murine study into different whey protein levels and (ii) the first stage of a lactoferrin trial that looked at the difference between OVX and Sham mice. In all studies we conducted the computational work independent of the clinical trial and were only given the key to decode and compare the data after all clinical work was finished. This was motivated by clinicians wanting more confidence in computational models. To further strengthen our modelling framework we also evaluated our computational model against a 3D printed biopsy of human bone that was fractured in an Instron test. Finally, we evaluated the influence that material properties have on computational simulations given that they can be measured from a number of sources including microindentation, nanoindentation, three point bending and micro-CT phantoms. The aim of this was to provide some guidance for computational modellers who wish to use this material property data in computational predictions.
1.2 CENTRAL AIMS AND SCOPE

The general aims for this thesis were to create a modelling framework to assess bone strength using a double blinded design, non-destructive, non-invasive and validated manner. They can be defined as follows:

1. Create a pipeline for efficiently converting 3D micro-CT into FE models.
2. Validate the modelling framework against a 3D printed biopsy as part of an Instron test.
3. Evaluate this framework as part of a human clinical trial into fluoride therapy. This is the first reported case we have observed in the literature.
4. Evaluate the influence of material properties that have been derived from different sources, including, nanoindentation, microindentation, three point bending and micro-CT phantoms, as part of a murine study into a variable whey protein diet.
5. Evaluate the mechanical difference between Sham and OVX mice as the first step of a larger lactoferrin study.

In addition we were fortunate to partake in a Vitamin D study with colleagues in the UK and were asked to evaluate our framework on bones with depleted Vitamin D to evaluate strength. The benefit in this case was that the bones could not be mechanically tested as they were too ‘soft’. This is included with permission in Appendix A.

1.3 NOVEL CONTRIBUTIONS

This thesis provides a number of novel contributions and important incremental achievements in the field of bone biomechanics. These include:

1. The computational evaluation of the effects of a fluoride treatment, as part of an actual human clinical trial using a double blinded design.
2. Evaluation of the effects of different sources of material properties on computational predictions from the same sample using a murine model.
3. Evaluation and validation of a computational model using a 3D printed bone biopsy setup.
1.4 BONE ANATOMY

Bone, a metabolically active tissue, is capable of adapting its structure to mechanical stimuli and is able to repair its structural damage through the process of remodelling (Robling, Castillo, & Turner, 2006). The skeletal system comprises approximately 18% of the weight of the human body and performs numerous functions, including support, protection, assistance in movement, mineral homeostasis, bone cell production and triglyceride storage (Tortora & Derrickson, 2009).

1.4.1 STRUCTURE OF BONE

Bone has a varied arrangement at different scales and these structures work in union to perform all its necessary functions. When referring to bone architecture, scale is of importance as the arrangement at different scales in bone can be thought of as hierarchical. Bone can therefore be divided at the macro, micro and nano levels, shown in Figure 1.1, and the homogenised material properties of bone varies between the different scales. Briefly, at the macro level, bone is simply divided into trabecular and cortical bone, at the micro level (ranging from 10 to 500 μm) the haversian systems, osteons and individual trabeculae are found and at the nano level (ranging from a few hundred nanometers to 1 μm): fibrillar collagen and embedded minerals (Müller, 2009; Rho et al., 1998).
BONE: MACRO SCALE

At the macrostructural level, bone is distinguished into either trabecular or cortical bone. A typical long bone (such as the femur or humerus) consists of the following components, shown in Figure 1.2. The diaphysis is the bone’s shaft/body, also considered the main portion of the bone. Epiphyses are the proximal and distal ends of the bone and the metaphyses are the regions between the diaphysis and the epiphyses. In a growing bone, the growth plate (epiphyseal plate) is found in this region. The growth plate is a layer of hyaline cartilage that allows the diaphysis of the bone to grow in length. The articular cartilage is a thin layer of cartilage covering the part of the epiphysis where the bone articulates with another bone, thus forming a joint. Among its functions, articular cartilage acts to reduce friction and absorb shock at joints that are able to freely move. The periosteum surrounds the external bone surface in areas where it is not covered by external articular cartilage. The periosteum can be broken down into an outer fibrous layer of dense, irregular, connective tissue and an inner osteogenic layer consisting of cells which enable the bone to grow in thickness (not length). The periosteal layer also protects the bone, assists in nourishing the bone tissue and provides an attachment site for ligaments and tendons. The medullary cavity (marrow cavity) is the hollow, cylindrical space within the diaphysis that
BONE ANATOMY

contains the bone marrow. The endosteum is a thin membranous layer that lines the internal bone surface facing the medullary cavity which contains a single layer of cells and a small amount of connective tissue.

The material properties of cortical and trabecular bone vary greatly from one another and it is dependent on the method of testing used. The Young’s moduli that have been reported for cortical bone, based on mechanical testing, range from 14 – 29 GPa. (Choi, Kuhn, Ciarelli, & Goldstein, 1990; Reilly, Burstein, & Frankel, 1974; Rho et al., 1998). The variability reported for trabecular bone is even greater and this is predominantly attributed to its porous anisotropic anatomy. The homogenised Young’s modulus of trabecular bone can range from 100 MPa – 4 GPa (Ashman & Rho, 1988; Dagan, Be’ery, & Gefen, 2004; Goldstein, 1987; Linde & Hvid, 1989). These values were all obtained from mechanical tests of long bones which included the tibia, femur and humerus from various animal models.
At the micro scale, mineralized collagen fibres in cortical bone form into planar arrangements and wrap in concentric layers around a central canal to form an osteon or haversian system. Trabecular bone, on the other hand, is an interconnected framework of trabeculae comprising of either a rod-rod, rod-plate or plate-plate structure (Rho et al., 1998). At this scale trabecular bone is highly porous, inhomogenous and irregular leading to difficulty in measuring its material properties. It cannot be assumed that the Young’s modulus at the micro level is the same as it would be at the macro level. However, this assumption, is made regularly. Osteonal segments
with the lamellar orientation in the transverse and longitudinal directions were reported to have Young’s moduli of 5.5 and 12 GPa, respectively in tension (Ascenzi & Bonucci, 1967). Isolated osteons have been shown to have Young’s moduli of 6 and 7 GPa in the longitudinal and transverse directions, respectively, in compression (Rho et al., 1998). Bending tests of a single osteon in both the longitudinal and transverse directions provide Young’s moduli of 2 and 3 GPa, respectively (Ascenzi, Baschieri, & Benvenuti, 1990) while torsion testing of a single osteon in both the transverse and longitudinal directions have shown Young’s moduli (in shear) values of 16 and 20 GPa, respectively (Ascenzi, Baschieri, & Benvenuti, 1994). Based on the values presented above, the Young’s modulus of bone at the micro scale is generally reported to range from 1 – 20 GPa. The reason for this spread in values is unclear and there is debate around whether the differences are attributed to the testing techniques (whether it be micro bending or microindentation) or whether the results simply represent a true difference in the properties at a microstructural level in the bone. One reasonable possibility for the large spread in values is the influence of microstructural defects, such as cement lines and voids (haversian canals, Volkmann canals, lacuna, osteocytes and canaliculi), in the areas that are measured (Rho et al., 1998). Other explanations include uncertainties in the geometry of the specimen, which could also have been intensified at smaller scales and difficulty aligning specimens in bending tests (Rho et al., 1998). These results highlight that the material behaviour of bone at the micro level depends on both the direction of testing and modes of loading. They also show that microindentation tests are able to provide information about the matrix, averaged across the canaliculi and haversian voids in cortical bone and the large porous spaces found in trabecular bone.

**BONE: NANO SCALE**

At the nano scale, the most pronounced structures are the collagen fibres which are embedded with crystals and non-collagenous proteins. There still remains inconsistencies in the literature regarding material properties of bone at this level. It has been proposed that because both trabecular and cortical bone are essentially just collagen fibres at this scale and there should be no differences in their material properties (Carter & Hayes, 1977). While some studies back up this proposal, others still show variations in the Young’s modulus at the nano level. Using both nanoindentation and acoustic microscopy, a study on one human cadaver showed that the Young’s moduli of trabecular bone falls between the Young’s moduli for cortical bone obtained in the transverse and longitudinal directions for both testing methods (Turner, Rho, Takano, Tsui, & Pharr, 1999). This study therefore concluded that the postulation by Carter and Hayes, is
generally applicable, as the Young’s modulus of trabecular tissue is similar to that of cortical tissue at the nano scale. This is further supported by another study which provided evidence showing that the elastic and yield properties of trabecular bone is similar to that of cortical bone in three loading cases (compression, torsion and shear), where the finite element (FE) analysis values were consistent with the experimental values (Niebur, Feldstein, Yuen, Chen, & Keaveny, 2000). On the other hand, nanoindentation experiments on the femoral neck of a 74 year old female provided a Young’s modulus value of 6.9 GPa for trabecular bone and 25.0 GPa for cortical bone (Zysset, Guo, Hoffler, Moore, & Goldstein, 1999). Nanoindentation studies of male cadaveric vertebrae have also shown variations in the values obtained, with a Young’s modulus of 13.4 GPa for trabecular bone and either 22.5 GPa for the osteons or 25.8 GPa for the lamellae (Rho, Tsui, & Pharr, 1997). These variations are thought to be attributed to differences in porosity or mineralisation of the extracellular matrix and the orientation and distribution of the collagen fibres. (Zysset et al., 1999). These results highlight that the material behaviour of bone does vary spatially and nanoindentation is able to provide information about the matrix properties in bone, irrespective of the geometry.

1.5 HISTOLOGY OF BONE TISSUE

Microscopically, like all tissue, bone contains an abundance of extracellular matrix and is surrounded by various cells. The extracellular matrix is composed of about 25% water, 25% collagen fibres and 50% crystallised mineral salts. The most abundant mineral salt is calcium phosphate which combines with another mineral salt calcium hydroxide, to form hydroxyapatite. As these hydroxyapatite crystals form, they combine with other mineral salts such as calcium carbonate and ions such as magnesium, fluoride, potassium and sulphate. These salts deposit in the collagen fibres of the extracellular matrix, causing the tissue to crystalise and harden, a process termed calcification (Robling et al., 2006; Seeman & Delmas, 2006; Tortora & Derrickson, 2009).
Four types of cells are present in bone tissue: osteogenic cells, osteoblasts, osteocytes and osteoclasts. These are shown in Figure 1.3. Osteogenic cells are unspecialised stem cells and are the only bone cells that undergo cell division which results in the formation of the osteoblasts. Osteoblasts are bone-building cells which synthesise and secrete collagen fibres and other organic components needed to build the extracellular matrix of bone tissue. These cells are also able to initiate calcification. As osteoblasts surround themselves with the extracellular matrix they become trapped within their own secretions and become osteocytes. Osteocytes are therefore the mature bone cells and are the predominant cells in bone tissue. They are responsible for maintaining daily processes such as exchanging nutrients and waste with the blood. Osteocytes are also the primary candidates for mechanosensing of solid strain and fluid shear that initiates bone growth and loss (Tortora & Derrickson, 2009). Osteoclasts are the fourth type of bone cell and are responsible for the breakdown of the bone extracellular matrix, a process termed resorption. This is part of the normal development, maintenance and repair of bone.
Bone can be categorised as either cortical (compact) or trabecular (spongy or cancellous) bone (see Figure 1.4). Approximately 80% of the skeleton is cortical and 20% is trabecular bone.

1.5.1 CORTICAL BONE

Cortical bone is the strongest form of bone tissue and is found directly beneath the periosteum of all bones and makes up the bulk of the diaphysis of long bones. The purpose of cortical bone is to provide protection and support and resist the stresses produced by weight and movement.

Figure 1.4a is a section through a long bone and shows the various components found within bone. Nerves, blood and lymphatic vessels from the periosteum penetrate the compact bone through the transverse, Volkmann’s canals. The vessels and nerves of the perforating canals connect with those in the medullary cavity, periosteum and haversian canals (central canals) which run longitudinally through the bone. Around the central canals are the concentric lamellae, which are rings of calcified extracellular matrix. Lacunae are small spaces between the lamellae that contain osteocytes and radiate in different directions. Found within the lacunae are tiny canaliculi, which are small channels filled with extracellular fluid. Within the canaliculi are osteocytes (Figure 1.4c), which communicate with neighbouring osteocytes via gap junctions. The canaliculi connect the gap junctions with one another and with the central canals, thus forming an interconnected canal system within the bone.

All the components mentioned above are arranged into repeating structural units called osteons (see Figure 1.4a). Each osteon consists of a central canal with its concentrically arranged lamellae, lacunae, osteocytes and canaliculi. In compact bone, osteons are aligned in the same direction along lines of stress. The areas between osteons contain interstitial lamellae, which also contain lacunae with osteocytes and canaliculi.
1.5.2 TRABECULAR BONE

In comparison to cortical bone, trabecular bone does not contain osteons but consists of lamellae arranged in an irregular lattice of thin columns called trabeculae (see Figure 1.4b). The spaces between the trabeculae ensure that the bone remains light. Within each trabeculae are lacunae that contain osteocytes. Canaliculi radiate outward from the lacunae and the osteocytes receive nourishment from the blood circulating within the blood vessels located in the spaces between trabeculae.

Trabecular bone makes up the majority of the interior bone tissue and whilst it may appear that trabecular bone has a disorganised arrangement compared to the systematic arrangement seen in cortical bone, this in fact is not the case. Trabecular bone is precisely oriented along trajectories of stress which is a characteristic that helps bone resist stresses and transfer loads without breaking (Robling et al., 2006; Tortora & Derrickson, 2009). Specifically, they lie along the directions known as ‘principal components’ which are contour lines that have zero shear stress.
The remodelling process works to define the skeleton, maintain appropriate ion levels in the blood and repair regions of bone as required. In the mature skeleton, bone remodelling occurs via the following sequence: activation–resorption–formation (Parfitt, 1979; Robling et al., 2006). It occurs at areas called basic multicellular units (BMUs) in a cycle of resorption (removal of old bone) and deposition (replacement with newly formed bone) (Frost, 1991). At any one time around 20% of the trabecular bone surface is undergoing remodelling (Hill, 1998). This remodelling process is regulated by osteoblasts and osteoclasts and it should be noted that these...
two cell types make up the BMU (Canalis, Giustina, & Bilezikian, 2007). In the resorption phase, osteoclasts remove bone over a period of two to three weeks. In cortical bone, the bone is tunnelled away whereas in trabecular bone, a lacunae is excavated on the surface. In the deposition phase, osteoblasts lay down new bone in the areas where resorption occurred. The deposition of new bone can last up to 100 days. Mechanically, this has been formalised as a ‘Mechanostat’, defined by Frost (Frost, 1994, 2003) and based on the ideas of Wolff (Frost, 1994). In brief, the ‘Mechanostat’ describes bone growth and loss in response to local mechanical deformation due to peak loading caused by the surrounding muscle. Therefore, bone is able to adapt its mass, geometry and strength according to its needs (Frost, 2003) Virtually all computational models that evaluate remodelling use the mechanostat in some form (Fernandez et al., 2013; McNamara & Prendergast, 2007).

An imbalance in the resorption and deposition phases can lead to either an increase or decrease in bone growth. During growth and development, the healing of fractures or an increase in mechanical stimuli, the process of bone deposition by osteoblasts is up-regulated and thus a lot more bone is formed than removed. On the other hand, when osteoclast activity is higher than that of the osteoblasts and the rate of bone resorption exceeds the rate of bone deposition, bone loss results. A rapid increase in bone loss can be attributed to either hormonal influences or an absence in mechanical stimuli (Parfitt, 1982). Abnormal bone remodelling occurs in a number of common diseases which include, osteoporosis, periodontitis, arthritis and tumour induced osteolysis (Hill, 1998). Osteoporosis, is a condition (that occurs primarily in old age) in which the increase in bone voids and reduced ability to form new bone leads to bone fragility and fractures. It will be the focus of this work.

1.7 OSTEOPOROSIS

Osteoporosis, characterised by the loss of bone mass and strength and therefore leading to fragility fractures, has most likely existed throughout human history. However, it has only recently become a major clinical problem with the rapid growth in the global aging population due to our increasing lifespans.
1.7.1 BRIEF HISTORY

- Egyptian mummies from over 4000 years ago have been found to have had a dowager’s hump, an indication of osteoporosis (Raisz, 2005).
- 18th century English surgeon, John Hunter, discovered the process of remodelling, which 100 years after his death has been shown to play a critical role in osteoporosis (Raisz, 2005).
- In the 1830s, French pathologist, Johann Lobstein, described osteoporosis (porous bone) when he noticed some patients’ bones were riddled with larger than normal holes (Raisz, 2005).
- In 1940, American endocrinologist, Fuller Albright, described postmenopausal osteoporosis and proposed that this could be attributed to oestrogen deficiency. Subsequently, the concept of two different forms of osteoporosis was proposed, whereby one was related to oestrogen deficiency and the other due to aging (Raisz, 2005).
- That theory has now been replaced by the current concept that osteoporosis represents a continuum in which multiple pathogenic mechanisms can lead to the loss of bone mass and deterioration of the architecture of the skeleton (Raisz, 2005).
- At present, osteoporosis is a global health problem and it has been estimated that over 200 million people worldwide are affected by it (Reginster & Burlet, 2006; WorldHealthOrganization, 2004). In New Zealand alone, around 80,000 bone fractures are attributed to osteoporosis per annum, with the total cost of treatment approximating $1.15 billion yearly. Three quarters of those affected are women (Brown, McNeill, Radwan, & Willingale, 2007). With a progressively increasing aging population, osteoporosis presents itself as an economic and social burden, globally.

1.7.2 DIAGNOSIS

As mentioned, osteoporosis is associated with a decrease in bone strength and many studies have shown significant correlations between low bone mineral density (BMD) and the frequency of bone fractures (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 1994; Reginster & Burlet, 2006). Currently osteoporosis is diagnosed based on BMD measurement using dual energy X-ray absorptioniometry (DXA) of either the hip or lumbar spine. A low T-score (the amount of bone mineral matter per square centimetre), defined by the World Health Organisation, is currently the method by which osteoporosis is diagnosed. (Raisz, 2005; Reginster & Burlet, 2006).
Factors that predispose individuals to osteoporosis include: age, low starting BMD measurements, history of bone fractures, family history, early onset menopause and the use of oral corticosteroids (Kanis et al., 2004; Reginster & Burlet, 2006). Postmenopausal women are also at high risk of developing osteoporosis due to a dramatic decline in oestrogen levels. Evidence from prospective studies of postmenopausal women shows severe decline in bone strength and an increase in bone fragility, thus leaving them at risk of developing bone fractures (Harris et al., 1999; Kanis, 1994; Raisz, 2005). Postmenopausal osteoporosis and the associated decline in bone strength was the overall focus of this work, particularly in Chapter 5.

1.7.3 THERAPIES

Therapies for osteoporosis can be split into two categories: antiresorptive and anabolic. Antiresorptive therapies decrease bone remodelling by suppressing osteoclast function and thus suppressing bone breakdown (Stepan, Alenfeld, Boivin, Feyen, & Lakatos, 2003). Antiresorptive agents include bisphosphonates, selective oestrogen receptor modulators, raloxifene and calcitonin (Black & Schafer, 2013; Canalis, Giustina, & Bilezikian, 2007; Raisz, 2005; Reginster & Burlet, 2006). The majority of available therapies target the osteoclasts in order to decrease bone resorption but are limited in their ability to restore bone mass and therefore reduce the incidence of osteoporotic fractures. Subsequently, there is a lot of interest in developing anabolic compounds to promote the activity of osteoblasts and in turn build bone (Grey et al., 2004). Parathyroid hormone 1-34 (PTH), or teriparatide, as it is referred to in the clinic, lactoferrin and fluoride are examples of anabolic therapies used to treat osteoporosis that have been investigated in New Zealand. Specifically, fluoride use in postmenopausal women is an anabolic examined in detail in this thesis.

PARATHYROID HORMONE

Recombinant human parathyroid hormone (rhPTH) is currently the only, food and drug administration (FDA), approved treatment for osteoporosis (Canalis, 2010). Trials conducted in mice models, postmenopausal women and men have shown improvements in bone mass when administered daily (Lane & Kelman, 2003). A large scale clinical trial was conducted where rhPTH, over a 21 month period, was used to study the effects of the drug on bone mass. The
placebo group experienced a higher occurrence of new vertebral fractures compared to the groups that were given either 20 or 40 μg of rhPTH. Additionally the treatment groups had increases in their BMD in the lumbar spine and femoral neck compared to the placebo group (Neer et al., 2001). Daily injections of PTH have been shown to increase bone mass in both humans and mice models (Morley, Whitfield, & Willick, 2001). The drug takes effect in different ways in the two bone types. The effect is most pronounced in trabecular bone, where the trabeculae have been shown to thicken and increases in the trabecular number and connections were also evident. In cortical bone, an increase in the cross sectional diameter was shown (Lane & Kelman, 2003; Zhou et al., 2003). PTH, administered in low doses in rats have shown an increase in the calcium content and in the dry weight of the rat bone (Hock, Gera, Fonseca, & Raisz, 1988). While the effects of PTH are potently anabolic on bone, its effectiveness at increasing bone mass varies. One hypothesis is that PTH treatment differs between the axial and appendicular skeleton and also differs depending on the integrity and health of the bones. In mice with genetically low trabecular bone volume, PTH treatment stimulated bone formation in the proximal tibia and vertebral body and as previously published (and mentioned above) the effect was greater in the trabecular bone compared to the cortical bone. The findings from this study showed that in mice models that were genetically altered to have a lower trabecular bone volume, PTH increased trabecular bone volume in the vertebral body to a greater extent than in the proximal tibia, even though the proximal tibia had the lower trabecular volume. The same finding was shown in an ovariectomised (OVX) mouse model (Zhou et al., 2003). In addition there was a greater reduction in fractures occurring at vertebral sites compared to the tibia (65% and 54%, respectively) (Canalis et al., 2007; Zhou et al., 2003). This therefore suggests that the effects of PTH varies between sites and appear to have a greater effect on the axial compared to the appendicular skeleton.

Reports of adverse effects of PTH treatment include; headaches, nausea and hypercalcemia (an elevation in serum calcium levels) (Canalis et al., 2007; Neer et al., 2001; Vestergaard, Jorgensen, Mosekilde, & Schwarz, 2007). Animal studies have shown the development of osteosarcomas due to prolonged exposure to high-dose of PTH and therefore is not recommended for use over two years (Canalis et al., 2007; Hodsman et al., 2005). PTH is also expensive and inconvenient, as it needs to be administered as an injection, daily.
LACTOFERRIN

Lactoferrin is an iron binding glycoprotein which is found in high concentrations in colostrum (milky fluid from the mammary glands of humans, cows and other mammals after the first few days of giving birth) and in milk whey (Levay & Viljoen, 1995; Takayama & Mizumachi, 2008). Lactoferrin is also produced by immune cells such as neutrophils, the epithelial cells of several exocrine glands and is also found in secretions from the mucosal epithelium, salivary, lacrimal, biliary and pancreatic glands (Lonnerdal & Iyer, 1995). It has been shown to have anabolic effects in bone and, in vitro, stimulates mitogenesis, differentiation and the survival of osteoblasts and inhibit osteoclast activity (Grey et al., 2004; Guo et al., 2009). Present on osteoblastic cells are the low density lipoprotein receptor-related proteins 1 and 2 (LPR1 and LPR2), which mediate the internalisation of lactoferrin into cytoplasmic membrane bound vacuoles. However, it has been shown that only LRP1 mediates the proliferative effects of lactoferrin (Grey et al., 2004). Studies on 3 week cultures of primary rat osteoblasts have shown that a dose-dependent treatment of bovine lactoferrin (bLF) increased bone matrix deposition, mineralisation and the area of mineralised bone (Cornish et al., 2004; Cornish & Naot, 2010). In addition to promoting osteoblast activity, bLF, has also been shown to have an inhibitory effect on osteoclast development (in rabbit bone cell cultures) while simultaneously having no effect on bone resorption (Cornish et al., 2004; Cornish & Naot, 2010; Lorget et al., 2002). These results therefore indicate that lactoferrin does not affect the activity of mature osteoclasts to resorb bone but does reduce overall bone resorption by reducing the development of osteoclasts. These findings were further supported by a study that looked at the effect of bLF on an osteoblastic cell line which showed a dose-dependent increase in cell growth (Blais, Malet, Mikogami, Martin-Rouas, & Tome, 2009). In addition, this study also showed the inhibition of osteoclastic cell activity when treated with bLF.

