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UNIVERSITY OF SOUTHAMPTON

# Understanding Skeletal Development Across the Life Course

PhD Thesis

FINAL VERSION: CORRECTED

*Student:*

Tsiloon LI

PhD Candidate: Faculty of Medicine and  
Faculty of Engineering and the Environment

*Supervisor(s):*

Prof. Philipp J. TURNER  
Faculty of Engineering and the Environment  
Bioengineering Research Group

Dr. Stuart A. LANHAM  
Faculty of Medicine  
Bone and Joint Research Group

Prof. Richard O.C. OREFFO  
Faculty of Medicine  
Bone and Joint Research Group

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ABSTRACT

FACULTY OF ENGINEERING AND THE ENVIRONMENT AND FACULTY OF  
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Doctor of Philosophy

UNDERSTANDING SKELETAL DEVELOPMENT ACROSS THE LIFE COURSE

by Tsiloon Li

Health can be influenced by a number of factors and there is evidence to suggest that environmental cues during early-life stages greatly affect disease susceptibility in adulthood. Incidences of bone disease are becoming increasingly prevalent and it is believed that bone health in later life may be determined during foetal and neonatal stages. Currently, surrogate measures of bone strength (bone mineral density and content) are used to assess the risk to fracture, but are acknowledged to predict only a proportion of clinical cases. Therefore, it is important to understand and develop supplementary fracture risk to augment traditional tools.

There has been difficulty in characterising the mechanical strength and toughness of bone due to the complexity of the hierarchical structure and compositional material properties. The bone quality framework describes the material and structural contributions to bone mechanical performance and hence utilisation of parameters associated with these contributors, alongside conventional bone mass measurements through densitometry, may improve the accuracy of fracture risk assessment. A myriad of factors have been suggested to affect bone health and therefore the current challenge is to identify the most influential. At present, there lacks a model that fully describes how material and structural factors act together throughout the bone hierarchy to affect the mechanical properties and fracture toughness of a whole bone, as well as how environmental factors may adapt these features.

Within this project the relationship between biological alterations in bone formation and how this adapts material and microscale architecture are explored, with a view to assess the effect on whole bone mechanics. An initial pilot study on the effect of maternal low dietary protein during pregnancy on second generation female rat bone health was conducted to establish methodological protocol. Specifically, this project investigated the effect of maternal vitamin D, a known contributory factor in bone health, on offspring skeletal development and health. It was hypothesised that cellular activity can influence organ-level bone properties through control of the bone

matrix and that subtle environmental assaults, such as low maternal protein or vitamin D, can alter this highly regulated process.

Results from measuring bone i) gene expression, ii) micromechanics, iii) composition, iv) architecture, v) fracture toughness and vi) whole-bone mechanics in murine models have shown increased expression levels to be significantly correlated to an increase in microindentation distances at multiple locations along the femur and a reduction in cortical bone thickness and mechanical competence at the femur diaphysis. In particular, *Runx2* expression was indicative of bone structure and mechanics, emphasising the importance of exploring the link between biological and mechanical bone environments further to understanding skeletal development and health.

Investigation into the effect of maternal low protein status during pregnancy on female second generation offspring bone health at 70 days of age demonstrated no significant differences between low protein background and control rats. Although a trend of lower mean osteogenic gene expression levels, lower mean fracture toughness, lower mean maximum load in whole bone mechanical testing and increased micromechanical indentation distances were observed in low protein animals, no significance was reached suggesting no persistent change is present from grand-maternal dietary protein status in second generation offspring.

The effect of vitamin D deficiency during *in utero* life on offspring bone development was subsequently assessed using this multi-disciplinary experiment strategy in rats. Although the importance of vitamin D in childhood and adulthood bone health is established, the role of vitamin D *in utero* towards post-birth bone health remains contentious. Vitamin D deficient offspring at 21 days of age (childhood) were observed to have reduced diaphyseal cross-section area and reduced mechanical capability in males. No further differences were found in gene expression, composition or material properties and no differences were identified in females. At 140 days of age (adulthood), negligible differences were found between control and vitamin D depleted animals in any bone health outcomes. These results indicate vitamin D depletion during *in utero* life has limited impact on skeletal health of rats at 140 days old. Critically, the detrimental effects of bone caused by vitamin D depletion at 21 days of age in male rats appears to have been recovered in adulthood after resuming a vitamin D sufficient diet after birth. Therefore, these results suggest vitamin D sufficiency during childhood is essential for skeletal development.