The studies mentioned above focus on administering bLF to isolated cells in culture, but in order for lactoferrin to be an effective treatment for osteoporosis it needs to be administered in an oral formulation. In an OVX mice model there was an increase in the total, femoral and lumbar BMD with oral bLF treatment. There were also improvements in biomechanical parameters with bLF treatment, assessed using three point bending of the femur, where the stiffness (yield load) and failure load (peak load) showed improvement (Blais et al., 2009).

In addition, orally administered bLF in mice has been shown to be absorbed from the intestine into the blood and localised within various tissues completely intact, suggesting that it is resistant to proteolytic degradation. (Blais et al., 2009; Fischer et al., 2007; Kuwata et al., 1998). Its rapid uptake can also be explained by the presence of lactoferrin receptors in the intestinal brush border.
of the mouse intestine (Kuwata et al., 1998). Being resistant to degradation and its rapid absorption in the intestine suggests that lactoferrin would be an ideal drug as it is able to be administered as an oral formulation. However, it has also been shown that repeated ingestion of lactoferrin reduces its uptake in the intestinal lumen (Fischer et al., 2007), suggesting that chronic treatment may not be suitable due to the possible development of a tolerance.

**FLUORIDE**

Elemental fluoride has been shown to have well established anabolic effects on bone at the level of the osteoblasts. Studies have shown that fluoride in low circulating levels allows for skeletal uptake. In brief, these effects include an increase in cellular proliferation and differentiation of osteoblasts in culture (Kanis & Meunier, 1984; Pak, Zerwekh, & Antich, 1995). On the other hand, upon exposure to high levels of fluoride, bone becomes poorly mineralised and mechanically defective (Pak et al., 1995). It has therefore been hypothesised that in order for fluoride to be effective as a treatment for osteoporosis it needs to be administered in a slow release and low-dose formulation in order to avoid toxic peaks in the blood serum (Pak et al., 1995).

When formulated into a drug, fluoride is administered as sodium fluoride (NaF). Many studies have been conducted using NaF to assess its effects on improving bone strength and as a treatment for osteoporosis. Several clinical trials have been conducted where NaF is administered in doses ranging from 40 - 80 mg/day for a period of 2 - 6 years. In the majority of these studies, NaF is supplemented with either Vitamin D or calcium, both of which have been shown to improve bone strength (Dawson-Hughes, Harris, Krall, & Dallal, 1997; Lips, Graafmans, Ooms, Bezemer, & Bouter, 1996; Tang, Eslick, Nowson, Smith, & Bensoussan, 2007). After one year of treatment with NaF, there were no increases in bone mass (Riggs et al., 1980), improvements in the vertebral fracture rate and additionally new fractures were also detected (Dambacher, Ittner, & Ruegsegger, 1986; Riggs et al., 1980). In the iliac trabecular bone, NaF treatment had a significant increase (43%) in the trabecular thickness but the overall bone mass and trabecular connectivity remained unchanged (Aaron, de Vernejoul, & Kanis, 1991). After three years of treatment, an improvement in the trabecular bone density was seen compared to the controls (Dambacher et al., 1986), suggesting that a year of treatment with NaF may not be sufficient in order to improve bone strength. All these studies also reported adverse effects which were attributed to NaF treatments which included: synovitis, anaemia, chronic nausea and vomiting.
NaF has also been studied in slow release formulations, which has been shown to maintain serum fluoride at therapeutic levels (Pak et al., 1989). Slow release sodium fluoride (SRNaF), is advantageous as it minimises some of the adverse side effects of the drug. SRNaF (25 mg, twice a day for three years) was used as a potential treatment for osteoporosis in a clinical trial and was administered on its own or in combination with Vitamin D and calcium supplements. Outcomes from this study showed a decline in the vertebral fracture rate and an increase in the mineral apposition rate in the vertebrae in the SRNaF group that was not given Vitamin D. It should be noted that there were no observed changes in the diaphysis of the radii or in the density of the femoral neck (Pak et al., 1989).

1.8 COMPUTATIONAL ASSESSMENT OF BONE

High resolution imaging methods, such as micro-computed tomography (micro-CT) and high resolution magnetic resonance imaging, have enabled the construction of detailed FE models that allow the modelling of bone, in particular trabecular bone structures, in detail. There are two primary approaches for generating FE models: geometry based and voxel based (Lengsfeld, Schmitt, Alter, Kaminsky, & Leppek, 1998). The voxel based method, which will be the approach used in this work, is achieved by converting each image voxel that is occupied by bone tissue directly into a finite element model (Morgan & Bouxsein, 2005). This type of meshing, first published in 1990 (Keyak, Meagher, Skinner, & Mote, 1990), has no geometric limitations, is fast and is fully automated. However one notable limitation is the potential for jagged inner and outer surfaces, which may cause numerical problems (Lengsfeld et al., 1998).

Computational modelling of trabecular bone has several potential research and clinical applications which include fracture risk assessment and the design and validation of prosthetic implants (Zannoni, Mantovani, & Viceconti, 1998). However, more relevant to this work is the ability of FE analysis to estimate the distributions of stress and strain within bone tissue in response to applied loads (Morgan & Bouxsein, 2005), determine the contribution of geometry to strength (independent of material properties) and conduct virtual compression tests (leaving the actual bones available for histology).

The microstructure of trabecular bone is one of the key factors in the prediction of the mechanical properties of bone, including bone strength and stiffness (Faulkner et al., 1991; Muller & Ruegsegger, 1995). Trabecular bone is characterised by its complex architecture (three dimensional, interconnected, open porous space which forms a solid structure) that results in
huge variations in its material properties (Keaveny, Morgan, Niebur, & Yeh, 2001). Models based on its microstructure allow for the study of its structure and function and are also able to provide insight into trabecular failure (Niebur et al., 2000). When comparative studies between the apparent yield properties for trabecular bone and an FE model of the same bone were carried out, it was shown that the model was able to predict areas of bone stress and strain at the same areas of failure as the actual bone sample (Keyak, Rossi, Jones, & Skinner, 1998; Niebur et al., 2000). The results were not statistically different for the same loading cases.

One advantage of computational modelling over conventional methods is the prediction of fracture loads by non-invasive means. Studies have shown that computational models are able to achieve precision that is comparable to densitometry data, along with being able to predict fracture loads up to an accuracy of 60% (Keyak et al., 1998).

Studies of the distal radius (in cadaveric specimens) have been used to analyse bone strength. Each bone was destructively loaded in compression to determine its linear elastic and failure regions. Subsequent FE analysis was carried out and showed strong correlations with the mechanical tests. Thus it was concluded that FE models are good predictors of bone strength (Macneil & Boyd, 2008). Another example of FE analysis is the assessment of trabecular bone. A mesh generator was used to create a three dimensional model of a bone biopsy obtained from a high resolution CT scan (consistent with the work described in Chapter 3). Following the mesh construction, a FE stress analysis test showed that the predicted Young's modulus values obtained were consistent with the range that was reported for compression testing of the same trabecular bone specimens (Muller & Ruegsegger, 1995). Another study looked at the correlation coefficients between assessing the femur using FE analysis and experimental validation using strain gauge measurements. There were good correlations between the stresses observed using the strain gauge technique when compared with the FE results, with R² values ranging from 0.84 – 0.9 (Lengsfeld et al., 1998).

In recent years, computational modelling has been incorporated into a few clinical studies. A study looking into the effects of bisphosphonates in Beagles used a combination of micro-CT, FE modelling and standard mechanical testing. To evaluate the influence of trabecular micro-architecture, a Young’s modulus of 1 GPa and Poisson’s ratio of 0.3 was applied to all models. The models were then scaled to match the apparent modulus from the mechanical tests to evaluate any changes to the material properties of the bone matrix (Day et al., 2004). This study showed that bisphosphonate treatments had an influence on the architecture of the bone but not on the material properties. Two separate, human, clinical trial-based studies looked at the
influence of bisphosphonates on bone strength. Findings from micro-CT showed significant architectural changes in the bone, FE analysis showed no mechanical differences in the strength of the bone (Tsai et al., 2015) and there were preferential increases in the trabecular but not cortical compartments (Keaveny et al., 2007). A three year long clinical trial assessed the influence of Denosumab (monoclonal antibody) in women with osteoporosis. This study showed significant improvements in vertebral body strength in axial compression and proximal femur strength in sideways fall configuration using QCT-based FE method (Zysset et al., 2015).

1.9 SUMMARY

This chapter describes the anatomy of bone at three different scales: the macro scale or whole organ scale (which is all that is visible to the naked eye), the micro scale (which include the osteons/haversian systems) and the nano scale (which predominantly includes the collagen fibres). The three main bone cells: osteoblasts, osteocytes and osteoclasts were mentioned, followed by a description of bone remodelling, a process that maintains balance between bone resorption and deposition. A condition resulting from an imbalance in this system, osteoporosis, was discussed. Three anabolic treatments for osteoporosis were then considered, followed by examples of FE analysis and its ability to predict values comparable to actual experimental bone tests and its incorporation into clinical studies.
1.10  **THESIS OVERVIEW**

This thesis is delineated by the following chapters:

**Chapter 2** provides a detailed description regarding the imaging modality (micro-CT) used as part of this thesis. Based on these high resolution images, 3D models of the digitised VOI’s of trabecular and cortical bones were created. A number of standard micro-CT indices were generated and used to analyse the bones. In addition to these indices, bone and tissue mineral densities for trabecular and cortical bone, respectively, were computed from micro-CT phantoms. To end this chapter, the process of digitally cleaning the bones will be explained.

Following on from the previous chapter where the bones were digitally cleaned, **Chapter 3** begins with the process used to voxel mesh the bone models. The meshed bones were then assessed using the FE method which was included as part of the modelling pipeline of this work. This chapter, outlines the material properties and boundary conditions assigned to the bone models followed by how the analyses were carried out. To finish, the set-up and results from the model validation experiment is discussed.

**Chapter 4** looks at different methods to evaluate material properties of bone at different scales. At the macro scale, three point bend testing and micro-CT phantoms are used to establish the material properties. At the micro scale, microindentation and at the nano scale, nanoindentation. Preparing the bones for the various tests and calculations that were carried out are covered in this chapter.

The influence of low-dose fluoride treatment on bone is discussed in **Chapter 5**. This fluoride work was carried out as part of a large scale, human clinical trial. Our modelling pipeline was applied to this work using a double blinded design and was able to predict the strength trends at different fluoride levels which was obtained from independent micro-CT analyses. To our knowledge, applying a modelling pipeline to a clinical trial is very limited in the literature.

**Chapter 6** discusses the influence of a whey protein diet in a murine model. One of the main aims of this study was to evaluate the influence on model predictions from material properties that had been measured from different sources, at different scales. This study shows that material properties do influence computational predictions of strength which was demonstrated when nanoindentation derived material properties were included in the FE model. Secondly we show that the effects were very localised between cortical and trabecular bone.
In experimental bone work, Sham operated and OVX mice are commonly used controls. **Chapter 7** assessed whether there was a mechanical difference in the bones from the two models. This was done by applying the modelling pipeline to this experimental data. We show that while there are morphological differences in the trabecular bone between the two groups (significant differences in the BMD and a number of micro-CT indices) there was no significant difference in the mechanical strength of the bones from either surgical groups. There were no changes in either morphology or mechanical strength in the cortical bone, between the two groups. This suggests that analysis of BMD, TMD and micro-CT indices alone do not reflect the complete mechanical integrity of the bone.

Finally, **Chapter 8** outlines the key findings of the thesis and critically assesses some of the limitations with this work and how they were overcome or compensated for. This chapter also discusses the three experimental techniques used (micro-CT analysis, FE modelling and assessment of material properties at different scales). The key outcomes from this thesis is the development and refinement of a pipeline which has been tested in real clinical situations.

An overview of the collaborative Vitamin D work where we tested our model on an external collaboration murine study is included in **Appendix A**.

3D cortical bone micro-CT indices are included **Appendix B**. These were originally included in Chapter 6 but have since been removed according to suggestions made by the reviewers of our paper.
1.11 LIST OF PUBLICATIONS

This thesis has been presented through the following collected works:

JOURNAL ARTICLES


CONFERENCE PAPERS AND EXTENDED ABSTRACTS


T. Li, T. Jenkins, **D. Sreenivasan**, J. Fernandez, R.O.C. Oreffo. Multiscale and Multidisciplinary Analysis of Murine Bone Health Following *In Utero* Vitamin D Deficiency: SkelGEN Collaboration. 4th TERMIS World Congress, September 8-11, 2015, Boston, Massachusetts, USA.

2 Micro-CT Model Creation

The following chapter covers the steps taken to image bone using micro-CT, determine the volumes of interest (VOIs) to be assessed, build trabecular and cortical bone models, analyse the data and obtain a number of micro-CT indices to be used as surrogate measures for bone strength. In addition, this chapter covers how the bone samples were digitally cleaned. These steps formed the basis for this body of work and were applied to human biopsy samples, obtained as part of a large scale clinical trial (Chapter 5), and to mice femur samples (Chapters 6 and 7).

2.1 OVERVIEW

Micro-CT has now become the “gold standard” for evaluating bone morphology and microarchitecture in small animal models such as mice (Bouxsein et al., 2010). Scanners are able to achieve an isotropic voxel size as low as a few micrometres, small enough to image mouse trabeculae which commonly have widths of around 30–50 µm (Bouxsein et al., 2010; Martin-Badosa et al., 2003). Using micro-CT to evaluate bone mass and morphology is highly advantageous over deducing measurements such as trabecular morphology, thickness and separation from standard 2D methods. The main advantages of using micro-CT is that the analysis encompasses a larger volume and the bone samples are assessed non-destructively so that they remain available for mechanical testing and histology preparations (Barbier et al., 1999; Muller et al., 1998). Furthermore, micro-CT is cost effective in comparison to histological analysis as it requires very little sample preparation (Holdsworth & Thornton, 2002).
One of the first studies that used micro-CT to assess bone morphology was the investigation of the subchondral bone structure in the femoral head of a guinea pig model of osteoarthritis (Layton et al., 1988). Since then, micro-CT has been used to evaluate bone growth and development (Alexander et al., 2001; Barbier et al., 1999; Laib, Kumer, Majumdar, & Lane, 2001), study genetically altered mice (Hankenson, Hormuzdi, Meganck, & Bornstein, 2005; Silva, Brodt, & Uthgenannt, 2004) and to assess disease states, such as studying OVX mice as an animal model of osteoporosis (Bouxsein et al., 2005; Jiang, Zhao, White, & Genant, 2000; Laib et al., 2001). Most importantly in regards to this body of work, micro-CT imaging allows the voxel based data, in combination with the high resolution morphology of the bone, to be converted into micro-FE models which can then be used to make inferences regarding bone behaviour (Bessho et al., 2007; Blanchard, Dejaco, Bongaers, & Hellmich, 2013; Lengsfeld et al., 1998).

This body of work applied micro-CT imaging to both human biopsy samples, obtained as part of a clinical trial (Chapter 5) and to studies involving mice models (Chapters 6 and 7), whereby femurs were obtained at end point and scanned. The process, from imaging to sample analysis, was almost identical for all three studies (see Chapters 5 - 7). For the purpose of this chapter, the mouse femur is discussed.

### 2.2 IMAGING BONE SAMPLES USING MICRO-CT

Each bone was scanned using a SkyScan 1172 micro-CT scanner (X-ray voltage 50 kV, 0.5 mm aluminium filter and voxel size 5 µm). The scanner was calibrated, with the alignment checked at both coarse (18 µm) and fine (3 µm) resolutions. The camera was set to medium and no filter was used. The alignment check was performed at 18 µm until no further adjustments were required and then at 3 µm and back to 18 µm until no adjustments were required at either resolution. Following the alignment check, a configuration file was prepared. This file specified the exposure time\(^1\), voltage and flat fields\(^2\) and a new configuration file was created at the beginning of each study. An exposure time of 12 minutes was chosen as it was the minimum time required to obtain a raw image once the x-ray source had stabilised. Following the stabilisation of the source, a flat field image was collected with uniform intensity and an average value of 90%.

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\(^1\) Sufficient time so that the signal was high enough to visualise the bone.

\(^2\) Flat field corrections are used to improve digital imaging. The goal is to remove artifacts from the images caused by pixel sensitivity of the detector or any disruptions in its optical path.
Bone samples were prepared for scanning as follows (refer to Figure 2.1):

1. The mouse femur was placed at the edge of a small piece of tissue, dampened with phosphate buffered saline (PBS) and wrapped tightly. PBS was used as the osmolarity of the solution is identical to the isotonic concentration of the human body and is non-toxic to cells.

2. The bone, wrapped in tissue, was then wrapped in a small piece of Glad Wrap (plastic wrap or cling wrap) to further secure it and prevent it from drying out.

3. The wrapped sample was then placed in a Styrofoam tube which had an attachment at the base to secure it onto the micro-CT stage.

Once the sample was secured onto the stage the X-ray source was allowed to stabilise for 12 minutes, as mentioned above. In that time period, the femur was visualised using the micro-CT camera to ensure the correct orientation of the bone sample (distal end of femur closest to the micro-CT stage) and the appropriate set up was performed. This include: adjusting the camera pixel size to 5 µm and checking that the height of the stage was sufficient so that the required region of the femur was scanned. Once the source had stabilised, a flat field image was captured and the scan was initiated. One complete scan took approximately one hour.
Once each scan was complete the alignment of the scans were checked using the SkyScan NRecon software and appropriate reconstruction parameters were selected. Beam hardening was set at 20% and the lower and upper contrast limits were set at 0.000 and 0.135, respectively. All the chosen reconstruction parameters were applied identically to each scanned sample. The only parameter that was unique to each scan was the post alignment correction, which was allowed to fall in the range of -6 to 6. When the parameters were set correctly, standardised reconstructions were carried out in NRecon.

In addition to the bone samples, two solid phantom rods for BMD and tissue mineral density (TMD) measurements were scanned. These rods, purchased from SkyScan and had a diameter of

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3 Most commonly encountered artifact in CT scanning which causes the edges of an object to appear brighter than the center even if the material is the same throughout
2 mm to approximately match the cross sectional thickness of the mice bones. The rods had BMD values of 0.25 and 0.75 gcm$^{-3}$ and were prepared for scanning identically to the bone samples. Reconstruction of the phantom rods were carried out identically to the bone samples, with all the reconstruction parameters kept constant.

### 2.3 DETERMINING THE TRABECULAR AND CORTICAL VOI

The reconstructed images were rotated using the SkyScan DataViewer software. The images were analysed in three views: cross sectional area, sagittal plane and coronal plane. The images were manually adjusted so that the bone slices were straight in each of the three planes mentioned. The purpose of this process was to create a final image of the scans where the shaft of the bone was straight.

The VOIs for both trabecular and cortical bone were determined as follows. Using the SkyScan CTAn (CT analyser) software, the growth plate of the bone samples was identified as a low density cartilage seam, running uninterrupted from one end of the bone slice to the other within the cross sectional plane (refer to Figure 2.2). The growth plate slice number was recorded for each bone scan. Note, a few slices either side of the growth plate showed an interrupted seam and thus, was no longer indicative of the presence of the growth plate.
Once the growth plate was identified for each bone sample, an offset was determined. The offset was the number of slices from the growth plate to the start of the volume of interest (VOI) for the trabecular and cortical bone. The offset number was kept constant for every bone sample. For trabecular bone, the offset number was 100 slices and for cortical bone 690 slices. This is diagrammatically represented by Figure 2.3. The offset was created so that only the secondary spongiosa (mature bone) was included in the VOI and was included in the analysis of the bone.

Once the offset had been established, the number of slices for both trabecular and cortical VOIs were decided. This is shown in Figure 2.3. Working out the trabecular VOI was particularly important, as the 2D images were used to determine the maximum possible volume to be included in the VOI of the trabecular bone. Therefore the trabecular VOI included 250 slices, which equated to a height of 1250 µm (5 µm per slice). The cortical VOI included 100 slices, equating to a height of 500 µm.

*Figure 2.2. Cross sectional slice through the bone. Red dashed lines show the continuous low density seam, indicative of the growth plate of the bone.*
2.4 DIGITISING THE VOI FOR TRABECULAR AND CORTICAL BONE

Using the CTAn software and the growth plate as a reference point, the trabecular and cortical VOI’s were manually digitised. The growth plate was chosen as the selection reference and the VOI slice number and the offset number, chosen as described in section 2.3, were also entered into CTAn. This created a vertical range of slices for the trabecular bone VOI to be analysed, relative to the growth plate. The same process was carried out for cortical bone. By setting these values, it ensured that the offset remained at 100 slices for trabecular bone and 690 slices for cortical bone) and the VOI for trabecular bone was 250 slices and for cortical bone, 100 slices.

The second stage involved using a drawing pad to draw shapes parallel to, but away from, the endocortical boundary and the remnants of the growth plate that were present at the corners of the slices (refer to Figure 2.4a and Figure 2.4b). This was done approximately every 15 slices or when the shape of the trabecular region of interest changed, until the entire VOI was digitised. The software interpolated between the drawn shapes to produce the three dimensional VOI. Figure 2.4c shows a slice through the digitised trabecular region within the VOI.
The cortical VOI was digitised similarly to the trabecular VOI with a few differences. The offset and VOI distance was set according to what had previously been identified. With the cortical bone, a hollow region of interest was drawn in order to exclude any remaining trabecular bone, see Figure 2.5a. As the shape of the cortical bone didn’t change as rapidly as in trabecular bone, approximately 3 - 4 drawings were sufficient for each bone in order to create the VOI. Figure 2.5b shows a slice through the digitised cortical region of the VOI.
2.5 CREATING A GLOBAL THRESHOLD VALUE

Analysis of the micro-CT images required that the previously digitised dataset be binarised. This was done using CTAn and involved transforming the grey scale images into black and white. For our study, global thresholding was used. One limitation of this method is that trabecular bone has both thick and thin structures. This makes it difficult to threshold, as the thin structures have a lower density compared to the thicker structures. This is shown in Figure 2.6. Figure 2.7 is an example of thresholding a slice within the trabecular VOI.

Figure 2.5. Digitising the cortical VOI. a. Manually drawing a shape inside the cortical VOI taking note to include any remnants of trabecular bone. b. Example slice through the VOI of the already digitised cortical VOI.
To ascertain the global threshold value, each dataset was analysed and a value obtained. Some trial and error was required in order to determine an appropriate value that could be applied to all of the bone samples. A value in the range of 90-255 is generally selected for bone.
2.6 BUILDING TRABECULAR AND CORTICAL BONE MODELS

Trabecular and cortical bone models were made using the SkyScan 3D-creator (Ant) software. To create the model, the previously digitised dataset was imported into the software and a drawing interchange/exchange format (DXF) file was created. The previously selected threshold value of 90 was entered and the models were built based on that value. The same process was used for both trabecular and cortical bone. Figure 2.8 shows an example of a trabecular and a cortical bone model.

![Bone models. a. Trabecular bone model b. Cortical bone model.](image)

2.7 ANALYSIS

2.7.1 CALIBRATION USING PHANTOM RODS

Calibration of the BMD measures was performed using two epoxy resin rods (phantom rods) made of calcium hydroxyapatite powder and had densities of 0.25 and 0.75 g/cm³ for the low and high density rods, respectively. Figure 2.9 shows a cross section through the two calibration rods. The lighter colour represents the lower density rod and the darker colour represents the high density rod. It should be noted that the phantom rods were scanned in exactly the same way as the bones (refer Figure 2.1) to ensure consistency.
A region of interest within the calibration rods was chosen using the circle drawing feature in CTAn. It excluded the outer edge and upper and lower boundaries of the two rods. The regions of interest included 150 slices for both the low and high density rods. The density of each rod was verified, by the software, as 0.25 and 0.75 gcm$^{-3}$ for the low and high density rods, respectively. BMD and TMD for each bone specimen was calibrated against the Hounsfield units of the phantoms, based on known densities.

### 2.7.2 Calculating Bone and Tissue Mineral Density

Having calibrated the software, the BMD and TMD for trabecular and cortical bone, respectively, was calculated. For trabecular bone, the dataset was loaded into CTAn and the VOI overlaid (see Figure 2.10). The process of digitising the VOI, explained previously (see section 2.4). BMD, is defined as the averaged bone mineral density of the marrow and trabecular bone within the medullary VOI and is only applied to trabecular bone. CTAn calculated the BMD for the VOI of the trabecular bone.
For cortical bone, the dataset was again loaded into CTAn and the TMD was measured. TMD only applies to cortical bone and is defined as the density of only the bone, excluding all the surrounding tissue. This type of analysis is applicable to cortical and not trabecular bone, as cortical bone is thick enough that the partial volume effect does not compromise the results. In brief, the partial volume effect occurs due to a loss of apparent activity in small objects of regions due to the limited resolution of the imaging system (Rittweger, Michaelis, Giehl, Wusecke, & Felsenberg, 2004). Unlike trabecular bone, where the VOI was used to analyse BMD, thresholding is used to analyse TMD for cortical bone. Cortical bone was binarised using the previously selected threshold value of 90 (see section 2.5). CTAn, then calculated the TMD of the cortical bone. This is represented by Figure 2.11.

The methods described for calculating BMD and TMD were applicable on an individual sample basis. However, the method was applied to the entire dataset at the same time using the custom processing feature in CTAn, Batch Manager (BATMAN).
The process of generating the standard micro-CT morphometric parameters in 3D was relatively straightforward for trabecular bone. The process involved using the BATMAN function of CTAn and selecting the required micro-CT indices to be exported.

Cortical bone, however, required more processing than trabecular bone and this was done in two parts. The first part provided the standard cortical morphometric indices and the second part involved calculating the porosity of the cortical bone. To calculate the standard indices, the previously digitised cortical bone (see section 2.4) was opened in CTAn. The bone was binarised using the selected threshold value of 90. A number of functions within the custom processing section of CTAn were then applied to the cortical bone. The shrink wrap function was used to delineate the VOI. It wraps the VOI around the periosteal boundary, allowing for the measurement of the periosteal and endosteal boundaries, which are the outer and inner bone surfaces, respectively. In addition, the shrink wrap function was re-run to ensure that the ‘stretch
over holes’ function was activated and any ‘holes’, which are generally where the blood vessels run through, were accommodated for by this process. Artefacts were removed using the ‘despeckle’ function, which removes any black and white dots. Once this process was complete, the 3D analysis was run. The second part of the cortical bone analysis involved establishing the porosity of the cortical bone. Again using CTAn, the bone was binarised using the threshold value of 90. Any remnants of the trabecular structures were removed using the ‘despeckle’ function. The key to analysing porosity in cortical bone is to base the binarised image on the volume of interest (see Figure 2.11), which shows the VOI overlaid on the binarised image. The bitwise operation allowed for the region of interest to become a copy of the image. So that the porosity could be analysed, the pores were filled in by applying the ‘despeckle’ function to the VOI. However, some of the pore spaces were still accessible to the outside and therefore were not filled in. An additional function, the morphological operation ‘close’, was applied to the VOI (see Figure 2.12). Finally, in order to ensure that there was no mismatch between the surface of the VOI and the surface of the original image, a single pixel thickness of the surface was removed from the VOI. This was achieved using the ‘morphological operations’ function, whereby a radius of 1 pixel was ‘eroded’ from the surface of the VOI. The analysis was then carried out, whereby the 3D analysis function is selected and run in CTAn as previously described for trabecular bone and the first part of the cortical bone analysis. This time, however, the output report file included the porosity measures for the cortical bone.
From the numerous values generated by CTAn, only a few were selected as part of the analysis for this work and were used as surrogate measures for bone strength. The selected indices are explained in Table 1 for trabecular bone and Table 2 for cortical bone. These indices were chosen as they provide descriptive measures for the amount of bone within the selected VOI.

Figure 2.12. Porosity calculations using CTAn and an example of the ‘despeckle’ function. a. VOI inclusive of all the pores within the cortical bone. b. Using the ‘despeckle’ and ‘close’ function all the pores were filled in. These two images show the differences between the original image (a) and the VOI (b) which has had all the pores filled in for porosity analysis.
Table 1. Definition and description of the 3D micro-CT variables used as surrogate measures for bone strength for trabecular bone microarchitecture

<table>
<thead>
<tr>
<th>Micro-CT variable</th>
<th>Description</th>
<th>Standard Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage bone volume</td>
<td>Ratio of the segmented bone volume to the total volume of the region of interest</td>
<td>%</td>
</tr>
<tr>
<td>Bone surface density</td>
<td>Ratio of the segmented bone surface to the total volume of the region of interest</td>
<td>( \text{mm}^2/\text{mm}^3 ) or ( \text{mm}^{-1} )</td>
</tr>
<tr>
<td>Trabecular thickness</td>
<td>Mean thickness of trabeculae</td>
<td>mm</td>
</tr>
<tr>
<td>Trabecular number</td>
<td>Measure of the average number of trabeculae per unit length</td>
<td>( 1/\text{mm} ) or ( \text{mm}^{-1} )</td>
</tr>
</tbody>
</table>

Table 2. Definition and description of the 3D micro-CT variables used as surrogate measures for bone strength for cortical bone microarchitecture

<table>
<thead>
<tr>
<th>Micro-CT variable</th>
<th>Description</th>
<th>Standard Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume</td>
<td>Volume of the region segmented as bone</td>
<td>( \text{mm}^3 )</td>
</tr>
<tr>
<td>Tissue Volume/Periosteal volume</td>
<td>A measure of bone deposition</td>
<td>( \text{mm}^3 )</td>
</tr>
<tr>
<td>Medullary volume/Endosteal volume</td>
<td>A measure of bone resorption</td>
<td>( \text{mm}^3 )</td>
</tr>
</tbody>
</table>

Note: In Chapter 6, based on suggestions made by the reviewers of the paper, the equivalent 2D indices were used for cortical bone. These include: mean total cross sectional tissue area (T.Ar), mean medullary area (Ma.Ar), mean cross sectional thickness (Cs.Th), mean total cross sectional bone area (B.Ar) and mean cortical area fraction (B.Ar/T.Ar).

### 2.9 RAPID FORM

The output DXF files from the SkyScan 3D creator software were imported into Rapid Form (Inus Technology Inc.). The data was digitally cleaned in order to remove any bone artefacts (any fragments of the bone that were not attached to the main body) and any redundant polygons. The mesh was then decimated (reduced in size) by 30%. This was done for computational efficiency but did not reduce the quality of the geometrical structures. Decimation of the mesh by 30% did not affect the root mean square (RMS) of the geometry by more than 1%. The files were exported as StereoLithograph (STL) files. Figure 2.13 and Figure 2.14 show the process of digitally
cleaning and creating the STL files in Rapid Form for trabecular and cortical bone, respectively. Figure 2.13a and Figure 2.14a show the DXF file imported into Rapid Form and Figure 2.13b and Figure 2.14b shows the redundant parts to be removed. The circled area (shown for trabecular bone only) represents some redundant parts of the mesh that were removed by the cleaning process (Figure 2.13c). The digitally cleaned images are shown in Figure 2.13c and Figure 2.14c. Note that while the same process was applied to both trabecular and cortical bone, the changes were a lot less noticeable in the cortical bone.
Figure 2.13. a. Shows the original DXF file for trabecular bone, imported into Rapid Form b. All the coloured parts some of which are highlighted by the circles in the images, are the redundant polygons and bone artefacts to be removed. c. Shows the final, decimated (not visible in image) mesh which was exported from Rapid Form as an STL mesh.
Figure 2.14. a. Shows the original DXF file for cortical bone, imported into Rapid Form. b. All the coloured parts are the redundant polygons and bone artefacts, to be removed. c. Shows the final, decimated (not visible in image), mesh which was exported from Rapid Form as an STL mesh.
The following chapter covers the methods by which the trabecular and cortical bone FE models were created and follows on from the previous chapter where the imaging and digital cleaning of the bones were discussed. This chapter details the process by which trabecular and cortical bones were voxel meshed, how the FE models were set-up (including assignment of material properties and boundary conditions) and the analysis that was carried out. The modelling pipeline was applied to this entire body of work (see chapters 5–7) to assess both human and murine samples. FE analysis based on high resolution imaging (such as micro-CT) of bone allows stress and strain patterns in bone tissue to be analysed. Bone strength estimates from FE analysis are comparable to experimental work.

This chapter also describes the validation experiment used for the FE bone models. Validation involved an Instron compression test of 3D printed bone samples (based on a human biopsy sample). The Instron process was subsequently FE modelled and areas of weaknesses identified in the model correlated with those identified in the 3D printed sample. This was then used to validate this entire body of work (see chapters 5–7).
3.1 TRABECULAR BONE

3.1.1 VOXEL MESHING

As described in Chapter 2, bone models were exported from Rapid Form as STL files. The native STL is a surface mesh which represents the surface as raw, unstructured triangles. These STL models were imported into Hypermesh (Altair Engineering Inc.) to be voxel meshed.

In early investigations, 3D extrusion was performed on the native STL files using irregular tetrahedral elements. While these tetrahedral elements produced smoother surfaces, the models were not as robust and repetitive for all the bone sets when performing FE analysis. This element type was therefore deemed unsuitable for use in the modelling pipeline, in part due to the complex haversian system (present in human but not murine cortical bones) and the abundance of inhomogeneous struts in the trabecular bone structure (present in both human and murine bones). The second attempt at meshing used hexahedral voxels. This provided meshes that were more robust for use in FE analysis, offered more repeatable results and were able to adequately accommodate the irregular architecture of trabecular bone. Specifically, different components were assigned to the various internal structures of the trabecular bone, so that the entire trabecular structure was filled and one solid structure was formed. The element size was chosen based on the convergence criteria. Element size was reduced until peak stresses did not change by more than 1-2%. The size of each element was also selected based on a trial and error process, with the objective, to best capture the original STL surface geometry. This is illustrated in Figure 3.1, where the meshing process was started by using voxels that were 50 µm in size. This created a block like structure that did not capture the architecture of the trabecular bone well. The voxels were reduced in size from 40 µm to 30 µm to 20 µm. Finally, a voxel size of 10 µm was chosen as the most appropriate size for these trabecular bone samples. From this solid structure, various filler components were deleted until the final mesh appropriately captured the geometry of the trabecular bone. The filler components are represented by the different coloured regions in Figure 3.1. The process of creating a voxel mesh from an STL mesh and removing the filler components is shown in Figure 3.2. It should be noted that the mesh size was sufficient for convergence, to effectively capture the geometry and the stress patterns of the bone.
Figure 3.1. The process of selecting an appropriate voxel size for the meshing process. The meshing process started at 50 µm and was refined until a final size of 10 µm was selected for all the bone samples. This was to ensure that the complex architecture of the trabecular bone was appropriately represented. Note, that the various colours are different filler components.
Figure 3.2. The process of creating a voxel mesh in Hypermesh for a trabecular bone sample. a. The native STL mesh from Rapid Form. b. The voxel mesh created in Hypermesh. c. Components of the mesh deleted with the final product showing a structure that appropriately represented trabecular bone. The enlarged segment of the mesh shows the individual voxels. Note, that the different colours are various filler components.
The material properties of the bones were modelled using linear elasticity, according to the following assumptions which were selected based on how bone behaves mechanically:

- Bone experiences very small deformations, $< < 10\%$, and therefore has very small strains.
- The bone did not undergo any large rigid body movements.
- The stress is proportional to the strain ($\sigma \propto \varepsilon$) within the bone which means that the applied load is proportional to the resulting deformation in a linear fashion.
- The material returns to its original shape when the loads are removed and the path of unloading remains the same as the loading path (elastic).
- There is no dependence on the rate of loading or straining. All simulations were quasi-static, which meant that the rate of loading was slow. Due to the quasi-static conditions, the system was in equilibrium, which meant that the constitutive law was followed.

Based on these assumptions the bone deformed according to the constitutive law defined as:

$$ e_{ij} = \frac{1 + v}{E} \sigma_{ij} - \frac{v}{E} \sigma_{kk} \delta_{ij} $$

where, $e_{ij}$ is the load in the x-direction, $v$ is the Poisson’s ratio, $E$ is the Young’s modulus, $\sigma_{ij}$ is the stress in the x-direction, $\sigma_{kk}$ is the stress in the other directions (y and z) and $\delta_{ij}$ is the Kronecker delta which is 0 if $i \neq j$ and 1 if $i = j$ (which was true in this case).

The bone was also assumed to be isotropic for each voxel in our microstructural model (this does not hold true when we homogenise the micro model and find the macro level properties), meaning that there were no preferred directions. Bone has been well documented to be stiffer in the longitudinal direction. For example, nanoindentation tests of osteons in the longitudinal direction have shown Young’s moduli of $19.4 \pm 1.7$ GPa whereas in the transverse direction, $16.6 \pm 1.1$ GPa (Rho, Roy, Tsui, & Pharr, 1999). All our bone models were loaded in the longitudinal direction. Since isotropy was assumed, only two parameters were required to
describe the model: Young’s modulus\(^4\), \(E\), and Poisson’s ratio\(^5\), \(\nu\). Young’s modulus of 1 GPa was assigned to the models. According to the literature, the Young’s modulus of trabecular bone can be anywhere between 1-14 GPa (although 14 GPa is a high value for trabecular bone) (Choi et al., 1990; Rho et al., 1997; Zysset et al., 1999). Poisson’s ratio was set at 0.3, a widely accepted value for trabecular bone (Wirtz et al., 2000; Zysset et al., 1999). Using a Young’s modulus of 1 GPa for all the models allowed the influence of only the architecture of the bone to be evaluated, since the material properties were constant across the population. To evaluate the influence of material properties, BMD and TMD values, obtained from the micro-CT phantom scans (as described in section 2.7) and these were used to estimate the bone’s material properties. This density was included in the model using the adapted (Carter and Hayes, 1977) power law (Currey, 1988) which relates elastic modulus to the apparent bone density. The power law was chosen as it naturally represents the experimental trends observed between density and Young’s modulus, provides a better transition between trabecular and cortical bone and also has been shown to distribute the residual errors better (Gupta & Dan, 2004). For this study we related apparent density from trabecular bone using the reported trabecular (cancellous) power law and the version for cortical (compact) bone (Gupta & Dan, 2004). A piecewise power law function was adapted for trabecular \((1050e^{-6}\rho^2)\) and cortical bone \((3.0e^{-6}\rho^3)\) as follows:

\[
E = \begin{cases} 
1050e^{-6}\rho^2 & \text{for } \rho \leq 350 \text{ kgm}^{-3} \\
3.0e^{-6}\rho^3 & \text{for } 350 \leq \rho \leq 1800 \text{ kgm}^{-3}
\end{cases}
\]

where, \(E\) represents the Young’s modulus of the bone and \(\rho\) is the apparent density of trabecular and cortical bone.

The FE modelling pipeline was developed and evaluated from a 3D printed bone test. The material properties reported by the manufacturer were 1.4-3.1 GPa, but material properties were found to be approximately 1 GPa from Instron testing. We adopted 1 GPa, which was at the lower end of the reported range and the von Mises yield value was also obtained from this test. The real bone failure load required scaling in order to reflect its true stiffness (which was much greater than the 3D printed bone). For example, cortical bone stiffness of murine bone was 10-25 GPa (from microindentation tests) hence the computed failure load was scaled by this factor. This linear scaling was permissible as we assumed linear elasticity, which is consistent with other

\(^4\) The Young’s modulus is the linear part of the stress vs. strain curve.

\(^5\) The Poisson’s ratio is the deformation out of plane relative to the direction of deformation, with a value of 0 being fully compressible and 0.5 being completely incompressible.
studies (Ulrich, Van Rietbergen, Laib, & Ruegsegger, 1999) and was applied to all studies in this thesis.

3.1.3 BOUNDARY CONDITIONS

In order to prevent displacements and rigid body rotations, the following boundary conditions were applied to the models (shown in Figure 3.3):

- The load was applied to a plate to cover the complex surface of the strut and beam structure, a typical characteristic of trabecular bone. In addition, the plate also functioned to disperse the load evenly across the bone.

- The top 10 rows (100 μm) of the bone were fixed and a tie constraint was created between the load on the plate and the top of the bone. This was done in order to apply the load evenly to the bone.

- The bottom 20 rows (200 μm) of the bone were fixed and was prevented from moving or rotating in any direction to simulate quasi-static conditions.
Figure 3.3. Model set-up in Abaqus. a. Plate with a reference point (RP-1) where the load was applied in the z-direction. The top of the bone (yellow) was tied to RP-1. The arrow represents a specified distance between the top of the bone and RP-1. b. Model showing the fixed bone base (orange). Fixing the base of the bone ensured that the bone model was not permitted to move or rotate in any direction in order to simulate quasi-static conditions.
3.2 CORTICAL BONE

3.2.1 VOXEL MESHING

The process of voxel meshing cortical bone was almost identical to that described for trabecular bone. Only the murine cortical bones were analysed (chapters 6 and 7) due to sample availability. This was advantageous, however, as murine cortical bones lack a haversian system, making the meshing process significantly simpler (Mills & Simpson, 2012). Hexahedral voxels with a size of 40 μm were used to mesh cortical bone, which was a different element size in comparison to the one used for trabecular bone. This element size was also chosen based on the convergence criteria and the element size was reduced until peak stresses did not change by more than 1 - 2%. A coarser mesh was chosen because, unlike trabecular bone with its complex structure, mice cortical bone is simply a solid bone with a hollow middle. Since the geometry was easier to capture, the voxel size was not required to be as fine (see section 3.1.1). Using the coarser mesh meant that the cortical models were significantly more efficient, computationally, compared to the trabecular bone models. Another advantage of evaluating cortical bone compared to trabecular bone, is that the regular surface made it easier to apply loads uniformly at the surface. Similar to trabecular bone, the meshed bone had filler elements which were removed (central inner structure) before being exported into Abaqus. This is shown in Figure 3.4.
Figure 3.4. The process of creating a voxel mesh in Hypermesh for a cortical bone sample. a. The native STL mesh from Rapid Form b. The voxel mesh created in Hypermesh c. Components of the mesh deleted with the final product showing a solid outer structure and a hollow inner structure, representing cortical bone appropriately. The enlarged segment of the mesh shows the individual voxels.
3.2.2 MATERIAL PROPERTIES

In a similar manner to trabecular bone, the cortical bone models were all assumed to be linear elastic and isotropic. The Young’s modulus was set to 1 GPa with a Poisson’s ratio of 0.3 (Dong & Guo, 2004). At the macro level, cortical bone has a much higher homogenised Young’s modulus compared to trabecular bone, with values reported in the range of 10 – 25 GPa (Choi et al., 1990; Fan, Swadener, Rho, Roy, & Pharr, 2002; Silva, Brodt, Fan, & Rho, 2004; Zysset et al., 1999). Once a failure force was determined we scaled this value 10 times to represent the yield load for cortical bone. This was due to the computational model being simulated with the normalised Young’s modulus value of 1 GPa. As a check, the model was evaluated with a Young’s modulus of 1 GPa and 10 GPa, with the latter showing a failure load that was exactly 10 times the former due to the linear elastic assumptions. TMD values were obtained from the micro-CT (discussed in section 2.7) and these values were used to provide a subject-specific estimate of the material properties for each cortical bone sample. Using the power law (Equation 3.2), the TMD values were used to calculate a Young’s modulus for each cortical bone sample, with the process repeated as it was for trabecular bone.

3.2.3 BOUNDARY CONDITIONS

The boundary conditions for cortical bone were set slightly differently to the trabecular bone models. A plate wasn’t required for cortical bone as it is a solid structure and did not have the struts and voids that are characteristic of trabecular bone. This made the model considerably more stable than the trabecular models. The following boundary conditions were applied to the cortical models and are shown in Figure 3.5:

- Top surface nodes were constrained to a reference point where the load was applied. By fixing the entire top row, it allowed the load to be dispersed evenly across the bone. The top row was only given freedom to move in the z-direction. All other displacements and rotations were fixed
- Bottom surface nodes were fixed and no displacements or rotations were allowed. Again, this was to simulate quasi-static conditions.
This modelling framework was applied throughout this thesis, as will be discussed in chapters 5 - 7. Early solutions took an average of 6 - 8 hours, solving in parallel, using an Intel Xeon four core processor at 2.67 GHz with 6 GB of memory. Towards the end of the project, simulations were run on an Intel Xeon six core processor at 3.50 GHz with 32 GB of memory, leading to solution times in the order of 15 minutes. It should also be noted that by reducing the number of voxels in the cortical model, the time taken to run the models reduced from several hours to approximately 5 minutes. The significant decrease in solution times has made this modelling pipeline more tractable and suitable for inclusion as part of clinical trials.
3.4 ANALYSIS

Representative examples of stress distributions in trabecular and cortical bone models are shown in Figure 3.6 and Figure 3.7, respectively, indicating the contours of von Mises stress. In trabecular bone, the stress pattern was inhomogeneous in distribution and did not exhibit any distinct patterns that could be characterised as repeatable. The principal stress pattern in the longitudinal direction (the loading direction/\(z\)-direction) was most similar to the von Mises pattern, which is expected as the von Mises reveals the dominant principal direction. Further, stress concentrations were identified at regions of high curvature and sharp corners, which is where engineering peak stresses tend to cluster. It should be noted that some of these stress concentrations may be due to numerical artefacts associated with the use of voxel elements however the element size chosen minimise these. In contrast, cortical bone is more circular and ellipsoidal in shape and the stress pattern was consistent with this almost analytic shape. Stress was observed to be circular and varied radially. The stress pattern peaked at the outer circumference, reduced to a minimum away from the cortex and increased again towards the inner circumference. This is consistent with engineering structures, which show higher stresses at the boundaries. Finally, as the model shown is of murine bone, which does not exhibit haversian canals, the stress pattern is more uniform. That would not have been the case for human cortical bone.

Von Mises stress determines whether the stress combination at a given point will cause failure. It gives the combination of three principal stresses into an equivalent stress, which is then compared to the yield stress of the material. If the von Mises stress of the material exceeds the von Mises yield stress then the material will undergo failure. The von Mises stress in 3D is defined as:

\[
\sigma_v = \frac{1}{\sqrt{2}} \left[ (\sigma_1 - \sigma_2)^2 + (\sigma_1 - \sigma_3)^2 + (\sigma_2 - \sigma_3)^2 \right]^{1/2}
\]

where, \(\sigma_v\) is the von Mises stress, \(\sigma_1\) is the principal stress in the \(x\)-direction, \(\sigma_2\) is the principal stress in the \(y\)-direction and \(\sigma_3\) is the principal stress in the \(z\)-direction. The models were solved quasi-statically with each increment in force leading to a new homogenised von Mises. Post analysis then identified the force that was associated with a von Mises value that exceeded the yield value. This force was then defined as the failure force.
Figure 3.6. Abaqus output file showing stress distributions within one trabecular bone model in three different views. Areas in dark blue are regions with zero stress.
To validate the FE model, an Instron compression test was carried out on one 3D printed (repeated three times) human bone biopsy sample from the fluoride study (Chapter 5). The equivalent FE simulations were carried out in Abaqus to establish a yield criteria/failure criteria which was then applied to the bone samples across the treatment groups.