In summary, these results highlight the importance of the relationship between bone biological mechanisms and bone structure/mechanics across different length scales. Appreciation of this link enables comprehension of how skeletal development is established and the consequent effect of any challenges caused by disease. Furthermore, uncovering the aetiology of bone disease will enable the development of improved prophylactic measures, diagnosis and therapeutic strategies.

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## Declaration of Authorship

I, Tsiloon Li, declare that the thesis entitled *Understanding Skeletal Development Across the Life Course* and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed:.....

Date:.....



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For my Auntie Helen.



*“Coming back to where you started is not the same as never leaving.”*

*“If you trust yourself... and believe in your dreams... and follow your star... you'll still get beaten by people who spent their time working hard and learning things and weren't so lazy.”* - Terry Pratchett



# Nomenclature

$\mu$ CT	Micro-computer tomography
$\nu$	Poisson's ratio
Alp	Alkaline phosphatase
<i>BMC</i>	Bone mineral content
<i>BMD</i>	Bone mineral density
BMU	Basic multicellular units
BSP	Bone sialoprotein
BV	Bone volume
cDNA	Complementary deoxyribonucleic acid
<i>CID</i>	Creep indentation distance
<i>Co. Th</i>	Average cortical thickness of femur midshaft
Col1	Collagen type 1
DEXA	Dual-energy x-ray absorptiometry
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
<i>E</i>	Young's modulus
<i>ED</i>	Energy dissipation
FEA	Finite element analysis
FGF	Fibroblast growth factor
HATs	Histone acetyl transferases
HDACs	Histone deacetylases
<i>IDI</i>	Indentation distance increase
IGF-1	Insulin-like growth factor 1
$k_{init}$	Crack initiation fracture toughness
mRNA	Messenger ribonucleic acid

NCPs	Non-collagenous proteins
Ocn	Osteocalcin
Opn	Osteopontin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
$r$	Pearson correlation coefficient
$r^2$	Coefficient of determination
$r_o$	Average outer radius of femur midshaft
r18s	Ribosomal 18s
RNA	Ribonucleic acid
ROI	Region of interest
RPI	Reference point indentation
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Runx2	Runt-related transcription factor 2
SSCs	Skeletal stem cells
SEM	Scanning electron microscopy
STL	Stereolithography format
$Tb.N$	Trabecular number
$Tb.Sp$	Trabecular separation
$Tb.Th$	Trabecular thickness
TGF- $\beta$	Transforming growth factor beta
$TID$	Total indentation distance
$TMD$	Tissue mineral density
$TV$	Tissue volume
VDR	Vitamin D receptor

# Chapter 1

## Introduction



## 1.1 Introduction

Human health and development can be influenced by exposure to a host of environmental factors throughout the course of a lifetime within the area of bone. The incidence of bone diseases is becoming an increasingly prevalent problem within the global population and evidence is accumulating to suggest that skeleton development and maintenance during the course of life is affected by pre-birth (*in utero*) and early postnatal experiences [1–3].

Though the skeletal system constitutes a significant part of the human body, the precise process and mechanism of bone adaptation and maintenance to fulfil a wide range of roles remains to be fully characterised. While in broad terms the structural significance towards mechanical demands and cellular activity to generate form and function has been uncovered [4, 5], current ideas on bone material capabilities and osteogenesis do not fully incorporate the subtle details and influences that allow for exact understanding on bone formation and functionality. This similarly affects the understanding of bone disease development and subsequent diagnosis and treatment.