The bone models were printed in 3D (Dimension Elite 3D printer, Stratasys) and were scaled to 10x the size of the original sample. The sample had an approximate width of 4 mm and the scaled up model, 40 mm. In addition, a plate was designed in SolidWorks (Dassault Systemes Pty. Ltd.)
and placed on the top and bottom of the printed bone sample (see figure 3.8). The top plate fits the load plate of the Instron and its main purpose was to distribute the load, uniformly. The base plate acted as a specimen clamp. The Instron 5800 series has a position accuracy of 0.02 mm and a load sensor accuracy of 0.1 N, which is 1/100 of the 10 N capacity of the cell chosen for these experiments. The Young’s modulus of the ABS plastic ranges from 1.0 to 3.1 GPa (according to the manufacturer’s specifications), depending on the direction in which the testing occurs. The material properties of trabecular bone can range from 1.0 to 14.0 GPa (Choi, Kuhn, Ciarelli, & Goldstein, 1990; Rho, Tsui, & Pharr, 1997; Zysset, Guo, Hoffler, Moore, & Goldstein, 1999). However, 14 GPa is at the high end for trabecular bone and is a value closer to cortical bone. Most values for trabecular bone are around 1-3 GPa. Therefore, ABS plastic was an ideal material to use as a validation test for trabecular bone modelling since its Young’s modulus falls within the lower range of those reported for trabecular bone.

The framework for this experiment is shown below in Figure 3.9. Figure 3.9a and Figure 3.9b shows the 3D printed sample subjected to an Instron compression test and the areas where the sample failed (red circles). For the Instron compression tests, the 3D printed model was loaded at a rate of 0.01 mm/s (to simulate quasi-static loading) and a force versus deformation curve was obtained (Figure 3.10). This graph showed that the model started to yield at around 2000 μm and therefore the linear region was approximately 1000 N/μm. The 8 points where failure occurred are shown on the graph by the red circles. Areas where the sample failed were also noted using a high speed, digital, SLR Nikon camera. The Instron compression test was carried out three times on three 3D printouts of the same sample.

Figure 3.9c and d show the equivalent FE set-up, which was simulated in Abaqus. Material properties of the FE model were set to 1 GPa and a Poisson’s ratio of 0.3. A Young’s modulus of 1 GPa allowed the assumption of a normalised material property which permitted the scaling of the yield criteria for both trabecular and cortical bone and for bones from different species (human and murine). The model was able to accurately predict 7/8 failure sites, which were shown as peak von Mises damage yields in the general vicinity of the surface cracks. These were seen in the 3D model that was subjected to the Instron compression test and are shown in Figure 3.9b and Figure 3.9d. The von Mises strain in those failure regions was approximately 0.8%. This was the value chosen as the failure criteria for the bone models used in this thesis, where we normalised the material properties. The equivalent von Mises stress for these failure regions was approximately 3 MPa in murine bone and this value was used in the yield prediction criteria. In addition to establishing a failure value, the validation experiments ensured that the boundary conditions and mesh resolutions were appropriate for these FE models.
Figure 3.8. A bone biopsy sample printed out in 3D with a plate attached to the top and bottom of the bone to function as a specimen clamp for the Instron compression tests.
Figure 3.9. Study framework for the validation of the FE models. a. 3D model with known material properties was printed and an Instron compression test was performed on it. b. Zones of micro-failure from the compression tests are highlighted by the red circles. c. The equivalent FE simulation was carried out in Abaqus (load direction shown by the black arrow). d. The predicted failure zones (areas of high stress, shown by the red circles) from the FE simulations showed strong correlations with the Instron compression test. (Sreenivasan et al., 2013).
Figure 3.10. Force vs Deformation curve from the Instron compression test of a 3D printed bone sample. The red circles represent “cracks” that occurred in the 3D printed sample during the Instron test.
In the previous chapter, the FE models were run with a uniform Young’s modulus of 1 GPa (to isolate bone architecture, only). Then, Young’s moduli were calculated using BMD and TMD that were specific to each sample by using the power law. This chapter provides an overview of the various methods used to obtain material properties for murine femur samples that were treated with a whey protein diet (Chapter 6). Three point bending, microindentation and nanoindentation were used to obtain the material properties of the murine bones at the macro, micro and nano scales, respectively. These testing methods provided additional material property information about the bone samples, which were then used in the FE models.

4.1 OVERVIEW

Bone is arranged in a hierarchical manner, meaning there is a lot of variation at the different scales, both structurally and in the material properties. Due to its anisotropic nature, the mechanical properties are determined by both composition and microstructure (Fan et al., 2002; Ritchie, 2011). This arrangement allows all the structures at the different levels to provide structural support, protection and mineral ion homeostasis (Rasoulian et al., 2013; Rho et al., 1998; Ritchie, 2011). When considering bone architecture, scale is of importance. Figure 1.1 illustrates the complex structure of bone, previously discussed in Chapter 1. Briefly, at the macro level bone is divided into trabecular and cortical bone. The microstructure includes the haversian systems, osteons and individual trabeculae. Lamellae are found at the sub-microstructural level, with the
fibrillar collagen and embedded minerals at the nano level (Rho et al., 1998). Beyond this is the sub-nanostructural level, where the molecular structures such as collagen and non-collagenous proteins are found (Rho et al., 1998).

Three point bending, microindentation and nanoindentation tests are techniques used to assess material properties and were used to assess the murine femur samples as part of the whey protein diet aspect of this work (see Chapter 6). The rationale behind this work was to provide a level of confidence to modellers that their conclusions would not change from choosing material properties from different sources. Three point bending measures the behavior of materials subjected to a simple beam loading. Microindentation and nanoindentation tests measure the mechanical response of a material to an applied load (Lucca, Hermann, & Klopfstein, 2010). While three point bending assessed only the cortical bone, microindentation and nanoindentation were carried out in the regions previously identified as the VOI (see section 2.3). This was to ensure that the measurements were consistent with the regions used for micro-CT analyses.

4.2 THREE POINT BENDING

Bend testing is commonly used to test the flexural stress and modulus of a material. For the purpose of this study only the flexural modulus was used. Three point bending was employed to assess the bulk material properties of the mice femurs. A pre-existing rig (see Chapter 6) was used to hold the femurs firmly in place at two constant points (either ends of the bone) to prevent twisting during testing. A wedged tip was pushed into the centre of the femur at a constant loading rate of 1 mm/min by a 1 kN load cell. This rate was chosen after trials were conducted on test specimens of an apple stalk and a wood chip. These were selected as they were both organic materials which were equivalent in size and geometry to the mouse femur sample. The loading rate was also consistent with other three point bending studies (Jämsä, Jalovaara, Peng, Väänänen, & Tuukkanen, 1998; Kamal et al., 2015). The bone specimen was loaded until failure and the force and displacement recorded. Figure 4.1 is a representative load vs displacement curve from a three point bending test of a mouse femur. The graph shows the bone reaching peak load at approximately 17 N. Three point bending was carried out for all 29 mouse femur samples.
Following three point bending, a number of measurements were taken in order to calculate the flexural elastic modulus of each of the samples.

The elliptical second moment of inertia was calculated as follows:

\[ I = \frac{\pi}{64}(b_2b_1^3 - a_2a_1^3) \]  

where,  \( I \ (m^4) \) is the elliptical second moment of inertia,  \( a_1 \) is the internal diameter in the load direction,  \( a_2 \) is the internal diameter perpendicular to the load,  \( b_1 \) is the external diameter in the load direction and  \( b_2 \) is the external diameter perpendicular to the load. A pictorial representation of these measurements is shown in Figure 4.2. All diameters were measured in metres.
Using the elliptical second moment of inertia, the flexural elastic modulus was calculated using:

\[ E = \frac{FL^3}{48dl} \]  

where, \( E \) (Pa) is the flexural elastic modulus, \( F \) (N) is the peak load (maximum load for each sample), \( L \) (m) is the support span distance, \( d \) (m) is the displacement at the peak load and \( I \) (m\(^4\)) is the elliptical moment of inertia (obtained from Equation 4.1).

The flexural elastic modulus provided a material property value for the cortical bone of the mouse femur. The above calculations were performed for all 29 samples.
4.3 NANOINDENTATION AND MICROINDENTATION

4.3.1 MARKING THE VOI FOR TRABECULAR AND CORTICAL BONE

Before the indentation tests were carried out, the femur samples were physically marked so that the VOI’s were clearly demarcated (see Figure 4.3). Both trabecular and cortical bone indentation tests were performed on a slice of the sample in the middle of the VOI.

Chapter 2 explained how the VOI’s for the cortical and trabecular bones were ascertained. However, for these experiments the center of the VOI was chosen for indentation, as it was a good representation of the overall VOI. Samples were stored in PBS at 4ºC until they were tested. Note that the VOI was selected based on the individual samples and therefore varied between studies.

The bones were prepared for both microindentation and nanoindentation testing in exactly the same way. Samples were mounted in low temperature curing epoxy resin (EpoFix cold setting resin, Struers) and were left to set for 24 hours. Once the samples had set, the curing blocks were put through a series of silicon carbide grinders; P120 and P220 coarse grinding, followed by finer grinding with P500 and P1200. The cured blocks included a layer which was an uneven and rough surface, present due to sample preparation. After coarse grinding (using the P120 grade silicon carbide graph...
carbide paper), this layer was approximately 120 µm thick. The P220 grinders then reduced the layer to 60 µm. After finer grinding (P500 and P1200) only 15 µm remained. Diamond polishing further reduced the depth of it to 1 µm, which was the surface upon which indentation tests were performed. The process of diamond polishing involved a diamond bead solution (a solution consisting of fine particles of diamond in a suspension) deposited onto polishing cloths to smooth out the surface of the samples. The fine diamond particles in the solution filled in the irregularities on the surface of the samples in order to create a smoother surface for indentation. Between polishing, the diamond beads that remained on the samples were removed using regular household detergent and 70% ethanol.

4.4 NANOINDENTATION

The Hysitron TI-950 TribolIndenter uses a diamond Berkovich tip (three-sided pyramid geometry), and is generally used in small scale indentation studies using the Oliver and Pharr method of analysis (Oliver & Pharr, 1992). This indenter uses a three plate capacitive transducer, which acts as both the actuator and the sensor of the instrument. The force is applied electrostatically, while the displacement is simultaneously measured by the change in capacitance. This device has a dual head testing capability, providing an available force range of 30 nm to 10 nm. The indenter also offers in-situ Scanning Probe Microscopy (SPM) imaging. This feature is critical for precise test placement and the identification of structures. It allows the indenter probe to be positioned within ten nanometers of the desired testing location. This type of analysis determines the mechanical properties of a material from the indentation load and the displacement measurements (Oliver & Pharr, 2004). Nanoindentation testing indirectly measures the contact area by determining the penetration of the surface by an indenter of known geometry (Fischer-Cripps, 2011). This type of testing has now become the primary means of determining the mechanical properties of small structural features (Oliver & Pharr, 2004) and is thus ideal for testing mice femurs. The basic principle of this method is to measure hardness and the elastic modulus of a material from indentation load- displacement data, obtained during one cycle of loading and unloading (Oliver & Pharr, 1992, 2004).

For nanoindentation, the bones were tested using a four step method. The indenter tip was first calibrated using a fused quartz sample, which has a Young’s modulus of 69.6 GPa (Zhang, Niebur, & Ovaert, 2008). First, the tip was held at 1 µN to allow the tip to equilibrate with the bone sample. A constant loading rate of 200 µN/second was applied until a maximum load of
2000 µN was reached. This peak load was held for 3 seconds before the sample was unloaded at 200 µN/second. These parameters were standard for the lab we worked with (Williams et al., 2011). Ten indents were carried out for each sample, approximately 10 µm apart. This was consistent for both trabecular and cortical bone. The values from the 10 indents were averaged to give one reduced/combined modulus value, which is the combined modulus of the indenter and the specimen (Fischer-Cripps, 2011), for each trabecular and its corresponding cortical bone sample. The indentation tests were carried out in the longitudinal direction on the transverse surface of the bone (Rasoulian et al., 2013). This is schematically represented in Figure 4.4. Hardness and reduced modulus values of the measured samples were obtained and used to calculate the Young’s modulus for each bone sample. In addition, SPM images were obtained for all of the samples to visualise and confirm that the indents were actually made on the bone. Figure 4.5 shows a sample pre-indentation and post-indentation (shown by boxes).

![Figure 4.4. Schematic of the cross section of a bone sample at the micro scale showing the osteons. Arrows indicate the direction in which the indentations were made (longitudinal direction). The indentations were equally spaced and in a straight line within the VOI. Adapted from (Rasoulian, Raesi Najafi, Chittenden, & Jasiuk, 2013; Ritchie, 2011).](image-url)
The following load-displacement curve, Figure 4.6, was obtained from one nanoindentation cycle on one mouse femur sample. The shape of the curve provides information about the response of the tested material, the mouse femur. The graph shows a steady rate of loading, with a peak indentation load of approximately 1970 µN. This load was held for 3 seconds (to account for creep) followed by a period of unloading.
The following equations were used to determine the Young’s moduli of the mouse femur samples. To determine the contact area of the Berkovich indenter, the following equation was used (Fischer-Cripps, 2011):

\[ A = 24.5h_c^2 \]  \hspace{1cm} 4.3

where, \( A \, (m^2) \) is the contact area for the Berkovich indenter and \( h_c \, (nm) \) is the vertical depth along which the contact is made. To calculate the reduced modulus, which is the combined modulus of the tip and the sample, the following equation was used (Oliver & Pharr, 1992):

\[ E_r = \frac{\sqrt{\pi}}{2} \frac{S}{\sqrt{A}} \]  \hspace{1cm} 4.4

where, \( E_r \, (GPa) \) is the reduced modulus of the sample, \( S \, (N/m) \) is the experimentally measured stiffness of the upper portion of the unloading data and \( A \, (m^2) \) is the contact area of the Berkovich indenter tip (calculated using Equation 4.3). Reduced modulus values were converted into Young’s modulus values using (Fischer-Cripps, 2011; Ma, Ong, Liu, & He, 2004; Oliver & Pharr, 1992):
\[ E = \frac{(1 - v^2)}{\left[ \frac{1}{E_r} - \frac{1 - v_i^2}{E_i} \right]} \]

where \( E_r \) (GPa) is the reduced modulus of the bone sample (specific to each sample), \( E_i \) is the Young’s modulus of the diamond indenter tip (1140 GPa), \( v \) is 0.3 (Zhang et al., 2008), the Poisson’s ratio of the bone sample, and \( v_i \) is also 0.3, the Poisson’s ratio of the indenter tip.

4.5 MICROINDENTATION

For microindentation tests (Vickers hardness test), the MTS Nano-indenter XP was used. A diamond Berkovich tip and the Oliver and Pharr method of analysis (Oliver & Pharr, 1992) was also used for these tests. This system uses electromagnetic actuation, meaning that the force is imposed on the indenter shaft by passing a current through a coil that sits within an annular magnet. The MTS Nano-indenter XP has a force resolution of 50 nN and a maximum available force of 500 mN. Displacement is sensed using the three-plate capacitive arrangement. The indenter has a displacement resolution of 0.01 nm and a travel of 2 mm. The indenter tip was calibrated using a fused silica sample, which has a Young’s modulus of 72.2 GPa. A peak load of 20 mN was applied (10x the force applied for nanoindentation) with a linear increase of 5 seconds. Peak load was held for 3 seconds to monitor creep, followed by a linear decrease of 5 seconds. These parameters were standard for the lab we worked with. Ten indents, 100 \( \mu \)m apart, were performed on each trabecular and cortical sample and the values were averaged to obtain a reduced modulus value for each sample. The indentation tests were all performed in a straight line (approximately equal distance apart) in the longitudinal direction, as shown in Figure 4.4. The reduced modulus values obtained from microindentation testing using Equation 4.4, were converted into Young’s modulus values using Equation 4.5. The only difference being the Young’s modulus of the micro-indenter tip was 1141 GPa and the Poisson’s ratio, 0.07.
The Influence of Fluoride on Bone Strength

The following is a reproduction of the article:


5.1 ABSTRACT

In this study we evaluate the influence of low-dose fluoride treatment on 23 patient biopsies. Computational finite element (FE) models of each biopsy were subjected to a range of non-destructive loads including compression, shear and torsion. The modelling framework was validated against a 3D printed model with known material properties subjected to compression till failure using an Instron machine. The primary outcomes from this study were that mechanical strength was not significantly correlated to low- dose (less than 10 mg/day) of fluoride levels (one-way ANOVA, p-values of 0.78, 0.69 and 0.62 for compression, shear and torsion, respectively). However, when bulk bone material properties were derived from DXA bone mineral density (BMD) from each patient’s proximal femur a non-significant linear decline in
mechanical strength with increase in fluoride was predicted. When the same material property was used for all bones (to evaluate bone architecture influence) then mechanical strength showed a characteristic concave upwards trend, consistent with the variation of micro-CT derived percentage bone volume (BV/TV). The secondary outcomes from this study were that in compression, BV/TV was observed to be a strong surrogate measure for mechanical strength ($R^2=0.83$), while bone surface density ($R^2=0.6$), trabecular thickness ($R^2=0.5$) and intersection surface ($R^2=0.6$) also explained the variation of mechanical strength well. However, trabecular separation and trabecular number were mildly correlated with mechanical strength ($R^2$ of 0.31 and 0.35, respectively). Compression was the loading mode most strongly correlated to micro-CT indices. Material properties adapted from the proximal femur reduced the CT index correlations by up to 58% indicating that bulk density from a near proximity is a poor representation of specific localised density. Substituting the 3D micro-CT indices with 2D histomorphometric data decreased correlations by at least 33% indicating that structural identification on a plane is not representative of the full 3D architecture necessary for a complete bone strength analysis. The presented non-destructive computational framework may be used to assess the roles that bone architecture and loading modes play in bone quality, and which micro-CT indices are good surrogate measures for mechanical strength.

5.2 INTRODUCTION

Osteoporosis is a disease characterised by low bone mass, deterioration of bone microarchitecture, enhanced bone fragility and increased fracture risk (Sambrook & Cooper, 2006). In particular, postmenopausal osteoporosis is caused by a reduction in estrogen (due to menopause) primarily affecting trabecular bone and predisposes the individual to fractures (Cummings, Kelsey, Nevitt, & O'Dowd, 1985).

Currently, osteoporosis treatment is dominated by medications that reduce bone resorption. There is interest in developing therapies that primarily stimulate bone formation. Elemental fluoride stimulates osteoblast growth in vitro (Farley, Wergedal, & Baylink, 1983), and increases bone formation and trabecular bone mineral density (BMD) in vivo (Riggs et al., 1990). Previous clinical studies have reported mixed results including no improvement in cortical BMD, and increased rates of fracture (Kleerekoper et al., 1991; Riggs et al., 1990) from 34 mg/day, likely due to impaired mineralisation (Lundy et al., 1995), reduction in vertebral fracture risk (Pak et al., 1994; Reginster et al., 1998), no effect (Meunier et al., 1998) from 13 - 27 mg/day; and impaired
mineralisation (Reid et al., 2007) from 20 mg/day. This study is motivated by Meta-analysis of trials of doses of fluoride < 20 mg/day suggesting that treatment might increase axial BMD and reduce fracture risk (Vestergaard, Jorgensen, Schwarz, & Mosekilde, 2008). If this was so, low-dose fluoride might become an inexpensive and readily available treatment to reduce fracture risk. However, adequately powered and controlled trials of low-dose fluoride have not been performed.

The feature that distinguishes trabecular bone from other biological structures is its complex architecture (the three dimensional, interconnected, open porous space resulting in the solid structure) (Keaveny et al., 2001). Microstructure based computational models have been shown to facilitate the study of trabecular structure and function and also provide insight into predicting trabecular failure (Niebur et al., 2000). Previous high resolution FE models of trabecular bones were compared with apparent yield properties for the same type of bone, and model predictions of bone stress and strain at the point of failure were not statistically different under the same boundary conditions (Keyak et al., 1998; Niebur et al., 2000).

One advantage of computational modelling over conventional methods is that it is able to predict fracture loads by non-invasive means. It has been shown that it is able to achieve precision which is comparable to densitometry data and is able to predict femoral fracture loads up to 60% accuracy (Keyak et al., 1998). For example, destructive compressive tests of the distal radius showed strong correlation with equivalent FE models (Macneil & Boyd, 2008). FE models derived from high resolution imaging of biopsy specimens also predicted bone moduli consistent with mechanical compression tests (Muller & Ruegsegger, 1995) and offers the ability to perform yield risk assessment (Zannoni et al., 1998).

A computational evaluation of the microarchitecture of human biopsies may provide a virtual assessment of the influence of fluoride on mechanical resistance. Given a subset of patients who may volunteer biopsies in large clinical trials, a computational assessment can provide an additional measure of the mechanical effect using a non-destructive protocol, leaving the bone available for other histological analysis and future imaging modalities. Computational modelling can provide additional insight by attributing strength measures to structural and material contributions, and assess bone quality against different loading modes.

In this study we conducted a 1 year randomised, placebo-controlled trial of the effects of low doses of fluoride (2.5 mg/d, 5 mg/d, and 10 mg/d) on BMD. The current analyses are of samples obtained from a subset of participants in that trial who underwent transiliac bone biopsy at the conclusion of the study. This study aims to integrate micro architectural data derived from CT
with computational modelling to (i) evaluate the influence of fluoride on human biopsies (representative of the total skeletal effect); and (ii) evaluate how micro architectural indices correlate to mechanical strength. Micro-CT indices are used to characterise structural information on bone and in experimental animal studies to visualise 3D microarchitecture. Measures including BV/TV, bone surface density, trabecular thickness, trabecular number and trabecular separation are assessed. Whether these measures actually correlate (or how well they correlate) with mechanical strength is less conclusive as strength is a function of material properties, geometrical arrangement of bone architecture and resistance to different modes of loading.

5.3 METHODS

Twenty four participants volunteered (out of 180) to provide a biopsy from their iliac crest as part of a 12 month placebo-controlled randomised trial of low-dose fluoride on bone in postmenopausal women with osteopenia. This study had ethical approval from the Northern X Regional Ethics committee in Auckland, New Zealand (approval number NTX/06/12/152). Participants had a bone mineral density T-score between -1 and -2.5 SD at either the lumbar spine or proximal femur. The women were equally randomised, using a computer generated algorithm, into four groups; placebo, 2.5, 5 or 10 mg of fluoride daily for twelve months. No stratification was undertaken during randomisation. BMD was measured at baseline, 6 months and 12 months at the lumbar spine, proximal femur and the forearm, using dual-energy x-ray absorptiometry (DXA). The DXA from which the bulk material properties were adapted from was an average of all three scans for each of the samples. The primary endpoint of the trial was change in lumbar spine bone density. Low-dose fluoride did not significantly affect bone density at any skeletal site, but tended to increase osteoid volume, reflecting impaired mineralisation (Grey et al., 2013). After 12 months, mean (95% CI) changes from baseline in BMD at the total hip were -1% (-0.4, -1.6), -1.1% (-0.5, -1.7), -1.4% (-2.0, -0.8) and -1.2% (-0.6, 1.8) in the placebo, fluoride 2.5 mg, fluoride 5.0 mg and fluoride 10.0 mg groups, respectively (Grey et al., 2013). At the end of the trial (month twelve), biopsies were obtained from the iliac crest, after tetracycline labelling. Biopsy samples were fixed in 70% ethanol, serially dehydrated in ethanol and embedded in methylmethacrylate prior to sectioning. It should be noted that the iliac crest is a standard location for obtaining bone biopsies. One biopsy, from a participant in the 2.5 mg fluoride group, was not evaluable for technical reasons.
Figure 5.1 outlines the framework used in this study. Firstly, biopsies were scanned using a SkyScan 1172 micro-CT scanner (X-ray voltage 80 kV, 1 mm aluminium filter; isotropic voxel size 14 μm). After standardised reconstruction using SkyScan NRecon software the datasets were analysed using SkyScan CT-analyser software. Cylindrical volumes of interest (VOIs) of trabecular bone were selected. Each dataset was binarised using global thresholding and trabecular parameters were measured. Using the SkyScan imaging processing software, 3D micro-CT indices were measured including BV/TV, trabecular bone thickness, trabecular separation, trabecular number, bone surface density and intersection surface for the 23 biopsies. Standard dynamic and static indices of bone histomorphometry were also measured on undecalcified sections by an experienced histopathologist, according to recommended procedures (Parfitt, Drezner et al. 1987).

Secondly, the raw digital data was exported in a StereoLithograph (STL) format and digitally cleaned to remove bone artefacts (segments of bone not attached to the main body) and redundant polygons. The STL mesh was then decimated (size reduced) in Rapid Form (Inus Technology Inc.) for computational efficiency without reducing the FE mesh fitting error. The
solution time to fit a FE mesh to the STL triangle mesh (from micro-CT) depends on the number of triangles. Hence we optimised this process by finding the minimum density of triangles in the STL mesh such that the computed FE mesh strain varied by less than 0.1% compared to the original mesh density. It was found that a reduction of 30% in the number of triangles did not visually change the appearance of the bone and did not produce strain differences of more than 0.1%. Following this, the bones were imported into the mesh generation package Hypermesh (Altair Engineering Inc.). Isotropic voxel cubes (hexahedral elements) were fitted to the STL mesh to reduce the fitting error to less than 14 microns (1 voxel). The hexahedral voxel mesh was then exported to Abaqus (Simulia, Dassault Systems Pty Ltd) for FE analysis. Hexahedral elements were chosen as they have a robust and stable behaviour for elastic mechanics compared to tetrahedral meshes in FE simulations.

Thirdly, to establish the validity of the mesh resolution and boundary conditions, we conducted a validation study using a 3D printed version (Dimension Elite 3D printer) of one of the biopsies and compressed till failure using an Instron 5800 machine (see steps in Figure 5.2).

Figure 5.2. a. A 3D printed model of a bone biopsy with known material properties subjected to an Instron compression test. b. Zones of micro failure were noted (highlighted by red circles). c. An equivalent FE simulation was run with identical boundary conditions. d. Predicted zones of failure are highlighted using von Mises criteria and compared with actual failure sites.
The Instron 5800 series has a position accuracy of 0.02 mm and a load sensor accuracy of 0.1 N (which is 1/100 of the 10 N capacity for the cell we used). The 3D printed model was scaled upwards by 10 times and printed out with a resolution of 100 μm. Given that the material properties of the printed material were similar to trabecular bone we used this to establish the von Mises yield point for all the simulations (taking into account the scaled size). The model was loaded in a specially designed metal base to prevent rigid body movement and loaded at a rate of 0.01 mm/s to simulate quasi-static conditions until the yield point and 8 surface cracks were visibly noted using a high speed digital SLR Nikon camera. The same biopsy was 3D printed twice more and we loaded the printed models to test for repeatability and all results were identical. During the test we focused a high speed Nikon camera on the specimen to record the fractured bone struts. The yield curve was also recorded to note the points at which micro failures were occurring (indicated by spikes in the curve) until ultimate failure. We recorded the location and number of micro-failures. We then simulated the identical experimental setup in Abaqus using material properties of the printed material taken from the manufacturer’s website (Young’s modulus of 1 GPa and Poisson’s ratio of 0.3). The model accurately predicted 7 out of 8 sites of failure by showing peak von Mises damage yield in the vicinity of the surface cracks. The von Mises strain in those regions of failure was ~ 0.8%, which was then adopted as the failure criteria for the remaining biopsies. The high resolution Nikon camera was used to identify the sites of bone strut failure from undeformed to complete yield of the structure. Additionally, we accounted for an estimate of internal crack prediction by counting the total number of spikes in the Instron yield curve and examining the additional internal failure zones (through slices in the Abaqus model). Internally we predicted the timing of 4 out of 5 yield points but currently could not confirm the spatial location.

Fourthly, all 23 models were then computationally loaded to failure under modes of compression, shear and torsion according to the average von Mises damage criteria determined from the previous Instron tests leading to 69 computational simulations taking on average 2 hours each on a standard PC. The failure force and torque were normalised by specimen area and height (hence volume) to assess the yield strength due to each bones architecture independent of size. The first set of simulations used the same material property estimate for all models and predicted the influence of biopsy architecture on bone quality. We then repeated all 69 simulations with a bulk material property (Young’s modulus) adapted from DXA BMD of the proximal femur of each patient using a density to Young’s modulus relation proposed by Currey (Currey, 1988) and modified for trabecular bone based on power law studies by Morgan and colleagues (Morgan, Bayraktar, & Keaveny, 2003).
Finally, we assessed the computationally predicted failure loads (a measure of strength) across the fluoride dosage levels and also for correlation with the 3D micro-CT measures and 2D histomorphometric data.

5.4 RESULTS

The primary analysis of this study was the influence of fluoride on mechanical strength, as shown in Figure 5.3. Failure torques and forces per unit of specimen volume were in the range of 0.035 - 0.05 Nm/mm$^3$ and 0.075 - 0.125 N/mm$^3$ (16.7 N to 23.0 N) respectively. Treatment with low-dose fluoride did not significantly influence the mechanical failure force (using a one-way ANOVA with p-values of 0.78, 0.69 and 0.62 for compression, shear and torsion, respectively). This result was consistent with the BMD results by DXA from all 180 participants. There were consistent, small, but non-significant reductions in mean failure force with the fluoride groups. The mean mechanical strength for compression, shear and torsion consistently decreased with increase in fluoride level from 0 mg (placebo) to 10 mg. This was characterised by a linearly decreasing strength when material properties were derived from patient specific DXA BMD measures (red). When a constant material property (Young’s modulus of 1 GPa) was used for all biopsies then strength showed a concave upwards trend (blue).
Figure 5.3. Graphs of normalised failure force versus fluoride levels for compression, shear and torsion modes plotted for constant material properties (blue) and bulk material properties derived from patient DXA BMD data (red). The graphs show mean ± standard deviation for 23 specimens.

Figure 5.4. Graphs of BV/TV and intersection surface versus fluoride level showing characteristic concave shape consistent with computational simulations. The graphs show mean ± 1 standard deviation for 23 specimens.
RESULTS

Figure 5.4 shows the micro-CT parameters, BV/TV and intersection surface (surface of bone intersecting with the region of interest), versus fluoride level (mg per day) as measured during the initial clinical trial independently from the computational measures. Both of these geometrical parameters are independent of material properties and also present a concave trend consistent with the predicted computational strength.

Figure 5.5 and Figure 5.6 depict the secondary outcomes from this study, specifically, that in compression the micro-CT derived BV/TV is a strong surrogate measure for mechanical strength ($R^2=0.83$). This shows that 83% of the variation in mechanical strength can be explained by percentage bone volume (a purely structural parameter). One of the contributors to BV/TV, trabecular thickness (also from micro-CT) could also explain ~50% of the variation in bone strength ($R^2=0.5$), while bone surface density (micro-CT) may explain ~60% of the variation ($R^2=0.6$). Intersection surface, a parameter resulting from the bone surface intersecting with the selected region of interest also exhibited good correlation (even though not typically used except in bone remodelling studies) and accounted for 60% of the variation in strength ($R^2=0.6$). Compression was the strongest correlated loading mode for most micro-CT indices with shear and torsion slightly less. However, there were exceptions such as intersection surface under shear load (Figure 5.6), which was more correlated to strength than compression ($R^2$ of 0.6366 versus 0.6). Furthermore, including bulk material properties derived from the proximal femur of each patient reduced the correlations of each micro-CT index with failure force in general by 8% to 58%.
Figure 5.5. Graphs of correlation plots for BV/TV (top row) and bone surface density (bottom row) versus normalised failure force for compression (left column), shear (middle column) and torsion (right column). Results are shown for all bones with a 1 GPa stiffness modulus (blue diamonds) and bulk material properties derived from patient DXA BMD data (red squares).
Figure 5.6. Graphs of correlation plots for trabecular thickness (top row) and intersection surface (bottom row) versus normalised failure force for compression (left column), shear (middle column) and torsion (right column). Results are shown for all bones with a 1 GPa stiffness modulus (blue diamonds) and bulk material properties derived from patient DXA BMD data.
Figure 5.7 (bottom row) shows the trabecular number and trabecular separation from 3D micro-CT data versus compression loading (red). Trabecular number and separation were only mildly correlated with mechanical strength ($R^2$ of 0.35 and 0.31, respectively). Figure 5.7 also overlays four 2D osteomeasures as computed by a pathologist (in blue) for BV/TV, trabecular thickness, number and separation which highlights that substituting the 3D micro-CT indices with 2D histomorphometric data decreased correlations by at least 33%.

Figure 5.7. Graphs of correlation plots for BV/TV, trabecular thickness, trabecular number and trabecular separation versus normalised failure force for compression. Results are shown for 2D histomorphometric data (blue diamonds) and 3D micro-CT data (red squares).
5.5 DISCUSSION

This study considered the effect of (i) low-dose fluoride on the mechanical strength of bone and (ii) correlation of mechanical measures against structural indices by using FE modelling. These loads included: compression, torsion and shear tests on the bone sample in a virtual non-destructive environment. This study found that low-doses of fluoride had no significant effect on the mechanical strength, a result consistent with those from the bone density analyses in the same trial (Grey et al., 2013). However, there were non-significant reductions in mean mechanical strength in the fluoride groups, which may reflect a tendency for this treatment to impair bone mineralisation (Grey et al., 2013), a contributor to bone strength that may not be captured by densitometric techniques. The negative failure force trend (with estimated material parameters) is consistent with previous reported clinical studies that show a reduced effect with increasing dosage (Kuhn et al., 1989). Simulations that contained a constant Young’s modulus of 1 GPa for all 23 biopsies were used to explore the influence of bone architecture alone on strength. This is a benefit of computational modelling where we can isolate contributing geometrical factors to mechanical strength (by normalising for material properties). Our results predicted a concave upwards trend, which can be explained in part by two geometrical parameters measured as part of the clinical trials; BV/TV and intersection surface. Of all the parameters tested only these two had the same distinct shape and were also the most correlated with bone strength (R²=0.83 and 0.6, respectively). However, intersection surface is a parameter influenced by the choice of region of interest (ROI). It represents the surface of bone intersecting with the ROI and while this correlates well with mechanical strength of the tested specimen it can vary by site and location. Hence, BV/TV is a better choice as one would expect this to be more representative of a broader region of bone than the intersection surface.

Compression loading showed the strongest correlation in general followed by shear and torsion. Physiological loading on bones can be decomposed into compression, loading and shear modes, especially when muscle forces are introduced. Hence, all three loads will play a role in different dynamic human tasks such as sideways cutting manoeuvres and gradient ascents in contrast to cyclic walking. While compression is the dominant mode typically, in more complex tasks bone failure may not be as strongly correlated to compression alone but instead consist of a weighting of all three modes. The results presented show that compression, shear and torsion correlate inconsistently with the different clinical indices and therefore suggest a more complex surrogate measure that is a function of all 3 loading modes for different tasks. Moreover, the compressive failure force across the entire range of specimens was between 16.7 N to 23.00 N leading to a yield
stress of 0.67 MPa to 0.93 MPa (average specimen diameter of ~5.62 mm). This is within the range of yield stresses for iliac crest cancellous bone reported by Ebbeson et al. 1997 (Ebbesen, Thomsen, & Mosekilde, 1997) of 0.1 MPa to 5.0 MPa for women with apparent bone densities of 100 to 300 mg/cm³. Our results are within that range but on the lower side.

This study confirms that BV/TV is a strong surrogate measure of computational mechanical strength, especially in compression. More importantly we found that bone surface density and trabecular thickness are also moderate measures of strength and can account for around 50-60% of the variation observed. Since no one clinical index can explain all the variations, it is likely that an improved derived index is a better measure. Specifically, BV/TV is an integrated measure of trabecular number and thickness but may also be improved by consideration of information from different loading modes. The results from this study provide a foundation for this by decomposing these different loading modes into compression, shear and torsion.

The reduction in correlation from including an estimate of material properties suggests that the bulk values chosen from the proximal femur are not representative of those at the iliac crest. The current clinical study did not measure tissue mineral density (TMD) for each specimen and so we evaluated the influence of using a measured bone density close to the biopsy location. Given that material properties would be expected to improve the correlation this result suggests that bone density depends on a number of factors including TMD from the correct bone site and bone type (cortical or trabecular). Furthermore, spatially varying material properties is likely necessary to accurately estimate the bone behaviour. Due to the constraints of this study with specimens no longer available due to histology analysis, a BMD estimate was the best alternative. This is a current limitation but is addressed in our ongoing anabolic studies that include material properties based on biopsy-specific TMD measurements.

To validate the modelling experiments, a 3D printed model of the biopsy was compressed in an Instron machine. The current Instron setup was limited to compression testing, which we have inferred to shear and torsion. Further validation modes are the subject of ongoing work. This validation experiment showed that the points of failure were consistent with the computational tests ~88% of the time. While this method does not capture the spatially varying bone density it does validate a models ability to capture the geometrical features for any bone that undergoes CT. Recent advances in powder technology will also allow us to create 3D structures with a spatially varying density derived from Hounsfield units and is the focus of further validation. Regardless, this idea is a novel way to conduct a mechanical test without destruction of the bone. We also evaluated variation in the Instron estimated yield point on model correlations and found
that a perturbation of ±10% in yield point varied the correlations by less than 2%. Hence, for a
linear elastic assumption of less than 10% deformation the yield values do not change the
conclusions from this study.

The equivalent 2D histomorphometric and 3D micro-CT indices were compared against
mechanical measures from the computational simulations and for each test the 3D data was more
correlated with the failure force compared to the 2D data. Histomorphometry is useful for
comparison between a single 2D section of data and the equivalent 2D region from 3D CT data
as a validation mechanism. However, Figure 5.7 highlights that substituting the 3D micro-CT
indices with 2D histomorphometric data decreased correlations by at least 33% indicating that
bone structural identification on a plane is not representative of the full 3D architecture necessary
for a complete bone strength analysis.

One advantage of computational models is that they have reached a level accuracy that qualifies
them as surrogates for destructive mechanical testing of real specimens (Niebur et al., 2000). They
can isolate loading modes and structural effects (not possible with real bone). However, this study
has several limitations that must be considered when interpreting results. Firstly, our current
modelling framework only provides information about the initial point of failure for each biopsy
and does not account for fracture propagation, which requires a proper damage evolution law
and remodelling of material properties in the vicinity of the crack. Given that we classify strength
as the resistance to initial yield it does not affect the conclusions from this study.

Secondly, while the iliac crest is a common site for biopsy acquisition it should be noted that this
is an isolated piece of the entire skeleton that is being used to represent the effects of fluoride on
the skeleton as a whole. Further, we only had biopsies available at end-point. This was due to
biopsies being collected on a volunteer basis that only provided one sample at end-point.
However, we could evaluate the effect of fluoride against the control group who received no
fluoride and could gauge the effect on the bone architecture. It should be noted that a challenge
often encountered with collecting two biopsies (start and end of study) is that volunteer numbers
may decline by the end of the study as the process of obtaining a biopsy is invasive and painful.
The bone samples were also normalised by volume to ensure that we were evaluating the effect
of the architecture and not just the subject-specific bone size. In future applications of this
framework we aim to collect biopsies at baseline and end-point, which must be included in the
initial ethics and study design.

Thirdly, we were also limited by sample size, another product of volunteer based data collection.
Initially, 24 samples were collected (six from each group including controls), however, one biopsy
contained only tissue with no bone so was removed from the set. Since sample size is important for confidence levels and we could only obtain 24 volunteers from 180 participants, future studies should either increase the total study number or improve biopsy volunteer numbers. Regardless, our results illustrate a trend for the mean response of mechanical strength to fluoride that is consistent with previous studies and may become significant with increased patient numbers. Twenty three patients were found to be suitable for a modelling study to identify reasonable correlations between computational damage and micro-CT indices. Furthermore, this study was for humans, so we are seeing the influence on the target population instead of an animal substitute.

5.6 SUMMARY

This study has highlighted that at low-dose levels there was no evidence of a statistically significant benefit from fluoride treatment. However, the use of computational modelling as an assessment tool within a clinical study was promising and able to reproduce similar conclusion and add further insight into the effects of fluoride on bone quality.
The Influence of a Whey Protein Diet on Bone Strength

The following is a reproduction of the article:


6.1 ABSTRACT

The primary aim of this study was to evaluate the influence of a whey protein diet on computationally predicted mechanical strength of murine bones in both trabecular and cortical regions of the femur. There was no significant influence on mechanical strength in cortical bone observed with increasing whey protein treatment, consistent with cortical tissue mineral density (TMD) and bone volume changes observed. Trabecular bone showed a significant decline in strength with increasing whey protein treatment when nanoindentation derived Young’s moduli were used in the model. When microindentation, micro-CT phantom density or normalised...
Young’s moduli were included in the model, a non-significant decline in strength was exhibited. These results for trabecular bone were consistent with both trabecular bone mineral density (BMD) and micro-CT indices obtained independently. The secondary aim of this study was to characterise the influence of different sources of Young’s moduli on computational prediction. Different sources provide varying estimates of Young’s modulus and this study aimed to quantify the predicted mechanical strength in 3D from these sources and evaluate if trends and conclusions remained consistent. For cortical bone, predicted mechanical strength behaviour was consistent across all sources of Young’s moduli. There was no difference in treatment trend observed when the Young’s moduli were normalised. In contrast, trabecular strength due to whey protein treatment significantly reduced when material properties from nanoindentation were introduced. Other material property sources were not significant but emphasised the strength trend over normalised material properties. This shows strength at the trabecular level was attributed to both changes in bone architecture and material properties.

6.2 INTRODUCTION

Micro-finite element (FE) techniques based on high resolution images allow for the modelling of trabecular and cortical bone in detail. This process uses images from micro-CT scans and converts voxels into elements to produce meshes suitable for FE analysis (Morgan & Bouxsein, 2005; Ulrich, van Rietbergen, Weinans, & Ruegsegger, 1998). Computational modelling is able to non-destructively and rapidly evaluate the quality of bone in response to various treatments. This is advantageous as it is less costly, can reduce sample numbers in large clinical studies and leaves the bone available for future histological and mechanical testing. Computational modelling has been shown to achieve precision comparable to densitometry data and is able to predict fracture loads up to an accuracy of 60% (Keyak, Rossi, Jones, & Skinner, 1998). Additionally, destructive compressive tests on the radius has shown strong correlations with equivalent FE models (Macneil & Boyd, 2008). There have been studies that have used micro-FE models to assess bone strength in response to treatments (Keaveny et al., 2007; Tsai et al., 2015; Van Rietbergen, Majumdar, Newitt, & MacDonald, 2002). Those studies revealed that modelling isolates strength changes in cortical and trabecular bone separately, that subject-specific variations within groups are important and that modelling reveals whole strength measures that may be different from surrogate measures of strength (like micro-CT indices). The current study demonstrates all three aspects.
While FE modelling has been shown to play a useful role in evaluating bone, there are few studies in the literature that compare computer predictions of bone strength against experiments using a double blinded design. In this study we evaluate computational predictions of strength against traditional micro-CT surrogates and see if the conclusions drawn are consistent. Secondly, while it is known that material properties derived from different sources and scales produce different Young’s modulus estimates, it is of interest to know how much this influences computational predictions and conclusions drawn as a result.

At the macro scale, bone is simply divided into cortical and trabecular bone. Cortical bone is the hard, outer layer whereas trabecular bone is porous and predominantly fills the ends of long bones. Fundamentally, trabecular and cortical bone are made up of the same constituents, however mechanically, the homogenised mechanical response of trabecular and cortical bone vary greatly due to their site specific architecture and material properties. Homogenised Young’s modulus of cortical bone can be anywhere from 14 - 29 GPa (Ashman & Rho, 1988; Rho, Kuhn-Spearing, & Zioupos, 1998), while homogenised Young’s modulus of trabecular can be 100 MPa – 4 GPa (Ashman & Rho, 1988; Dagan, Be'ery, & Gefen, 2004). Mechanical properties of bone are determined not only by bone mineral composition but also the structural organisation at different sub scales (microstructural and nanostructural). Therefore, measurement of material properties at different scales incorporates different levels of porosity and homogenised underlying structure. Within bone tissue exists a complex hierarchical system. In humans, the haversian systems, osteons and individual trabeculae are found in the microstructural layer, which contribute to homogenised material properties at the macro level (Zysset, Guo, Hoffler, Moore, & Goldstein, 1999). It should be noted that while human bone consists of a haversian system, mice lack one (Mills & Simpson, 2012). The lamellae are found in the sub-microstructural layer. At the nanostructural level is where fibrillar collagen and embedded minerals are found and the sub-nanostructure contains the molecular structure such as the collagenous and non-collagenous organic proteins (Nalla, Kinney, & Ritchie, 2003; Rho et al., 1998). These structures contribute to properties measured through nanoindentation. Microindentation and nanoindentation tests are used to determine the hardness of bone tissue at different structural levels (Zwierzak, Baleani, & Viceconti, 2009), which are used to inform micro-FE models. Three point bending tests can also provide an indication of the axial mechanical strength of cortical bone (Jamsa, Jalovaara, Peng, Vaananen, & Tuukkanen, 1998). As treatment effects manifest at different levels of bone, using material properties derived from different scales may highlight the treatment effect that may otherwise be hidden.
The mechanical properties of bone are dependent on several diverse factors which can include but are not limited to age, diet, exercise and disease (Rasoulian, Raeisi Najafi, Chittenden, & Jasiuk, 2013). Bone can also be affected by treatments differently, in trabecular and cortical zones. This is the reason why computational models that isolate cortical and trabecular behaviour provide insight into compartmentalised bone response. It is also known that bone strength is dominated by the cortical response but still needs trabecular support to prevent internal buckling. Endocortical thinning is usually the last stage of bone degradation, following trabecular resorption (Thomas et al., 2009), hence, while net bone strength may not be compromised, evaluation of trabecular bone strength is an early indicator of future mechanical failure.

In order to test the use of computational modelling as an informative tool in bone treatment studies, we conducted a variable whey protein controlled murine study. Twenty nine mice were fed a varied cheese diet (with whey protein), later euthanised and their femurs obtained for testing. This study integrated micro-architectural indices from micro-CT, mechanically derived material properties and micro-FE modelling. Specifically, the objectives of this study were to determine (i) whether computer modelling can predict trends in treatment outcomes locally within cortical and trabecular bone by assessing the geometrical architecture alone (with normalised material properties) using a double blinded design; and (ii) are computational predictions of strength across treatments consistent when material properties are normalised and derived from nanoindentation, microindentation, three point bending and micro-CT phantom derived grey scale values.

6.3 METHODS

12 week old, female, C3H mice were divided into three groups: placebo = 9, 0.1% whey protein = 9 and 1% whey protein = 11. Animals were fed to the point of sacrifice with either the control diet (AIM-93M including 180 g of soy protein per kg), or a diet in which 10 g of soy was replaced by 0.1% or 1% of beta-glycan derived whey-protein. At 26 weeks, the mice were anesthetised with isoflurane and morphine was administered to avoid pain. Whole blood was collected by cardiac puncture, mice were then euthanised and femurs were excised, cleaned of the surrounding soft tissue and fixed in 70% ethanol. The distal end of the femurs were scanned using a SkyScan 1172 micro-CT scanner (X-ray voltage 50 kV, 0.5 mm aluminium filter, voxel size 5 μm). Standardised reconstructions were carried out using SkyScan NRecon software and the datasets were analysed using SkyScan micro-CT analyser software, CTAn (version 1.14.4.1). Figure 6.1 shows where the
volumes of interest (VOIs) of trabecular and cortical bone were selected from: the trabecular region of bone was 0.4 mm proximal to the growth plate and extended 1.5 mm in the proximal direction and the cortical region of bone was 3.25 mm proximal to the growth plate and extended 0.5 mm in the proximal direction. BMD and TMD values were obtained from calcium hydroxyapatite micro-CT phantoms and were averaged for the VOI. Each dataset was binarised using global thresholding (90-255). Using CTAn, 3D micro-CT indices were measured which included: percentage bone volume (BV/TV), trabecular thickness (TbTh), trabecular separation (TbSp) and trabecular number (TbN) for trabecular bone; and mean total cross sectional tissue area (T.Ar), mean total cross sectional bone area (B.Ar), mean medullary area (Ma.Ar), mean cortical area fraction (B.Ar/T.Ar) and mean cross sectional thickness (Cs.Th) for cortical bone for all 29 femur samples.