Interest in the role of maternal nutrition during pregnancy on adulthood disease status has prompted a number of studies [6, 7] and evidence is emerging that skeletal growth can be programmed due to adverse maternal nutrition during foetal and early life [3, 8]. Attempts to ameliorate this through hormonal rebalance and addition of osteogenic growth factors have been found to be insufficient in reversing the effects [9], highlighting the importance of considering the role of maternal nutrition during pregnancy on offspring development, skeletal health and risk of bone diseases in adult life. Equally, intrinsic bone toughness has attracted investigation due to the difficulty in quantitatively measuring the number of mechanisms present across the length scales and how these factors subsequently interplay to result in global bone toughness [10].

The osteogenic environment is reliant on activity at the molecular and cellular level and, as such, changes will modulate bone development. The precise mechanism is not completely understood, however it is believed that changes in gene expression, hormone and protein levels modulate bone structure [11–15]. The aim of this project will be

to explore the roles of bone matrix components towards material and structural bone quality parameters and formulate cause and effect relationships between genomic expression, protein deposition, micromechanics and organ level mechanics. It is believed that understanding the biological foundation of bone matrix mechanics and the mechanical interaction between different hierarchical levels will provide new insight into characterising bone function and disease status.

Applying this clinically, it is hypothesised that mothers with diets deficient in particular nutrients at critical stages of pregnancy cause changes in the course of the infant's skeletal development, such that mechanical properties are reduced due to architectural and compositional adaptations that persist through to later life. With regards to this thesis, the effect of vitamin D, a known requirement for general bone health [16], is of interest and it remains to be elucidated whether low vitamin D status during pregnancy results in programming of offspring skeletal development. Use of the techniques developed from the investigation into the bone matrix profile will inform on the importance of vitamin D on offspring bone health.

To quantify the impact of this, a host of experimental methods are required to gain an understanding of the biological, mechanical and material properties of bone and any changes that may occur subsequently. These include cellular and molecular biological protocols to measure patterns of gene expression and use of mechanical testing and high resolution imaging to determine morphological and functional adaptations. An appreciation of bone structure, material properties, bone development and modulation will first be necessary to realise the impact of changes that are evident.

This thesis details the current background on bone quality factors and mechanical behaviour, biological activity in bone formation and maintenance and the mechanisms behind programming of health that form the rationale behind the project aims. Methodology to define each of these aspects of bone is described before results from testing the project objectives are presented.

## Chapter 2

# Literature Review



## 2.1 Bone and the Skeleton

The skeletal system is a major component of the human body and comprises of around 20% of the total mass. Due to the clear medical importance, the structure and function of various elements that compose the skeleton have been well detailed [4, 17, 18]. Primarily composed of bone, cartilage and ligamentous tissue, the skeleton performs an important role in supporting and protecting vital organs and other soft tissue, facilitating movement and interaction with the surrounding environment, providing a source for calcium, and acting as a store for bone marrow. Being the most predominant tissue within the skeleton, this review will focus largely on bone.

### 2.1.1 Structure and Function of Bone: the Macroscopic Level

Following is an overview of the basic structure and function of bone that has long been established and can be found in summary references that are readily available, such as citations [17] and [18]. The larger scale features of bone are illustrated in figure 2.1.

The form of bones within the body varies greatly depending on the specific physical demands at each location and classification is commonly achieved through physical characteristics; long, short, flat, irregular and sesamoid (first column, figure 2.1). As mentioned previously, bone also acts as a reservoir for minerals (such as calcium) [19], growth factors [20, 21] and lipids, in addition to providing an environment for haematopoiesis [22]. This project however will be centred around the physical requirements of bone, so that the mechanical competencies (and how this is established biologically) will be emphasised over the endocrinological roles of bone.