The raw digital data in StereoLithography (STL) format was digitally cleaned and this process removed any bone fragments (any part of the bone not attached to the main body) and redundant polygons. The mesh was decimated (reduced in size) for computational efficiency without affecting the overall quality of the bone geometry, using Rapid Form (Inus Technology Inc.). The
solution time to fit a FE voxel mesh to the STL triangle mesh was dependent on the number of triangles present. The process was optimised by finding the minimum density of triangles in the STL mesh so that the resulting voxel mesh varied by less than 0.1% when computing strain compared to the original mesh density. A reduction of 30% in the triangle number did not affect bone geometry difference by more than 0.02 mm. Following from this, a hexahedral voxel mesh was created for each bone sample using a mesh generation package, Hypermesh (Altair Engineering Inc.). Voxel cubes were chosen as they have a robust and stable behaviour for elastic mechanics compared to tetrahedral meshes in FE simulations. On average, trabecular bone had between 300,000 to 400,000 elements and cortical bone had between 11,000 to 13,000 elements. Aspects of this framework are shown in Figure 6.2.

Figure 6.2. The modelling framework for this study. i. Micro-CT scanning and measurement of indices. ii. Generation of a geometric mesh. iii. Mechanical testing of the sample. iv. FE simulation of trabecular and cortical bone. v. Interpretation of results.

A previous validation study (see Figure 6.3) using a 3D printed trabecular bone model with a Young’s modulus of 1.4 GPa and Poisson’s ratio of 0.3, was subjected to an Instron compression test and subsequently, replicating the Instron test as an FE simulation resulted in a von Mises
failure criteria for the bone models. Three bone samples were scaled 10 times and printed out in 3D (Dimension Elite 3D printers) using ABS thermoplastic with a resolution of 100 microns. The models were loaded in an Instron (Instron 5800 series) at a rate of 0.01 mm/s. The models started to yield at around 2000 μm, with a linear region of approximately 1000 N/μm. In the failure regions, the von Mises strain was approximately 0.8% which had an equivalent stress of approximately 3 MPa. This was the value chosen as the failure criteria for this work and was used in (Eq 6.1). For further details, see (Sreenivasan et al., 2013).

Following this, the voxel meshed bone samples were imported into Abaqus (Simulia Dassault Systemes Pty Ltd) for FE analysis. Twenty nine models were then computationally loaded to failure under a compression loading mode according to the average von Mises damage criteria, determined from the Instron test. Von Mises stress determines whether the principal stress

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**Figure 6.3.** Study framework for the validation of the FE models. i. 3D model with known material properties was printed and an Instron compression test performed on it. ii. Zones of micro-failure from the compression tests are highlighted by the red circles. iii. The equivalent FE simulation was carried out in Abaqus (load direction shown by the black arrow). iv. The predicted failure zones (areas of high stress, shown by the red circles) from the FE simulations showed strong correlations with the Instron compression test.
combination at a given point will cause yield and gives the scalar combination of three principal
stresses which is then compared to the yield von Mises stress of the material, obtained from the
validation test. If the von Mises stress of the bone exceeded the von Mises yield stress then the
bone is considered to have failed in compression. Von Mises stress in 3D is defined in as follows:

\[ \sigma_v = \sqrt{\frac{1}{2} \left( (\sigma_1 - \sigma_2)^2 + (\sigma_1 - \sigma_3)^2 + (\sigma_2 - \sigma_3)^2 \right)} \]  

where, \( \sigma_v \) is the von Mises stress, \( \sigma_1 \) is the principal stress in the x-direction, \( \sigma_2 \) is the principal stress in the y-direction and \( \sigma_3 \) is the principal stress in the z-direction. The models were solved quasi-statically, with each increment in force leading to a new homogenised von Mises stress.

Two von Mises yield criteria were used, (i) a yield stress determined from the average von Mises of the whole bone at failure, defined as the average yield stress and (ii) yield stress determined from the peak von Mises values at the sites of micro failure only, defined as the peak yield stress.

Post analysis then identified the failure forces that were associated with an average and peak von Mises stress that exceeded the yield value.

All 29 models (trabecular and cortical were solved separately) were computationally loaded to failure under a compression load according to the average von Mises damage criteria which was determined from the Instron test. In total there were 58 (29 trabecular and 29 cortical) computational simulations that took on average 6 h per simulation on a standard PC. Failure force was then normalised by the finite element computed cross sectional area of the specimen to assess the yield strength attributed to the architecture of the bone model, independent of the size of the sample. All samples had the same height. The first set of simulations used a normalised Young’s modulus of 1 GPa to predict the influence of architecture on the mechanical strength of the sample. This permitted the use of a yield criteria determined from Instron tests on a 3D printed bone that also had a Young’s modulus close to 1 GPa (1.4 GPa according to the manufacturer). To evaluate the influence of material properties, BMD and TMD values were obtained from the micro-CT phantom scans and were used to estimate the BMD and TMD derived Young’s modulus of the bones. This density was included in the model using the adapted Carter and Hayes, 1977 power law (Currey, 1988), which relates elastic modulus to the apparent bone density. The power law was chosen as it naturally represents the experimental trends observed between density and Young’s modulus, provides a better transition between trabecular and cortical bone and also has been shown to distribute the residual errors better (Gupta & Dan,
For this study we related apparent density from trabecular bone using the reported trabecular (cancellous) power law (Carter & Hayes, 1977), and the version for cortical (compact) bone (Gupta & Dan, 2004). A piecewise power law function was adapted for the two ranges of densities for trabecular ($1050 \, \text{e}^{-6} \rho^2$) and cortical bone ($3.0 \, \text{e}^{-6} \rho^3$) as follows:

$$E = \begin{cases} 
1050 \, \text{e}^{-6} \rho^2 & \text{for } \rho \leq 350 \, \text{kg/m}^3 \\
3.0 \, \text{e}^{-6} \rho^3 & \text{for } 350 \leq \rho \leq 1800 \, \text{kg/m}^3
\end{cases}$$

where, $E$ represents the Young’s modulus of the bone and $\rho$ is the apparent density of trabecular and cortical bone.

Three point bending was also used to determine the material properties of cortical bone by fracturing the mice bone samples. A rig was built to hold the femur in place at two constant points ensuring the sample did not roll while a two-sided wedge shaped tip was pushed into the centre of the femur (anterior side up) at a constant loading rate of 1 mm/min (see Figure 6.2 iii), which was chosen after initial trials on test specimens. The forces and displacements were recorded during testing and were used to determine whole axial bone material properties. This graph (Figure 6.4) shows the bone reaching peak load at approximately 17 N. All 29 bones underwent three point bending.
Following the three point bending tests, a number of measurements were taken in order to calculate the flexural elastic modulus for each sample using the following Euler-Bernoulli equation (Wallace, Pankaj, & Simpson, 2014):

\[
E = \frac{FL^3}{48dl}
\]

where, \( E \) (Pa) is the flexural elastic modulus, \( F \) (N) is the peak load (maximum load for each sample), \( L \) (m) is the support span distance (15 mm), \( d \) (m) is the displacement at the peak load and \( l \) (m⁴) is the elliptical moment of inertia.

The bones were prepared for both microindentation and nanoindentation testing in exactly the same way. The trabecular and cortical VOIs were marked on the bone and the samples were mounted in low temperature curing epoxy resin (EpoFix cold setting resin, Struers) and were left to set for 24 hours. Once the samples had set, the curing blocks were put through a series of silicon carbide grinders; P120 and P220 coarse grinding, followed by finer grinding with P500 and P1200. The cured blocks included a layer which was an uneven and rough surface present due to the sample preparation. After coarse grinding (using the P120 grade silicon carbide paper), the layer was approximately 120 µm thick. The P220 grinders further reduced the layer to 60 µm. After
finer grinding (P500 and P1200) only 15 µm remained. Diamond polishing further reduced it to 1 µm, which is the surface upon which indentation tests were performed. The process of diamond polishing involved a diamond bead solution (a solution consisting of fine particles of diamond in a suspension) deposited onto polishing cloths to smooth out the surface of the samples. The fine diamond particles in the solution filled in the irregularities on the surface of the samples in order to create a smoother surface for indentation. Between polishing, the diamond beads that remained on the samples were removed using regular household detergent and 70% ethanol. Any remnants of the diamond beads would not have affected the indentation tests as they were easily visualised under the microscope and were avoided during testing. The Hysitron TI-950 TribolIndenter and MTS Nano-indenter XP, both equipped with a diamond Berkovich tip and using the Oliver and Pharr method of analysis (Oliver & Pharr, 1992) were used for indentation. The bones were tested using the Hysitron TI-950 TribolIndenter using force control with a peak force at 2000 µN with a linear loading time of 5 seconds. Indents were performed on a cross section of the trabecular and cortical bone surfaces. Peak load was held for 3 second to monitor creep and a linear unload of 5 seconds.

Microindentation utilised the MTS Nano-indenter XP but with a peak load of 20 mN which is 10x the force applied by the nanoindentation tests. Both nanoindentation and microindentation tests were carried out in the longitudinal direction (perpendicular to the structures within the bone sample). Reduced modulus values were obtained from the nanoindentation tests and were converted into Young’s modulus values using the following equation:

\[
E = \frac{(1 - v^2)}{\left[\frac{1}{E_r} - \frac{1 - v_t^2}{E_i}\right]}
\]

6.4

where, \(E_r\) (GPa) is the reduced modulus of the bone sample (sample specific), \(E_i\) is the Young’s modulus of the diamond nanoindenter tip (1140 GPa), \(v\), the Poisson’s ratio of the bone is 0.3 (Zhang, Niebur, & Ovaert, 2008) and \(v_t\), the Poisson's ratio of the indenter tip is 0.3. Formula obtained from (Ma, Ong, Liu, & He, 2004). Reduced modulus values obtained from the microindentation tests were converted into Young’s modulus values using Equation 6.4. The Young’s modulus of the diamond microindenter tip was 1141 GPa and the Poisson’s ratio of the indenter tip was 0.07.
RESULTS

Following material testing, the Young’s moduli from the nanoindentation and microindentation tests were applied to the model for both trabecular and cortical bone. Flexural elastic moduli from the three point bending were applied only to the cortical bone models.

Data was expressed as the mean value ± 1 standard deviation. A one-way Anova was performed using Microsoft Excel. A p-value of < 0.05 was considered statistically significant and < 0.01 was considered strongly statistically significant.

6.4 RESULTS

The treatment effect on mechanical failure strength, material property source choice and yield criteria on mechanical strength of trabecular bone is shown in Figure 6.5. The material properties for trabecular bone, derived from nanonindentation, microindentation and micro-CT phantom derived values are shown in Table 3. BMD derived Young’s modulus showed a range of 0.81 - 1.02 GPa. Nanoindentation had a range of 15.11 - 17.16 GPa and microindentation, 17.08 - 19.45 GPa. The range in Young’s modulus obtained from BMD and microindentation were not significantly different across the treatment groups, however, when derived from nanoindentation there was a significant difference.

The mechanical strength of trabecular bone (see Figure 6.5) when using the average von Mises yield criteria showed a significant decline in strength (p < 0.01) with increasing whey protein treatment when nanoindentation derived Young’s modulus were used in the model. The trend was also significant (p < 0.01) and more pronounced when the peak von Mises yield criteria was used. Mice specific Young’s moduli, derived from nanoindentation, microindentation and micro-CT phantom density were implemented into the model for each bone sample which resulted in a yield force for each bone sample. When microindentation, micro-CT phantom density and normalised Young’s modulus were included in the model a non-significant decline in strength was exhibited for both average and peak yield von Mises criteria, though peak yield emphasised the declining strength trend. Trabecular strength predicted with BMD derived Young’s modulus due to whey protein treatment showed more pronounced decrease in strength compared to normalised Young’s modulus.

The treatment effect on mechanical strength, material property source choice and yield criteria on mechanical strength of cortical bone is shown in Figure 6.5. The Young’s moduli derived from nanoindentation, microindentation, micro-CT phantom derived values and three point bending
are shown in Table 4. TMD derived Young’s modulus showed a range of 8.45 - 8.57 GPa. Young’s modulus derived from nanoindentation had a range of 20.83 - 22.03 GPa and values from microindentation a range of 27.50 - 30.93 GPa. Three point bending showed a range of 4.60 - 7.91 GPa. The range in values were not significantly different across the treatment levels for cortical bone for any of the four sources of Young’s moduli.

Mechanical strength of cortical bone (see Figure 6.6) as predicted by average von Mises yield criteria showed no significance and no trends as the whey protein treatment was increased (from placebo to 1%). The effect of including the Young’s modulus did not change the predicted trends compared to normalised material properties. Three point bending produced on average half the strength estimate of nanoindentation and microindentation. Using the peak von Mises yield criteria did not change the significance, but introduced higher variability in predicted failure between the material property sources.
RESULTS

Figure 6.5. Normalised average (blue) and peak (red) yield force for trabecular and cortical bone for: normalised Young’s moduli of 1 GPa, BMD, nanoindentation (significant differences between placebo and 0.1% and placebo and 1%, p < 0.01) and microindentation for placebo, 0.1% and 1% whey protein fed groups.
Figure 6.6. Normalised average (blue) and peak (red) yield force for cortical bone for: normalised Young's modulus of 1 GPa, TMD, nanoindentation, microindentation and three point bending for placebo, 0.1% and 1% whey protein fed groups.
RESULTS

Table 3. Averaged Young’s modulus with standard deviation for trabecular bone. Values from BMD derived, nanoindentation and microindentation.

<table>
<thead>
<tr>
<th>Young’s modulus: Trabecular bone [GPa]</th>
<th>BMD derived</th>
<th>Nanoindentation</th>
<th>Microindentation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td>1.02 ± 0.18</td>
<td>17.16 ± 2.16</td>
<td>17.79 ± 3.10</td>
</tr>
<tr>
<td><strong>0.1%</strong></td>
<td>0.99 ± 0.19</td>
<td>15.11 ± 3.03</td>
<td>17.08 ± 3.58</td>
</tr>
<tr>
<td><strong>1%</strong></td>
<td>0.81 ± 0.17</td>
<td>16.16 ± 1.98</td>
<td>19.45 ± 1.99</td>
</tr>
</tbody>
</table>

Table 4. Averaged Young’s modulus with standard deviation for cortical bone. Values from TMD derived, nanoindentation, microindentation and three point bending.

<table>
<thead>
<tr>
<th>Young’s modulus: Cortical bone [GPa]</th>
<th>TMD derived</th>
<th>Nanoindentation</th>
<th>Microindentation</th>
<th>3 point bend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td>8.45 ± 0.38</td>
<td>21.84 ± 1.92</td>
<td>30.93 ± 1.97</td>
<td>7.91 ± 4.83</td>
</tr>
<tr>
<td><strong>0.1%</strong></td>
<td>8.52 ± 0.30</td>
<td>22.03 ± 3.43</td>
<td>27.50 ± 3.10</td>
<td>4.60 ± 1.72</td>
</tr>
<tr>
<td><strong>1%</strong></td>
<td>8.57 ± 0.59</td>
<td>20.83 ± 3.18</td>
<td>27.90 ± 2.48</td>
<td>6.27 ± 3.84</td>
</tr>
</tbody>
</table>

The effect of whey protein treatment on BMD (trabecular) and TMD (cortical) is shown in Figure 6.7. BMD shows a decreasing trend with increase in whey protein treatment. A one-way ANOVA across the three treatment groups revealed a significant difference in BMD between placebo and 1% whey protein (p < 0.01) and a significant difference in BMD between 0.1% and 1% whey protein (p < 0.01). A one way ANOVA across the three treatment groups revealed no significant differences in TMD and the trend was uniform across the treatments.
The effect of whey protein treatment on BV/TV, TbTh, TbSp and TbN for trabecular bone is shown in Figure 6.8. BV/TV, TbTh and TbN showed decreasing trends with increase in whey protein treatment. In contrast, TbSp increased with an increase in treatment. A one-way ANOVA across the three treatment groups revealed a significant difference in BV/TV and TbTh between placebo and 1% whey protein treatment ($p < 0.01$) and between 0.1% and 1% whey protein treatment ($p = 0.014$). There was no significance in the trends for TbSp and TbN. Correlation between mechanical failure force and trabecular micro-CT indices revealed strong to moderate correlations and is shown in Figure 6.9. This correlation was significantly improved when BMD derived material properties were included in the strength prediction with TbTh being the exception. BV/TV showed a strong correlation with failure force ($R^2 \sim 0.9$) and TbSp and TbN showed moderate correlation ($R^2 \sim 0.42$ and $R^2 \sim 0.74$, respectively).
Figure 6.8. Comparing micro-CT indices between three treatment groups for trabecular bone. Placebo (blue), 0.1% whey protein fed (red) and 1% whey protein fed (green). Top left: BV/TV showed a strongly significant difference between placebo and 1% ($p = 0.011$) and between 0.1% and 1% ($p = 0.015$). Bottom left: TbTh showed a strongly significant difference between placebo and 1% ($p$-value 0.014) and between 0.1% and 1% ($p = 0.002$). Top right: there were no differences between the groups for TbSp. Bottom right: there were no differences between the groups for TbN.
The effect of whey protein treatment on the T.Ar, B.Ar, Ma.Ar, B.Ar/ T.Ar and Cs.Th of cortical bone is shown in Figure 6.10. With increasing whey protein treatment, both T.Ar and Ma.Ar increased in trend. A one-way ANOVA across the three treatment groups revealed a significant difference in Ma.Ar between placebo and 1% (p = 0.04) and between 0.1% and 1% (p < 0.02). However tissue area was not significant. In contrast, bone area showed no significant differences and was uniform. B.Ar/T.Ar showed a strongly significant difference between placebo and 1% (p < 0.01) and between 0.1% and 1% (p < 0.01). Cs.Th showed a significant decrease between placebo and 1% (p = 0.03).
The first objective of this study was to evaluate the influence of a varied whey protein diet on computationally predicted mechanical strength of murine bones in both trabecular and cortical regions of the femur. Our findings (summarised in Table 3 and Table 4) show consistent values with the literature (Akhter, Fan, & Rho, 2004; Choi, Kuhn, Ciarelli, & Goldstein, 1990; Silva, Brodt, Fan, & Rho, 2004). Specifically, our studies show the Young’s moduli of trabecular bone from...
microindentation ranged from 13 - 22 GPa (up to 43% maximum variation from the mean). Trabecular nanoindentation ranged from 12 - 19 GPa (up to 25% maximum variation from the mean), which is consistent with the literature (Akhter et al., 2004); cortical microindentation ranged from 24 - 32 GPa (up to 17% maximum variation from the mean) and nanoindentation ranged from 17 - 25 GPa (up to 19% maximum variation from the mean), a range consistent with the literature (Akhter et al., 2004). The range for three point bending was 2 - 7 GPa (up to 66% maximum variation from the mean) consistent with the literature (Choi et al., 1990; Silva et al., 2004). The difference in Young’s moduli between indentation and three point bending is well known and has been shown to differ by a factor of up to five (Silva et al., 2004), which is also consistent with our findings.  

Our BMD derived trabecular bone Young’s moduli range of 0.8 - 1 GPa was half that reported for the femoral neck ROI by Taddei et al. 2007 (Taddei, Schileo, Helgason, Cristofolini, & Viceconti, 2007), and our TMD derived cortical bone Young’s moduli range of 8.45 - 8.57 GPa was slightly less than the 10 GPa of the femoral diaphysis reported by Taddei et al. 2007 (Taddei et al., 2007).

There was no significant influence on mechanical strength in cortical bone observed with increasing whey protein treatment, which was consistent with cortical TMD and B.Ar obtained independently. Hence, further bone treatments would be needed to confirm the prediction confidence in our model for cortical bone. However, trabecular bone showed a significant decline in strength with increasing whey protein treatment when nanoindentation derived Young’s moduli were used in the model. When microindentation, micro-CT phantom density and normalised Young’s moduli were included in the model a non-significant decline in strength was exhibited. These results for trabecular bone were consistent with both trabecular BMD and trabecular micro-CT indices obtained independently.

The presented study revealed similar characteristics to another FE study, which showed different findings between computational FE analysis and micro-CT (Tsai et al., 2015). In that study micro-CT showed significant changes due to the anabolic treatment, whereas the FE predictions showed no mechanical differences. This highlights that micro-CT indices, which are surrogate measures of strength may not always correlate with actual strength (predicted using a model). Another FE study considering the effects of bisphosphonates on human vertebral strength showed how modelling was able to isolate the increase in strength in the trabecular component of bone (Keaveny et al., 2007). This demonstrates the benefit that modelling brings to clinical studies through highlighting spatially varying increases in strength. This is consistent with our study, which also showed variations in trabecular strength only. A further micro-FE study into the effects of Idoxifene on the mechanical properties of human bone showed no mean changes
between treatment groups but did show significant changes from baseline within treatment groups (Van Rietbergen et al., 2002). This highlights the importance of subject-specific variation to mechanical properties, which computational FE captures efficiently.

The second objective of this study was to characterise the influence of different sources of Young’s moduli (micro-CT phantom-derived, nanoindentation, microindentation and three point bending for cortical bone only) on computational prediction. It is well known that different sources provide varying estimates of Young’s modulus and this study aimed to quantify the predicted mechanical strength in 3D from these sources and evaluate if trends and conclusions on treatment effects remained consistent. For cortical bone predicted mechanical strength behaviour was consistent across all sources of Young’s moduli (despite different failure magnitudes), however, three point bending produced on average half the strength estimate of nanoindentation and microindentation. There was no difference in treatment trend observed when the Young’s moduli were normalised. In contrast, trabecular strength due to whey protein treatment significantly reduced when Young’s moduli from nanoindentation were introduced. Other material property sources were not significant but emphasised the declining strength trend over the normalised Young’s moduli. This shows strength at the trabecular level was attributed to both changes in bone architecture and Young’s moduli and the choice of Young’s modulus does change the findings. In summary, the whey protein treatment effect was observed solely in trabecular bone and the choice of material property changed the significance of predicted mechanical strength due to treatment effects.

This study had several limitations that were taken into consideration when interpreting the results. Firstly, the initial point of failure for each femur sample was taken into account and fracture propagation was not considered. This would have required a proper damage evolution law and remodelling of material properties in the areas of micro failure. However, since we classify strength as the resistance to the initial yield the overall conclusions are not affected. For the indentation tests, the small sample size (placebo n = 9, 0.1% = 9 and 1% = 11) was a limitation. With trabecular bone, structural issues such as the porosity of the sample meant that the location where the indentations were taken varied between samples to ensure testing took place on an area with large bone volume. This may have in part contributed to the significant failure forces predicted for trabecular bone in the model. In addition, thermal drift during nanoindentation and microindentation could have had an impact on the results in particular for the microindentation tests where the drift was not automatically corrected for but in order to minimise the effect of thermal drift, a certain limit was specified and had to be reached before the experiment could proceed. Additionally, sample size was a limiting factor as it was only 29 mice. A larger sample
size would most likely have emphasised the trends observed. Desktop micro-CT systems employ a polychromatic source which also present with several limitations. These have been compensated for in this study. In order to minimise the effects due to beam hardening, a low voltage and an appropriate filter were used in addition to a beam hardening compensation of 20% (standard for mice bones) which was used for the reconstructions. The partial volume effect is a key limitation that also needs to be addressed. This limitation is only applicable to trabecular bone as BMD measurements are used in this study. A few samples were omitted from the study due to issues encountered during the scanning and indentation process. Regardless, the small sample size was suitable for a modelling study as we were able to identify trends in the data and correlate the computational data with micro-CT indices.

The decrease in trabecular mechanical strength (as predicted using the average von Mises yield criteria) with increasing whey protein treatment is unexpected but reflects some potential underlying process, whereby the whey protein treatment may have influenced bone mineralisation and hence reduced strength. This decline in strength is reflected in the micro-CT indices from Figure 6.8, which shows decline in BV/TV, TbTh and TbN, leading to increased TbSp (Ulrich, Van Rietbergen, Laib, & Ruegsegger, 1999). This does not affect the conclusions from this study which was to determine if a computational prediction of strength is consistent (in a double blinded design) to micro-CT indices, which was the case. When the peak yield criteria was used instead, the decline in trend was stronger and there were variability’s observed in the failure loads from difference material sources. The peak yield criteria uses the location in bone where micro-failure is occurring (shown by peak stresses identified from the 3D Instron test) as the threshold for failure and is more aligned to the specific features of trabecular architecture and BMD. Hence, it correlates better to the micro-CT indices concerning TbTh and BMD, which showed strong significant declines with increasing treatment and would emphasise the decline in mechanical strength better.