The archetypal bone structure observable by eye is typically an outer region of cortical bone surrounding an internal cavity of trabecular bone and marrow. The most distinguished difference between cortical and trabecular bone is in morphology, where the former is dense and compact in contrast to the open, porous and sponge-like nature of the latter. On a more subtle level, cortical bone tends to be more mature in terms of mineralisation as generally trabecular bone experiences a higher level of metabolic activity and remodelling [23]. These differences result in distinct mechanical characteristics

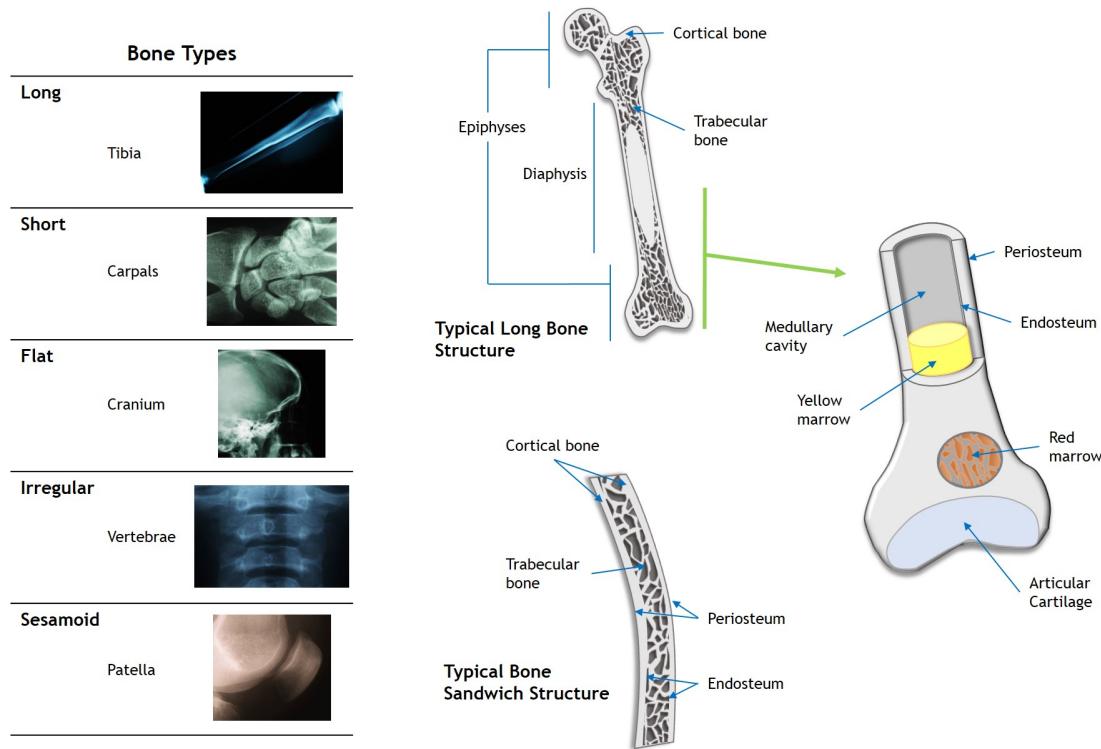


Figure 2.1: Large Scale Bone Structure. The first column within this figure displays x-ray images of the five types of bone found within the human body. The second column shows the common structure of long bones and the internal sandwich structure of the other bone types. Lastly, in the third column is a more detailed view of long bone anatomy.

from the two respective morphologies (section 2.2.1).

When viewed in more detail, the external surfaces of many bones are covered by periosteum, a connective membrane that is served by a number of blood and lymphatic vessels, and nerve fibres. The periosteum also provides areas of attachment (insertion points) for ligaments and tendons so that one bone can be joined to another or to muscle. At the interface between the periosteum and external bone surface is the osteogenic layer, containing bone forming cells (osteoblasts), bone resorbing cells (osteoclasts) and skeletal stem cells. Additionally, the internal surfaces have a finer connective membrane layer covering the trabecular bone, termed the endosteum.