In contrast, the uniform failure force (as predicted using the average von Mises yield criteria) shown in cortical bone across multiple sources of Young’s moduli is consistent. This can be explained by the B.Ar and TMD also being uniform across treatments. However, the failure from using three point bending Young’s moduli was approximately half for each treatment and is likely due to the violation of the assumptions behind the Euler-Bernoulli equation (Wallace et al., 2014). Wallace and colleagues, highlighted, that when the three point bending sample is short and not long or slender then there are shearing effects that are ignored and not considered in the Bernoulli equation. Hence, the Young’s modulus in the axial direction is underestimated and care should be given to Young’s modulus derived using three point bending. When the peak yield
criteria was used instead there were increased variability observed in the failure loads from different material sources, though not as pronounced as trabecular bone. This is likely due to the variations in peak stress due to the presence of canaliculi. However, murine bone does not have haversian canals (unlike human cortical bone) (Mills & Simpson, 2012) so these larger pores would not contribute to variations in peak stress. However, translation to the human condition may likely lead to larger variations in cortical bone.

BMD showed significant decreases with increasing treatment and is likely due to the nature of BMD measurement, which accounts for both bone tissue and pores. Porosity is increasing due to the increased TbSp, and reduced TbTh and TbN shown in Figure 6,8, but there is still uncertainty about the bone mineral. Ideally, TMD of trabecular bone would address this issue but requires finer resolution, which was not performed in this study. In contrast, TMD showed no change and was uniform across treatments for cortical bone. TMD measures only bone and is a better representation of the actual bone mineral content but was not possible for trabecular bone in this study due to the higher micro-CT scanning resolution required. The fact that no difference was observed highlights that the mineral density in cortical bone is not influenced by treatment effects and the very small standard deviation highlights that there was very consistent TMD across samples and is a reliable parameter, compared to BMD, which had larger variability within a group.

The reduced BV/TV with increasing treatment effect highlights that the whey protein diet was interfering with bone size, however, cross sectional area is normalised when reporting mechanical failure force and all specimens had the same height. The fact that there is still a decreasing trend for mechanical failure indicates that it is due in part to bone architecture. This finding is further elaborated by the significant reduction in TbTh, and non-significant decline in TbN, which are measures of trabecular bone architecture. The increasing separation is consistent with the decline in strength. In order to check confidence in model predictions, the normalised average failure force was checked for correlation with BV/TV, TbSp, TbTh and TbN and was found to be strongly to moderately correlated, which has been reported numerous times in the literature (Müller et al., 1998; Newitt et al., 2002; Teo, Si-Hoe, Keh, & Teoh, 2006).

While TMD, failure force and B.Ar exhibited a non-significant uniform trend across treatments the localised behavior of cortical bone showed significant change. Medullary area (Seeman, 2003b) increased significantly with increased whey protein treatment. This was revealed in a thinning of the internal cortical boundary and increased area on the periosteal surface with increasing treatment but which was not significant. This highlights an expanding outer cortex
(Seeman, 2003a). However, the increased T.Ar coupled with the increased Ma.Ar produced a net area change that is fairly uniform, which was shown in the B.Ar. In addition a significant decrease in the cross sectional thickness and very significant decreases in the cortical area fraction with increasing whey protein treatment further reinforces the localised treatment effects within the cortical bone.

### 6.6 SUMMARY

In conclusion, our modelling pipeline was able to predict strength trends in cortical and trabecular bone due to a whey protein treatment that was consistent using a double blinded design to TMD, BMD and micro-CT indices. It was shown that the source of Young’s moduli is important, especially in the case of trabecular bone, where treatment effects were isolated for this study. Inclusion of nanoindentation derived Young’s moduli produced a declining strength trend with increasing whey protein that was significant, highlighting its ability to detect subtle property changes in small volumes. Three point bending results produced estimates of strength around half that of nanoindentation and microindentation, however, this is expected due to likely violations in the Euler-Bernoulli equations. This study highlights that computational predictions are sensitive to the source of Young’s moduli but in general the strength prediction trends due to treatment are consistent regardless of the source of Young’s moduli. Models can therefore play a useful role in helping to deduce likely trends and treatment effects as part of pilot studies before commencing large clinical trials.

### 6.7 NOTE

Cortical bone was originally analysed in 3D and the parameters chosen were: bone volume, periosteal volume and endosteal volume. Based on suggestions from the reviewers of the manuscript, these indices have been replaced by the equivalent 2D parameters. The original 3D analyses can be found in Appendix B.
Mechanical Differences between Sham Operated and Ovariectomised Mice

The following is a reproduction of the article:


7.1 ABSTRACT

Ovariectomised (OVX) and Sham operated (Sham) mice are standard controls in experimental bone work. These controls are often used together in reported murine studies, but it is unclear what mechanical differences exist between these groups. This information may be useful when choosing controls for evaluating bone treatment effects. To evaluate the mechanical influence of
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OVX compared to Sham mice, a validated micro-finite element (FE) analysis technique of virtual loading of micro-CT derived murine femurs was carried out. Twenty two femurs, \( n = 11 \) in each group, were obtained, 30 weeks post-surgery. Trabecular and cortical bone were isolated and assessed separately. There was a significant difference in the bone mineral density (BMD) between the OVX and Sham groups \( (p = 0.02) \). In addition there were also significant differences in percentage bone volume \( (BV/TV) \) \( (p = 0.02) \) and trabecular thickness \( (TbTh) \) \( (p < 0.01) \). However there were no significant differences in either the average yield or peak yield force in the trabecular bone. Cortical bone showed no differences in tissue mineral density (TMD) or in the average or peak yield force. Therefore, this study suggests that there are no significant differences in cortical bone in either the OVX or Sham groups and while there were significant differences in trabecular BMD and \( TbTh \) from micro-CT based bone indices, mechanically, there were no differences between the two groups. This study suggests that either OVX or Sham mice are suitable controls for comparing mechanical differences with bone treatment effects.

7.2 INTRODUCTION

Placebo operated (termed Sham) and OVX mice, who have had their ovaries removed, are commonly used control models in experimental bone work. Sham mice are used to eliminate any biases due to the placebo effect while OVX female mice are used to evaluate changes in trabecular bone architecture, attributable to oestrogen deficiency and to study the effects of different treatments at various stages of oestrogen deficiency (Laib et al., 2001). They are also used to remove any oestrogen effects that may confound a study. OVX mice are well accepted models of osteoporosis as it has been well documented that following OVX surgery, mice lose a significant amount of trabeculae (Thompson, Simmons, Pirie, & Ke, 1995). Sham mice are commonly used as controls in experiments involving surgical procedures. On the other hand, OVX mice are not used solely as controls but together with Sham mice. OVX mice models are most commonly used when assessing treatments for bone strength and associated clinical conditions such as osteoporosis (Rhee et al., 2009).

OVX mice exhibit an initial phase of rapid bone loss followed by a slower phase. The greatest loss occurs in the trabecular bone, rather than in the cortical bone (Kalu, 1991). Mice that underwent OVX surgery have shown a significant decline in \( BV/TV \), \( TbTh \) and trabecular number \( (TbN) \) when compared to the Sham mice (Bouxsein et al., 2005). BMD is one of the main risk factors used clinically for bone fragility and is thus a widely accepted surrogate for determining bone strength.
(Bouxsein et al., 2005; Laib et al., 2001). This measure, however, averages bone and pores together and does not take into consideration specific architectural changes in the trabecular bone which is a limitation, as a deterioration in the trabecular architecture, significantly contributes to the fragility of the bone mechanically (Dempster, 2000). Bone mass, microarchitecture, turnover, micro-damage and mechanical properties are all contributing factors to bone quality (Rhee et al., 2009). While it has been shown that cortical bone predominantly bears the majority of the load (up to 45 - 75% of the axial load) (Rockoff, Sweet, & Bleustein, 1969), trabecular bone is able to resist buckling movement by buttressing the cortical bone (Thomas et al., 2009). As BMD is an averaged measure of bone quality (includes the trabecular bone, soft tissue and voids) it is therefore limited in regards to specific information about the bone itself. A FE model, however, is able to isolate features of the bone like architecture and material properties and is thus able to evaluate the mechanical strength of the bone and assess the contributions of these specific features to the overall strength of the bone. This feature of computational models allows us to reveal which aspects of bone, changes manifest in, and secondly whether this makes a difference to the homogenised strength at the end.

Therefore, the aim of this study was to use a previously validated modelling pipeline to give insight into the differences that are attributed to murine bone following Sham or OVX surgery. The aims of this study were to (i) evaluate bone architecture differences between OVX and Sham mice through computational mechanical assessment and through micro-CT indices and (ii) evaluate material property differences between OVX and Sham mice through BMD (trabecular bone), TMD (cortical bone) and its influence on overall computationally predicted bone strength. These differences will inform the bone community on the choice of control (and whether both OVX and Sham mice are necessary) when evaluating the effects of bone treatments on mechanical strength.

7.3 METHODS

Female, 6 wk-old, C3H/HeN strain mice (n = 22) were purchased from Harlan. They were housed in a room controlled for temperature (22 ± 1°C) under a 12:12 h light-dark cycle and were given free access to a standard-pellet diet and water. All experimental procedures used during these experiments complied with institutional guidelines and policies to prevent pain and distress under license from the French Veterinary Service (A75-05-19). Eleven, 12-wk-old, female, C3H mice underwent OVX surgery and 11 mice were Sham operated. Mice were anaesthetised with
keta
min (100 mg/kg) + xylazin
(10 mg/kg) and morphi
was administered to prevent pain. At 30 weeks post-surgery, mice were anaesthetised and euthanised. The right femur was cleaned from muscle and fixed in ethanol, for future testing.

The femurs were scanned using a SkyScan 1172 micro-CT scanner (X-ray voltage 50 kV, 0.5 mm aluminium filter, voxel size 5 μm). After standardised reconstructions using the SkyScan NRecon software, the datasets were analysed using the SkyScan CT-analyser software. Volumes of interest (VOIs) of trabecular and cortical bones were selected in the distal end of the femur. The VOIs were selected in two regions: trabecular region which was 0.5 mm proximal to the growth plate and extended 1.25 mm in the proximal direction. The cortical region of bone was 3.45 mm from the growth plate and extended 0.5 mm in the proximal direction. Each dataset was binarised using global thresholding and bone parameters were measured. Using the SkyScan image processing software, standard, 3D micro-CT indices were measured, some of which included BV/TV, TbTh, TbSp and TbN for trabecular bone and bone, periosteal and endosteal volumes for cortical bone, for all 22 bone samples. The framework for this work is shown in Figure 7.1.

The raw digital data was exported in a StereoLithograph (STL) format and cleaned digitally. This removed any erroneous bone artefacts (parts of the bone that were not attached to the main body) and redundant polygons. The mesh was then reduced in size (decimated) for computational efficiency without reducing the geometric integrity of the structures using Rapid Form XOR, now Geomagic (Inus Technology Inc). The solution time to fit a FE voxel mesh to the STL triangle mesh depends on the number of triangles. The process was optimised by finding the minimum density of triangles in the STL mesh, such that the resulting voxel mesh varied by less than 0.1% when computing bone strain compared to the original mesh density. It was found that a reduction of 30% in the triangle number did not significantly affect the bone shape or quality and additionally, did not produce computed bone strain differences of more than 0.1%. A hexahedral voxel mesh was then created for each bone sample using a mesh generation package, Hypermesh (Altair Engineering Inc.). As part of the meshing process, voxel cubes were chosen due to their robust and stable behaviour in elastic mechanics compared to tetrahedral meshes.
A validation study (see section 3.5) using a 3D printed bone model, subjecting the model to an Instron compression test and subsequently, replicating the Instron test as an FE simulation resulted in a failure criteria for the bone models. This was the value chosen as the failure criteria for this work and was used in (Equation 7.2).

Following this, the voxel meshed bone samples were imported into Abaqus (Simuli Dassault Systems Pty Ltd) for FE analysis. Twenty-two models were then computationally loaded to failure under a compression loading mode according to the average von Mises damage criteria, determined from an Instron test. Von Mises stress determines whether the principal stress combination at a given point will cause yield and gives the scalar combination of three principal stresses, which is then compared to the yield von Mises stress of the material, obtained from the validation test. If the von Mises stress of the bone exceeded the von Mises yield stress then the bone is considered to have failed in compression. Von Mises stress in 3D is defined as:

$$\sigma_v = \sqrt{\frac{1}{2}[(\sigma_1 - \sigma_2)^2 + (\sigma_1 - \sigma_3)^2 + (\sigma_2 - \sigma_3)^2]}$$

where, $\sigma_v$ is the von Mises stress, $\sigma_1$ is the principal stress in the x-direction, $\sigma_2$ is the principal stress in the y-direction and $\sigma_3$ is the principal stress in the z-direction. The models were solved...
METHODS

quasi-statically, with each increment in force leading to a new homogenised von Mises. Two von Mises yield criteria were used; (i) a yield stress determined from the average von Mises of the whole bone at failure, defined as the average yield stress; and (ii) yield stress determined from the peak von Mises values at the sites of micro failure only, defined as the peak yield stress. Post analysis then identified the forces that were associated with an average and peak von Mises stress that exceeded the yield value.

Simulations took approximately 6 hours each on a standard PC. Failure force was then normalised by the cross sectional area of the specimen to assess the yield strength attributed to the architecture of the bone model, independent of the size of the sample. The first set of simulations used a normalised Young’s modulus of 1 GPa to predict the influence of architecture on the mechanical strength of the sample. This permitted the use of a yield criteria determined from Instron tests on a 3D printed bone that also had a Young’s modulus close to 1 GPa. To evaluate the influence of material properties, BMD and TMD values were obtained from the micro-CT phantom scans and were used to estimate the material properties of the bone’s. This density was included in the model using the adapted (Carter & Hayes, 1977) power law (Gupta & Dan, 2004) which relates the elastic modulus ($E$) to the apparent bone density ($\rho$) of trabecular bone according the following equation:

$$E = 1050e^{-6}\rho^2$$

The primary analysis of this study was the assessment of Sham or OVX operated mice, from a mechanical perspective, of trabecular and cortical bone. Each model was loaded in compression until the von Mises yield criteria was reached and a damage failure force recorded. Comparisons between OVX and Sham mice were carried out for: BMD and TMD for trabecular and cortical bone, respectively, the failure force of each of the two bone types (trabecular and cortical) using average and peak von Mises yield criteria; a number of standard micro-CT indices and the homogenised Young’s modulus for trabecular bone only.
7.4 RESULTS

A representative cross-section through the trabecular regions of both OVX and Sham mice femur samples (Figure 7.2a and Figure 7.2b, respectively), visually demonstrate the differences attributable to the two surgical procedures. The OVX cross section shows less trabeculae, more voids and thinner struts in comparison to the Sham cross section.

![Figure 7.2. Representative cross section through the mouse femur. a. OVX operated mouse femur. b. Sham operated mouse femur.](image)

Figure 7.3 shows comparisons between BMD, average and peak von Mises yield force and homogenised Young’s modulus. For trabecular bone, BMD of OVX mice was significantly lower than Sham (p = 0.008). However, when computing the strength (via the average von Mises yield criteria) due to architecture alone there were no significant differences between OVX and Sham mice. When the maximum von Mises yield criteria was applied, there was an increase in strength in the Sham group but the difference was not significant. The homogenised Young’s modulus following mechanical tests also showed no significant difference between OVX and Sham mice.
Figure 7.3. Comparisons between OVX and Sham for trabecular bone. Top left: strongly significant difference ($p = 0.008$) in BMD between OVX and Sham. Bottom left: no significant difference in the homogenised Young’s modulus between OVX and Sham. Top right: no difference in the normalised average yield force between OVX and Sham. Bottom right: no difference in the normalised peak yield force between OVX and Sham. Blue represents OVX and red represents Sham.

Figure 7.4 shows comparison of trabecular micro-CT indices between OVX and Sham mice. BV/TV, TbTh, TbSp and TbN. While all indices showed a trend of lower OVX values compared to Sham mice, BV/TV was significantly lower ($p = 0.02$) and TbTh was significantly lower ($p < 0.01$). TbSp and TbN, were not significant but OVX mice showed trends of always being lower.
In contrast, cortical bone exhibited no differences between TMD values of Sham compared to OVX. Further, the average and peak von Mises failure force also showed no significant difference between the two groups (Figure 7.5). This is visually depicted in Figure 7.6a and Figure 7.6b, a representative cross section through the cortical regions of both OVX and Sham mice femur samples, respectively. There were no significant changes between the perisoteal and endosteal volumes which are indices indicative of bone formation and resorption, respectively (see Figure 7.7). While the changes were not significant, there was an increase in the endosteal volume in the OVX group compared to the Sham. This is indicative of a thinning of the internal cortical boundary. There was minimal to no change in the periosteal volume between the groups which shows that the outer cortex exhibited no significant bone deposition. The net change in bone volume between groups remained consistent with the perisoteal volume. In addition, there was a strong correlation between the normalised failure force and bone volume ($R^2 = 0.94$), which is expected and used as a check of the predicted forces (See Figure 7.7).
RESULTS

Figure 7.5. Comparisons between OVX and Sham in cortical bone. Top left: TMD shows no differences between OVX and Sham. Top left: no differences in the normalised peak yield force between OVX and Sham. Bottom right: no differences in the normalised average yield force between OVX and Sham. Blue represents OVX and red represents Sham.

Figure 7.6. Representative cross sections through the mouse femur. a. OVX operated mouse femur. b. Sham operated mouse femur.
The aim of this study was to computationally evaluate whether there was a difference, mechanically, between Sham and OVX mice, both of which are used as standard experimental control models. We found no significant differences between OVX and Sham mice in cortical bone for either TMD or computational strength (via failure) due to bone cortical architecture in either the average or peak yield force. For trabecular bone we found significant differences between BV/TV, TbTh and BMD between OVX and Sham, but no significant difference between TbN, TbSp and computational strength (via failure) due to trabecular architecture in either average or peak yield force. However, trabecular failure force using the peak stress failure criteria did show reduced mean strength in the OVX bones, which is consistent with the significantly reduced TbTh observed. Peak stress is highly influenced by local changes in trabecular architecture and the reduction in TbTh would increase the localised stresses in those regions. This suggests that the peak yield stress criteria is more aligned with the observations seen in micro-CT than using an average stress criteria.

Figure 7.7. Comparisons between OVX and Sham in cortical bone. Top left: periosteal volume shows no difference between OVX and Sham. Bottom left: endosteal volume shows no difference between OVX and Sham. Top right: bone volume shows no difference between OVX and Sham. Blue represents OVX and red represents Sham.
Significant differences in trabecular bone micro-CT indices for OVX and Sham mice have been reported and are consistent with the findings in this study. A decrease in OVX BV/TV and TbN (our results showed a non-significant decrease for TbN) is consistent with patterns of trabecular bone loss (Rhee et al., 2009). A decrease in OVX TbTh, but not in comparison to a Sham group but one that underwent treatment for bone loss has been shown (Ito et al., 2002). These are therefore consistent parameters that decreases when there is bone loss. We also showed significant reduction in trabecular BMD of OVX compared to Sham mice which remains consistent with studies using OVX mice (Binkley et al., 2003; Bouxsein et al., 2005). This implies that differences between OVX and Sham mice are likely to manifest in the trabecular structure but this is unlikely to contribute to whole bone strength as shown by our model predictions.

Minimal or insignificant change in the cortical bone is consistent with studies involving OVX and Sham mice in the literature whereby a much greater loss in bone is evident in the trabecular region compared to the cortical compartment (Bouxsein et al., 2005; Ito et al., 2002). Our study was consistent in that we showed no differences in TMD, failure (due to average von Mises Yield criteria) and failure (due to peak von Mises yield criteria). Endosteal volume, a measure of bone resorption (Seeman & Delmas, 2006) increased with OVX surgery. However, this was a non-significant increase, but nevertheless is showing an increase in resorption in the OVX group with no change in the periosteal volume (a measure of bone formation). It has been suggested that bone resorption occurs rapidly in OVX mice and there may be a delay in formation which may have been a possibility in our study (Sims, Morris, Moore, & Durbridge, 1996). The predicted results were also highly correlated ($R^2=0.94$) against standard micro-CT derived bone volume, which was expected and improves confidence in our modelling predictions (Sreenivasan et al., 2013). This implies that the overall strength of OVX and Sham mice are unlikely to be much different as most of the strength in bone is dependent on the cortical shell.

Computational models have reached a level that allows them to act as surrogate measures for destructive mechanical tests (Niebur et al., 2000). The advantage of using FE models is its ability to isolate features of the bone that may contribute to bone strength (which is not possible in more traditional measurements). While the BMD, BV/TV and TbTh all show significant differences between the OVX and Sham groups, these differences were not sufficient to compromise the strength of the bone. This is supported by a study which showed that the trabecular structure changed from plate-like to rod-like as early as one week after OVX surgery but the mechanical integrity of the bone may not be affected by these changes (Laib et al., 2001; Yamazaki & Yamaguchi, 1989).
There were a few limitations associated with our study that should be considered when interpreting the results. First, we predicted trends in trabecular bone using a sample size of only 22 mice where OVX mice exhibited significantly reduced TbTh and trends towards reduced TbN and TbSp. TbN and TbSp may also lead to significance with increased sample size. Secondly, trabecular failure due to peak von Mises yield criteria was not significant but showed a trend of reduced OVX strength that may become important with increased sample size as well. Thirdly, as only one time point was assessed, 30 weeks post-surgery, the conclusions are only representative of this time point and the behaviour may vary for young bone or much later time points. For example, we showed changes in trabecular bone but a longer time point may have shown changes in the cortical region, which tends to undergo endocortical loss with aging (Buenzli, Thomas, Clement, & Pivonka, 2013).

7.6 SUMMARY

In summary, our results have shown that from a computational perspective, there were no significant differences in the mechanical strength in either trabecular or cortical bone due to either OVX or Sham surgery, even though there was a difference in the BMD and micro-CT indices in trabecular bone. This study suggests that either OVX or Sham mice are suitable controls for comparing mechanical differences with bone treatment effects in the age range tested as part of this study.
Conclusions and Future Directions

The aim of this thesis was to develop an efficient and validated computational pipeline to be used alongside clinical bone treatments. While computational models are currently not able to replace clinical trials altogether, they are able to provide insight into the influences of isolated bone components and its strength by highlighting anabolic effects on bone architecture and material properties on cortical and trabecular bone, separately. The advantage of FE modelling is that it is inexpensive, fast to solve as multiple simulations can be run in parallel and reduces the sample size required for testing prior to large scale clinical trials. To that end, this thesis looked at approaching clinical studies from a computational perspective with the aim of reproducing trends observed using a double blinded design and to provide further insight into bone strength using virtual strength tests.

The developed modelling pipeline was applied to three clinical studies:

2. A murine study, looking at the influence of a whey protein diet on bone strength by evaluating material properties from different sources at different scales (macro to micro to nano).
3. The first stage of a wider lactoferrin trial. Assessing the differences between Sham and OVX mice which are used as standard experimental control models.
This modelling framework was also validated against a 3D printed biopsy sample (obtained from the fluoride study) that was fractured in an Instron compression test. To our knowledge, this approach was a novel method of validating our modelling pipeline.

The following chapter covers conclusions and limitations of the three studies (Chapters 5 - 7), which were the focus of this thesis. This will then be followed by a similar discussion regarding the three experimental chapters (Chapters 2 - 4) and to finish, the potential future directions of this work. A summary of work carried out in this thesis is shown in Figure 8.1.