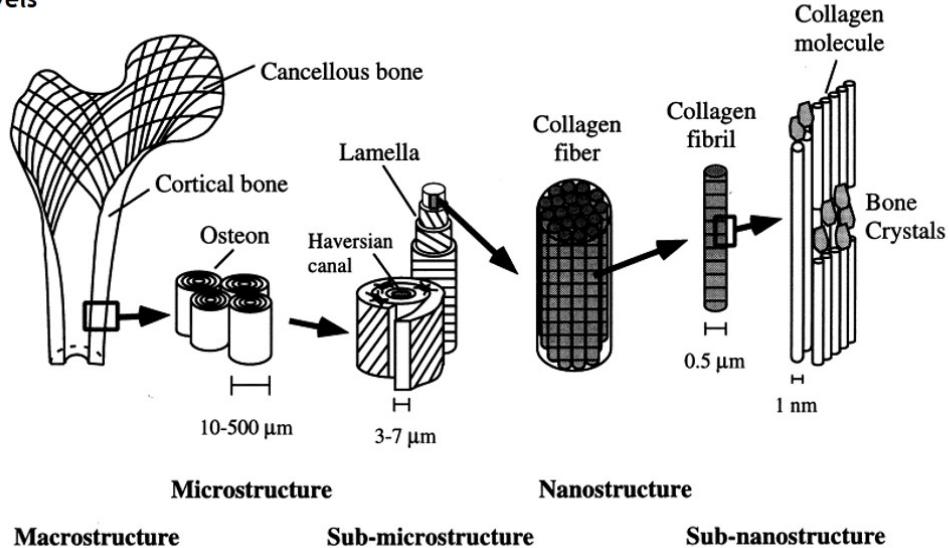
The different classes of bone mentioned earlier all vary in structural composition; short, flat and irregular bones have a relatively simple sandwich construction (second column,

figure 2.1) of a trabecular core surrounded by cortical bone, with endosteum and periosteum covers respectively. Bones of this nature typically assume a protective role and this form improves the impact resistance.

In contrast long bones display a more complex form (second and third columns, figure 2.1). The diaphysis is an annular of cortical bone that composes the middle portion of long bones, with the inner space, the medullary cavity, filled with yellow marrow and acts as a lipid store. At either end of this are the epiphyses, volumes of trabecular bone covered by an outer cortical bone layer, which also houses red marrow and is the site of blood cell production. In many cases the outer surfaces of the epiphyses are also covered by an articular cartilage layer that assists in movement by providing low friction surfaces and also act as shock absorbers. A significant proportion of the appendicular skeleton is made up of long bones and as such, this class of bone is highly important to environmental interaction and quality of life.

### **2.1.2 Structural Hierarchy of Bone**

Bone itself as a material is highly complex and consists of hierarchical levels whereby the structure differs along the length scales. Significant amounts of research have been conducted into the format of bone at each of these scales to determine how the hierarchy contributes towards the final behaviour of bone at the observable organ level. This research has uncovered a multitude of properties and reviews on this subject from Weiner and Wagner [4] and Rho *et al* [23], both in 1998, have proved influential in defining the various organisational levels found within bone as presented in figure 2.2.

**Rho et al Bone Hierarchy Levels**


<b>Level 7: Whole Bone</b>	<b>Level 5: Cylindrical Motifs: Osteons</b>	<b>Level 3: Fibril Array</b>	<b>Level 1: Major Components</b>
<b>Level 6: Spongy vs Compact Bone</b>	<b>Level 4: Fibril Array Patterns</b>	<b>Level 2: Mineralised Collagen Fibril</b>	

**Weiner and Wagner Bone Hierarchy Levels**

Figure 2.2: Hierarchical Structure of Bone. The hierarchical structure of bone as described by Weiner and Wagner [4] and Rho *et al* [23]. Along the length scales there are different structural features that enables bone to fulfil a wide range of mechanical demands. This hierarchical structure is also the basis of the heterogeneity and anisotropy of bone material and mechanics. (Please note that cortical bone is often referred to as compact bone, and trabecular bone is often referred also as spongy and cancellous bone depending on the author.) Top diagram reprinted from Medical Engineering and Physics, Vol 20, Issue 2; J.-Y. Rho, L. Kuhn-Spearing and P. Zioupos, Mechanical properties and the hierarchical structure of bone, Pages 92-102, Copyright 1998, with permission from Elsevier.

Both of these reviews (and subsequent follow-up work, such as citation [24]) consider the origin of bone material to be collagen and mineral phases, such that, ascending through to larger scales, the hierarchy levels are:

1. Collagen, carbonated hydroxypapatite mineral, non-collagenous proteins (NCPs) and water,
2. Mineralised collagen fibrils,
3. Collagen fibres formed from arrays and bundles of mineralised collagen fibrils,
4. Lamellae, woven bone, dentin etc. that are formed from groups of collagen fibres,
5. Osteons and individual trabecula,
6. Cortical and trabecular bone,
7. Whole bone organs.

### **The Mineralised Collagen Fibril: Collagen and Hydroxapatite**

The mineralised collagen fibril is commonly identified as the building block of bone and is essentially a two-phase composite of collagen protein reinforced with hydroxyapatite (HA) crystals. Collagen is attributed as the most abundant protein in the human body and is biochemically complex with a number of different variants [25]. Collagen type I (COL1) is the most prevalent type found in bone material and the molecule is a triple helical structure of two  $\alpha 1(I)$  chains and one  $\alpha 2(I)$  chain (different types of collagen can often be distinguished at the molecular level by different formative chains). These collagen I molecules are measured at around 300 nm and 1.5 nm in length and thickness respectively and self-assemble to form a collagen fibril. Due to the nature of the molecular binding, collagen fibrils present a characteristic banding of 67 nm lengthways as a result of the gap (35 nm) and overlap (32 nm) zones [26] that are present. A representation of collagen fibril form and formation can be seen in figure 2.3.

With respect to bone formation, collagen forms the framework of bone matrix by provision of binding sites for mineral crystals. There are two mineralisation stages; primary

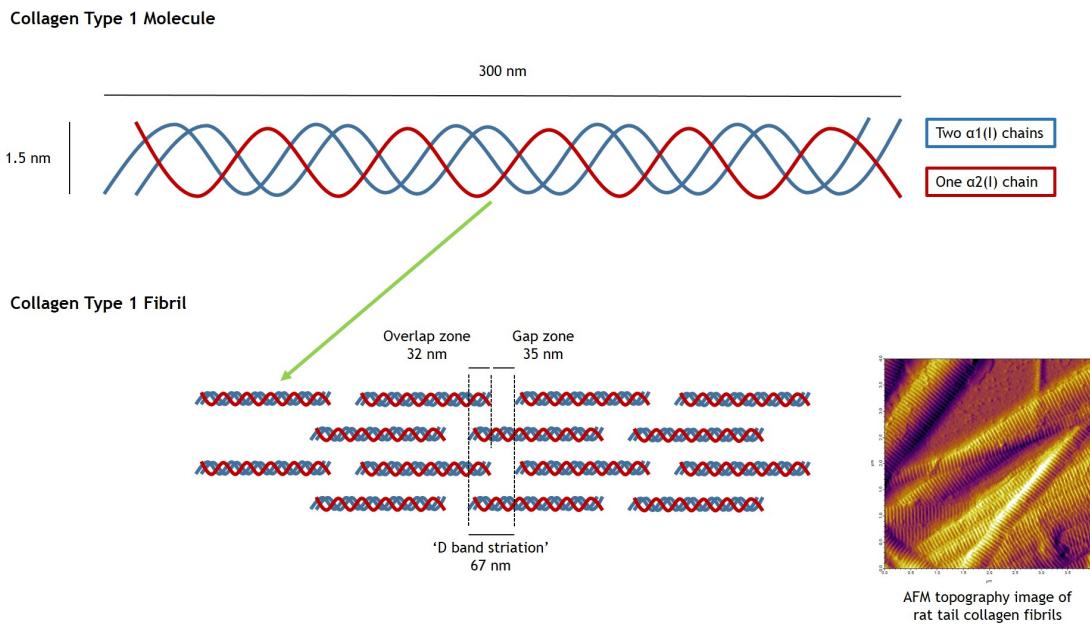


Figure 2.3: Collagen Fibril Formation. This figure provides representations of a collagen molecule with triple helical structure and then how these molecules are grouped together to form a collagen fibril. An atomic force microscopy image of collagen from rat tail displays the striated banding pattern characteristic of collagen.

mineralisation where the crystals are laid down and secondary mineralisation where the crystals begin to grow [27]. Specifically, it has been demonstrated that amorphous calcium phosphate (negative net charge) is attracted to the gap regions of collagen (positive net charge), whereupon the mineral nucleates through the fibril [28]. Crystal growth is directed along the long axis of collagen fibrils whilst additional mineralisation also occurs between fibrils (extrafibrillar mineralisation), with crystals found to strongly attach and form rings around each fibril [29–31].