![Figure 8.1. Framework summarising the work covered in this thesis. a. Micro-CT imaging and VOIs of trabecular and cortical bone. b. Voxel meshing trabecular and cortical bone. c. Validation test and material testing (Chapter 6 only). d. FE simulation of trabecular and cortical bone. e. Stress distribution patterns in trabecular and cortical bone. f. Evaluating the influence of various anabolic treatments on bone strength.](image)

**8.1 INFLUENCE OF LOW-DOSE FLUORIDE ON BONE**

This study evaluated the effects of low-dose fluoride on postmenopausal women for a 12 month period. Twenty four out of 180 participants volunteered biopsy samples (taken from the iliac crest) at the conclusion of this study. Bone density analysis showed that low-dose fluoride treatments had no significant effects on any skeletal site but showed a non-significant decline in
strength with increasing fluoride level. The modelling pipeline was applied to this study using a double blinded design and while the results also predicted that fluoride treatment had no significant effect on the mechanical strength/failure of the bone (consistent with the bone density analyses), it exhibited the same non-linear decline in strength trend. While fluoride treatment had no effect on bone strength, the modelling work was able to provide a number of other insights. When the architecture of the samples were isolated and evaluated, the trend observed was consistent with the geometrical parameters, BV/TV and intersection surface. In addition to the two indices mentioned, the failure force was also strongly correlated to the bone surface density and TbTh for the three different loading modes studied. This showed that BV/TV, bone surface density and TbTh are strong surrogate measures for bone strength. When a bulk estimate of material properties was applied to the model (to evaluate the effects of material properties on the failure force), the correlations decreased suggesting that a bulk estimate was not an appropriate substitute for a site specific measurement. The final finding from this study was the comparison of 2D histomorphometric analysis with 3D micro-CT indices. We showed that traditional histomorphometric measures were not able to substitute for the complete 3D architecture of bone which is necessary for complete analysis.

Several limitations of this study will now be addressed. Biopsy samples were only obtained at the end of the study and therefore we were not able to compare between the start and end points. This however could not have been rectified as the process of obtaining biopsies is invasive and painful. We were also limited by sample size but the same rationale applies. Regardless of sample size, the trends predicted by computational modelling were consistent with clinical work and it is possible that with an increase in sample size, the results may have been significant. To assess material properties, bulk values were used in this study. This was improved upon in the two following studies where BMD and TMD values were used as estimates of the material properties and in addition, we had indentation derived material properties for the whey protein diet study. While this study was limited by several factors, it was a unique and novel application of our modelling pipeline to a human clinical trial into fluoride therapy and to our knowledge, has not been presented in the literature before.

8.2  INFLUENCE OF A WHEY PROTEIN DIET ON BONE

This study looked at the influence of a whey protein diet on bone in a murine model. Unlike the fluoride study, the secondary aim of this part of the thesis was to evaluate the influence of
INFLUENCE OF A WHEY PROTEIN DIET ON BONE

Material properties measured from different sources at different scales. Material properties were assessed using: BMD/TMD (at the level of the VOIs) with power laws, three point bending (whole bone level), microindentation and nanoindentation. Young’s modulus values obtained from these tests were included in the model to see how material properties from different sources influenced the outcomes of the failure prediction of the bone samples. As with the fluoride work, the models were also run with normalised material properties in order to study the influence of architecture only. There was no significant influence on the computed mechanical strength of cortical bone with increasing whey protein treatment. This result was consistent with cortical TMD and micro-CT based bone volume. Trabecular bone on the other hand showed significant decline in strength as the whey protein treatment increased when material properties from nanoindentation were used in the model. When material properties from microindentation, micro-CT phantom density and normalised material properties were included in the model, the decline in strength was non-significant. These findings were consistent with trabecular BMD and micro-CT indices. The second part of this study was to evaluate the influence of different sources of material properties on computational predictions. For cortical bone, predicted mechanical strength behavior was consistent across all sources of material properties (although the magnitudes of failure, varied). The exception to this was three point bending which on average predicted half the strength estimates of nanoindentation and microindentation. The trend did not change when material properties were normalised. Trabecular bone strength was significantly reduced when material properties from nanoindentation were introduced. While the other material property estimates did not show a significant reduction in strength, the trend was emphasised. Therefore, this study shows that in trabecular bone, changes were attributed to both architecture and material properties and the choice of material properties does change the findings.

As with all studies, there were limitations that needed to be considered. Indentation tests on trabecular bone proved difficult due to structural issues. The porous nature of trabecular bone meant that the sites at which the indents were done varied between samples. While this was more pronounced in the trabecular bone, microscopic pores within cortical bone may have also contributed to variations in the values. An increase in sample size in each of the whey protein groups would have strengthened the material property values obtained. However, as this was a modelling study, the small sample size was suitable as trends were identified and correlations with standard micro-CT indices were made to provide confidence in the model predictions. In addition, this study used BMD and TMD values to obtain an estimate of the material properties
of the bone, an improvement on the bulk material properties (from DXA) used in the fluoride study.

To summarise, the whey protein treatment effect was observed only in trabecular bone and the choice of material property changed the significance of the predicted mechanical strength due to treatment effects. Due to large fluctuations in values, experimental work that require the input of material properties need to be carefully considered as the values are so different at different scales. This means, for example, BMD/TMD values cannot substitute material properties at the micro level. Furthermore, to our knowledge, this is the first study that has integrated material properties from three point bending, BMD and TMD, microindentation and nanoindentation for trabecular and cortical bone and applied that information to a model.

8.3 INFLUENCE OF SHAM AND OVX SURGERY ON BONE

Sham and OVX mice are commonly used control models in mice experimental studies. This part of the thesis looked at evaluating mechanical differences between Sham and OVX mice. The aim of this study was to apply the modelling framework to provide insight into differences attributed to murine bone following either surgical procedure. This is of interest to the bone community as this has not been evaluated as part of a validated pipeline before. Our results showed that neither surgical procedure had significant effects on either the normalised averaged yield force or the normalised peak yield force of either trabecular or cortical bone. Although the peak load yield force showed an increase in the Sham group, this difference was not significant. Consistent with the whey protein diet work, cortical bone showed no difference between the two surgical groups. In addition, there was no change in the TMD between the two groups. While there were no changes seen in the mechanical strength of the trabecular bone between the two groups, there were a number of significant differences in a few other parameters. There was a significant difference ($p = 0.008$) in BMD between the two surgical groups. There was also a significant difference ($p = 0.02$) in the BV/TV and in the TbTh ($p < 0.01$). In addition, while the differences were not significant, TbSp and TbN showed the same trends. These significant parameters are consistent with the strong correlations with these micro-CT indices shown in the fluoride and whey protein diet studies. Therefore, our work has shown that even with the significant differences in the BMD, BV/TV and TbTh, the differences were not enough to have an effect on the mechanical strength of the bone, which is dominated by cortical bone.
The major limitation with this work is that only one time point was assessed to see the effects of the two surgical procedures on bone. Since the conclusions drawn only represent one time point, the behavior of younger bone may be different and since it is well known that cortical bone undergoes endocortical loss with aging, the same applies for older bones. As mentioned previously, the peak yield force for trabecular bone showed an increase compared to the OVX group. While this was not a significant increase, a larger sample size may have yielded a significant result.

In summary, the Sham/OVX study showed that these two surgical procedures had no effect on cortical bone. Trabecular bone, however, showed changes in BMD, BV/TV and in the TbTh. While there were significant changes in these parameters, there were no differences between the two groups when it came to the mechanical strength of the bone for either the average or peak von Mises yield force. This therefore suggests that the changes in the bone parameters were not significant enough to have an effect on the overall mechanical strength of the bone.

### 8.4 MICRO-CT IMAGING AND MODEL CREATION

Micro-CT was the ideal imaging modality for the type of work investigated as part of this thesis as it is compatible with standard bone study practice (research and clinical trials). It has been described as the “gold standard” for evaluating bone morphology and microarchitecture in small animal models (Bouxsein et al., 2010). As this work looked at imaging small human bone biopsy samples and murine bones, micro-CT was the best imaging mode. With that said, there were aspects of this process, particularly in creating the bone models that need to be discussed as potential limitations. Determining the growth plate, which was the starting point of the VOI of the bone sample, was a process that required care. The growth plate was identified as a low density, uninterrupted, cartilage “seam” running through a cross section of the bone. The “seam” was only uninterrupted for a few slices either side of the growth plate and therefore the process of selecting it was a subjective process. The growth plate also served as the starting point for the offset which was created so that the VOI only included the secondary spongiosa (mature bone). Selecting the offset was also a subjective process as it was at the discretion of the individual carrying out the analysis to decide what was categorised as primary and secondary spongiosa. However, once the offset number of slices was decided upon, it was kept consistent for every bone sample in that experimental group. Once the offset was established, the distance/number of slices of the VOI was determined. Like working out the growth plate and offset, this process
was also dependent on the person carrying out the analysis. Selecting the VOI for trabecular bone required more care than selecting the VOI for cortical bone. This is because the same VOI distance was applied to all of the samples and therefore in order to ensure that the majority of the trabeculae in every sample was included in the VOI, a distance/number of slices was selected, which was applied to every bone. The general rule employed was to include as much of the secondary spongiosa as possible until the upper limit of the VOI started to change from trabecular to only cortical bone. On the other hand, selecting the cortical bone VOI didn’t require as much attention to detail as cortical bone is simply bone throughout the VOI. However, just like the trabecular bone, the same distance/slice number was applied to every bone in the experimental set. The biggest limitation is the process of manually segmenting the trabecular bone. As this was a manual and not an automated process, decisions such as identifying the trabecular bone away from the endocortical boundaries and choosing how often to create the “drawings” were left up to the individual. The same limitations applied to creating the cortical bone models but the process was significantly more straightforward in comparison to the trabecular bone. The final limitation would be using a global threshold value to build the models. The process of selecting a value involved going through a large number of the digitised images and selecting an appropriate value to transform the grey scale images into black and white. This process required some trial and error and was particularly challenging as trabecular bone has both thin and thick struts which made it difficult to threshold. The chosen value was applied to both trabecular and cortical bone and this selected value was specific to each study.

Having listed all the limitations with this method it is important to mention that all our work was checked and approved by an experienced micro-CT technician. In addition, only one individual went through the process from scanning to building the models in order to maintain consistency in this work. It should be noted that although this process appears long and involved due to the manual decisions of the technician, this would not influence the computational pipeline as this is always completed as part of standard practice and does not add any extra time to the analysis due to computer modelling inclusion.

8.5 MATERIAL PROPERTIES AT DIFFERENT SCALES

As mentioned in previous chapters, bone has a hierarchical arrangement, meaning that at different scales the structure and in turn the material properties vary hugely. (Rho et al., 1998). In order to assess the material properties of bone at different scales, a number of experimental
techniques were employed. For whole bone testing, three point bending, for the VOIs of the bone samples, micro-CT based BMD and TMD measurements, for the micro scale, microindentation and for the nano scale, nanoindentation. A number of limitations with this work will now be discussed, starting with how the bones were marked. The indentations tests were carried out in the areas that were associated with the trabecular and cortical VOIs and therefore these areas needed to be physically marked on the bones (see figure 4.3). Physically marking the bones involved using digital calipers to measure and mark the VOI (using the previously obtained distances) by using the condyles as a landmark. This process was not perfectly accurate but was the best way to identify the VOIs of both, trabecular and cortical bone. To ensure consistency, the measurements and markings were done by the same individual for the bones in this experimental group. Another key limitation were the difficulties in carrying out the indentation tests due to the structural complexities of the trabecular bone. The porous nature of the bone meant that the “line” of indents varied between samples. With cortical bone, the presence of microscopic pores or canaliculi, may have also been a contributing factor in the variation in the data obtained. However, unlike trabecular bone, cortical bone pores would not have been as great a variable as the porous trabecular structure. In addition, the data from this shows that there is little variability in the material properties of cortical bone compared to trabecular bone, meaning the microscopic pores within cortical bone did not play as big a part as the pores in trabecular bone. This is especially true for murine bone that does not contain haversian canals unlike humans.

Even though there were limitations involved in these experimental techniques, all the testing was carried out by one individual to maintain consistency. All the work was also checked by an experienced co-worker.

8.6 FINITE ELEMENT MODEL SET-UP

Using the FE method, assessing mechanical properties of bones and in turn predicting their failure has been discussed previously. In this thesis, we have developed a replicable pipeline which involved converting 3D micro-CT bone models into voxel based FE models. Hexahedral voxels were chosen to create the FE model over tetrahedral elements. While tetrahedral elements captured the geometry of the bone well, computational stability was lacking due to issues concerning highly distorted elements and convergence. This was mainly due to the extremely complex nature of the haversian systems in human trabecular bone. Hexahedral voxels were then chosen as this element type was able to handle the irregular architecture of trabecular bone.
sufficiently. While it did not replicate “smooth” edges as with the tetrahedral elements, this was compensated for this by refining the mesh down to an isotropic individual voxel size of 10 μm to ensure the architecture of the bone was appropriately captured. The same process was carried out for cortical bone but did not require as much refinement as it lacked the complexities of its corresponding trabecular bone. An isotropic voxel size of 40 μm was used for cortical bone. The material properties of the model were described using linear elasticity and the bone samples were assumed to behave under several assumptions including: bone experiencing small deformations, no rigid body movements, stress being proportional to the strain and bone undergoing elastic deformation. Assigning the material properties as isotropic and linear elastic is acceptable for bone (Ulrich et al., 1999) at the micro scale. Boundary conditions were assigned in order to prevent displacements and rigid body rotations and also to simulate quasi-static conditions. To evaluate the failure of the bone, a failure criteria was established based on the Instron compression test of a 3D printed bone biopsy with similar material properties. Therefore, failure of the bone was described in terms of the von Mises stress of the material and exceeding the von Mises yield stress would predict bone failure. The limitation in adapting this failure criteria was due to basing it on the failure of the 3D printed bone. While the 3D printed bone has a Young’s modulus that fell in the same range as trabecular bone, it was still a 3D printed model and not an actual bone sample. Nonetheless the von Mises strain in the failure regions of the 3D printed model was approximately 0.8% equating to a von Mises stress of approximately 3 MPa which was implemented into the yield prediction code. Another limitation was that the failure criteria was based on the 3D printed bone that was scaled up 10x. This failure value was then scaled back down to be applied to the actual bone biopsy sample.

8.7 FUTURE DIRECTIONS

The Sham vs OVX study was part of a larger lactoferrin study (see Chapter 1 for details regarding lactoferrin). While assessing the effects of lactoferrin on bone was outside the scope of this thesis, the next step will be to evaluate the effects of the protein, lactoferrin, in a murine model. Our modelling pipeline will be applied to this study in a similar way to the studies included as part of this thesis. Much like we did with the Vitamin D deficiency study (see Appendix A), the modelling pipeline will also be applied to a subset of bones from an external adiponectin study. Briefly, adiponectin is an adipocyte derived hormone which plays a role in regulating energy homeostasis, glucose and lipid metabolism and inflammatory pathways (Williams et al., 2009).
Like lactoferrin, receptors for adiponectin, AdipoR1 and AdipoR2, have been identified on osteoblasts and osteoclasts (Berner et al., 2004) which have led to studies that have demonstrated the direct effects of adiponectin on bone cells (Williams et al., 2009).

BMD is the most common diagnostic tool of bone health and is an indirect indicator of osteoporosis and an individual’s susceptibility to fracture. It is however, a flawed measure simply due to how it is calculated. BMD is the average pixel intensity in an area of the scan identified by the scanner software (Heaney, 2005). It therefore does not capture the true volumetric density of bone as it only captures the length and width and not the depth. Even though BMD is not the best measure of bone density, it remains the “gold standard” in the clinic (Lewiecki, 2005; Miller, Hochberg, Wehren, Ross, & Wasnich, 2005). In research, as mentioned in Chapter 2, BMD is used to assess trabecular bone while TMD is used to assess cortical bone. BMD is a grouped measure which includes bone and all surrounding soft tissue while TMD on the other hand only measures bone. It is now possible to apply TMD measurements to trabecular bone. This would yield more accurate bone density measures compared to using BMD. It would be advantageous in the future to use TMD to analyse both trabecular and cortical bone. However, this would require special training in micro-CT analysis and the bones would have to be scanned at a much higher resolution and therefore a considerably smaller volume, scanned.

The overarching goal of this work will be to eventually incorporate computational modelling in the clinic and in clinical trials. While computational modelling is not yet at a level of sophistication where it can be implemented in the clinic, we have shown throughout this work that simulations that were initially run overnight were able to be cut down to fifteen minutes. There are many advantages of applying computational models to clinical trials. For example, before the large scale fluoride trial a smaller pilot study was carried out. This pilot study showed treatments with low-dose fluoride caused an improvement in bone strength and fluoride treatment had an anabolic influence on bone. This, however, was not the case when the large scale study was conducted. It is possible that if computational modelling was included as part of the pilot study, the decision to proceed to a clinical trial may have been different. In the Sham/OVX study, the clinical data (BMD and some micro-CT indices) showed a significant difference between the two groups but the modelling predictions of strength due to bone architecture showed no differences between the groups. This is supported in (Christen, Webster, & Müller, 2010) which mentions that skeletal stability is not only determined by the mineralisation of the bone (what is assessed in clinical tests) but also by the cortical and trabecular microarchitecture (which can be assessed by models). This may have been a possibility with the
low-dose fluoride pilot study which may have led to a reconsideration of progressing with the larger scale clinical study.

The fluoride study was the only one where three different loading modes: compression, shear and torsion were tested. The three modes were used as it was a clinical trial based study and the bone was obtained from the hip which experiences a range of loading modes during daily activities. This study showed that loading the bone in compression had the strongest effect on the mechanical force and was the dominant mode, followed by shear and then torsion. Based on this, the bones from the other two studies were only loaded in compression. For the future, in order to get a more complete understanding of the effects of different loading modes on bone and the effects of different anabolic treatments on bone strength, we suggest applying the other loading modes to the model, which is key to informing multiscale modelling strategies.

Multiscale modelling refers to a type of modelling in which multiple models at different scales and complexities are integrated to describe one system. Micro-FE models were used in this thesis in which the architecture was described in detail and the material properties were considered uniform. On the other hand, macro scale (organ-scale) FE models are not able to resolve the bone’s entire microstructure but are able to describe the complete continuum geometry of the bone in question (Christen et al., 2010). In light of this, we propose that the next step in this work would be to use the homogenised material properties at the micro scale to define the material properties at the macro scale. The advantage of this process is that actual computed material values from the micro level will be applied to the macro level in order to determine the toughness, stiffness and predict bone fracture at the whole bone level. Multiscale bone models can be applied to the understanding of anabolic treatments, bone diseases and effects of aging.

One disease in particular that highlights a good use of multiscale models is Paget’s disease, a chronic disorder caused by excessive bone remodelling due to abnormal osteoclast (bone resorption) and osteoblast (bone formation) activity. This consequentially results in the heterogeneous bone matrix becoming very regular (Chavassieux, Seeman, & Delmas, 2007; Roodman, 1996). Histologically, trabecular bone was shown to have a more organised but poorly mineralised matrix with no changes to the material properties of the bone. There were also changes in structural parameters such as the TbN, TbTh, spacing and connectivity when compared to control samples (Seitz et al., 2009). However, due to the more organised matrix structure, bones affected by Paget’s disease are susceptible to shear forces.

The progression of bone due to aging can also be studied using a multiscale model. It has been suggested that around 80% of bone turnover due to aging occurs in the trabecular bone even
though trabecular bone represents only 20% of the total bone mass (Shahnazari et al., 2012). The aging process is not as dramatic in cortical bone but does cause an increase in bone loss from the endocortical surface (Russo et al., 2006). However, it is well documented that cortical bone dictates the behavior of bone as a whole and is predominantly load bearing. As mentioned previously, BMD is a surrogate measure for the mechanical competence of bone and also is a measure of an individual’s fracture risk (Augat & Schorlemmer, 2006). A significant decline in trabecular bone in turn causes a decline in the BMD may not necessarily be indicative of an individual being at risk for a fracture. In these cases, cortical bone is still able to compensate for the loss of trabecular bone. This is a case where a multiscale bone model would be advantageous as it would be able to give a more accurate prediction of when the bone is likely to fail compared to a standard clinical measure. A multiscale model would also be able to integrate the material properties of both the trabecular and cortical bones to predict the point of failure more accurately than a measure of BMD.

8.8 CONCLUDING REMARKS

A computational pipeline (validated on a 3D printed Instron test) for assessing bone failure strength in anabolic treatments was developed in this thesis. The pipeline was evaluated as part of a large scale two year human clinical trial on fluoride therapy, in varied whey protein levels in murine models, two commonly used control murine models (OVX and Sham) and contributed to an international study looking at the effects of Vitamin D in murine models. In all cases we were able to predict the trends predicted by clinical indices using a double blinded design, whether they be non-significant trends, or significant differences (such as with Vitamin D). The computational pipeline reported failure loads based on the Instron derived yield criteria and isolated the effects of bone architecture from material properties for both trabecular and cortical bone. Material properties were estimated from BMD and TMD which were density values based on micro-CT phantoms and mechanical tests in the whey protein diet study (three point bending, microindentation and nanoindentation). This modelling pipeline is now planned for post-doctoral work as part of a lactoferrin anabolic and adipopenectin murine study. The key outcome of this thesis was the refinement of a pipeline, which is efficient, integrates with clinical work and has been tested in real clinical scenarios (using a double blinded design) to provide confidence to the bone community on the use of computational models.
As mentioned in Chapter 1, our modelling pipeline was applied to a Vitamin D study with colleagues at The University of Southampton, UK. This study provided an external test of the modelling pipeline developed in this thesis. It has been well established that doses of Vitamin D, surpassing physiological levels, stimulate bone resorption (Suda, Ueno, Fujii, & Shinki, 2003). It is also well known that Vitamin D deficiency leads to impaired bone mineralisation, leading to rickets in children and osteomalacia in adults (DeLuca, 1988). In brief, this study investigated a murine model of in utero Vitamin D deficiency. We were consulted on estimating failure load in young 21 day old bone (as a surrogate for strength) as it was felt the bones were too “soft” to conduct the range of mechanical tests normally employed.

Femora from 21 day old male rats were analysed (n = 5 for control and n = 5 for Vitamin D depleted) for osteogenic gene expression, microarchitecture and BMD by micro-CT scanning. The micro-CT scans were also used to generate FE models to computationally predict bone strength (the point in this study where our pipeline was applied).

The bone samples were virtually loaded under compression in Abaqus (see Figure A.1 for stress distribution pattern). Using a student’s t-test, we have shown that there is a significant difference (p < 0.05) in the failure loads between the control and Vitamin D depleted groups. The model predicted that the Vitamin D bones would fail at lower loads. The mean and standard deviation of the failure force are shown in Figure A.2. Following model predictions, one set of experiments were conducted to verify our findings (a three point bending test) that confirmed the Vitamin D deficient bones failed at lower loads in comparison to the control group. This study shows how murine in utero Vitamin D deficiency causes a reduction of bone health at 21 days of age.

These types of studies demonstrate the benefits and importance of computational modelling as in this case, the murine bones were too “soft” for mechanical indentation tests. It also highlights where computational models can give insight and a pre-experiment estimate to inform if mechanical tests should proceed.
This work has been included with permission from T.Li and R.O.C. Oreffo, Bone and Joint Research Group, University of Southampton, UK.
As mentioned at the end of Chapter 6, micro-CT indices for cortical bone were originally assessed in 3D. The indices have now been recalculated in 2D (based on suggestions from the reviewers). The original 3D indices are included below to show that the trends do not change when the equivalent 2D indices were presented. The equivalent 3D and 2D indices are as follows:

- Periosteal volume = Mean total cross sectional tissue area
- Endosteal volume = Medullary area
- Bone volume = Total cross sectional bone area

Figure B.1. Top row and bottom left: comparing micro-CT indices across whey protein treatment groups for cortical bone. Placebo (blue), 0.1% (red) and 1% (green). Top row: no differences in the periosteal volume or bone volume across the three groups. Bottom left: endosteal volume showed a strongly significant difference between placebo and 1% ($p < 0.01$) and between 0.1% and 1% ($p = 0.001$).
References