From here, these mineralised fibrils are used to form lamellae sheets and the basis for higher order structures. Also at this level, organic components such as blood vessels, nerves and cells can also be observed. Importantly, the interface between biological events and origination of bone structure/mechanics is most evident at this stage and key to understanding the influence and consequences of bone cellular activity on observable physical properties.

### Fibril Arrays, Lamellae and Osteons

Mineralised collagen fibrils are organised and aligned into fibril arrays and collagen fibres through chemical bonds (crosslinking), proteoglycans and glycoproteins [4, 24]. The role of non-collagenous proteins (NCPs) has been intensively researched due to suspected roles in promoting mineralisation and acting as adhesive agents between different fibrils, hence aiding matrix construction. For example, bone sialoprotein (BSP) have been observed to bind specifically to collagen due to the fibril triple helix structure and initiate nucleation of bone mineral [32]. In relation to bone, other recognised NCPs include; dentin phosphophoryn (DPP), osteopontin (OPN), matrix gla-protein (MGP) and osteocalcin (OCN) [30]. A worthwhile point to mention now is that many NCPs appear to be involved in both mineralisation and bone matrix formation, such that it has proved difficult to describe exactly the roles and mechanisms relating to bone health individual NCPs perform. Nevertheless, it is well established that mineralised collagen and NCPs are necessary for bone formation.

In ordered bone, these arrays form sheets of unidirectional collagen fibrils termed lamellae. In many cases, lamellae (measured at 3-7  $\mu\text{m}$  in thickness) are serially placed alongside each other in parallel: the constituent fibrils making up each lamellae have been found to range from all aligning in one overall direction to fanning round in sequential lamellae, resulting in a plywood-like structure [23, 33] (this is shown in sub-microstructure illustration in figure 2.2). In contrast to the highly ordered form of lamellae, woven bone features bundles of randomly orientated collagen fibrils and fibres. This type of bone is characteristically found in situations where rapidly deposited material is required, such as during development or the beginning of fracture healing, before more ordered bone can be formed [24]. Images of ordered and woven bone can be viewed in figure 2.4.

Moving up through the bone hierarchy, lamellae are utilised to construct both trabecular and cortical bone. Within trabecular struts, lamellar sheets in localised packets are each orientated in slightly different directions due to the faster rate of deposition (in comparison to cortical bone) of new lamellar bone at the surface of each trabecula [18, 24]. Consequently, trabecular bone is comprised of interconnecting struts of less

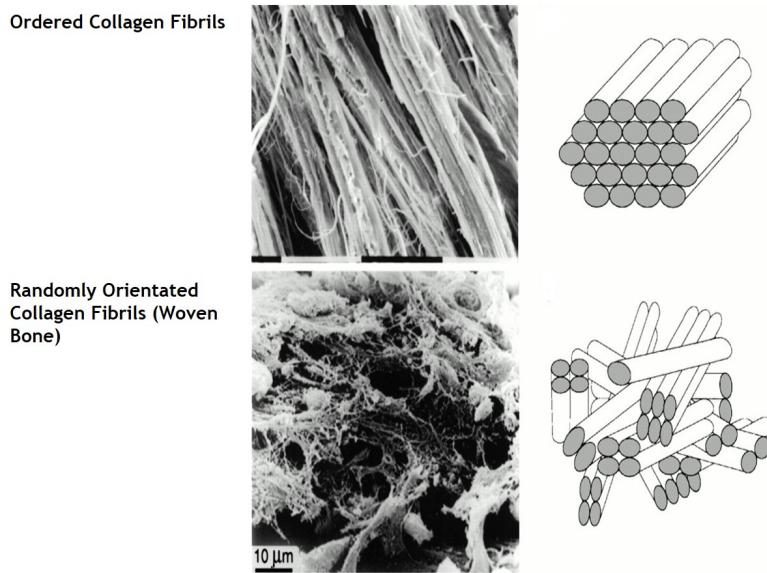


Figure 2.4: Collagen in Ordered and Woven Bone. SEM images and diagrams of both ordered collagen arrays (two upper images) and disorganised collagen found in woven bone (lower two images). Image adapted from Annual Review of Material Science, 1998, S. Weiner and H.D. Wagner, The Material Bone: Structure-Mechanical Function Relations [4].

ordered lamellae bone that are arranged irregularly.

In contrast, cortical bone is made up of smaller pillar-shaped subunits called osteons (also referred to as Haversian systems in humans) that align along the longitudinal bone axis. The osteons are composed from concentric rings of lamellae, with a high preferential orientation towards the prevailing stress conditions [34]. The middle of each osteon contains a central canal (also Haversian canal), where osteon blood vessels and nerve fibres are located. Overall, individual osteons have been measured at roughly 400 mm in height and 200 mm in width in the human body. The spaces formed between multiple osteons are filled with layers of interstitial lamellae bone, with this whole entire construct grouped together by an envelope of circumferential lamellae bone [18] (figure 2.5 depicts the basic structure of cortical bone made from osteons).

As described previously, the various whole bone organ shapes present in the human body are composed from both cortical and trabecular form, completing the structural hierarchy. It is extremely evident that the structure of bone has an important role in

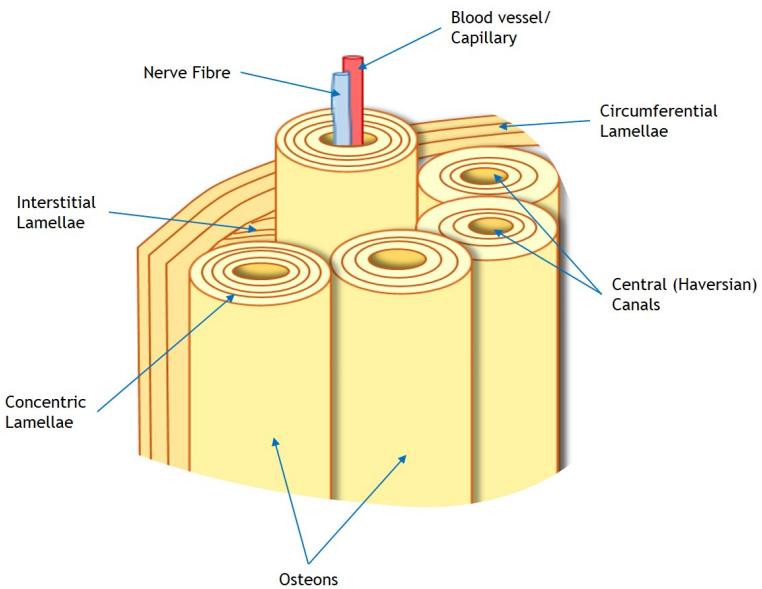


Figure 2.5: Osteons within Cortical Bone. The above illustration shows how cortical bone is composed of various lamellae forms: osteons are constructed from concentric sheets of lamellae with intervening spaces filled with interstitial lamellae. This whole form is enveloped by circumferential lamellae. At the middle of each osteon is a central canal where blood vessels and nerve fibres can serve cortical bone.

determining mechanical properties. Inherently, the geometry, bone mass and properties of the bone material all affect the functional capability of the whole bone [35].

## 2.2 Mechanics of Bone

Mechanical characteristics of structures and materials and can be described through a number of properties, most often by maximum strength, yield strength, stiffness and fracture toughness. Considering these properties can determine; the ultimate load a structure can experience before breaking (maximum strength), the load at which plastic deformation predominates over elastic deformation (yield strength), how much elastic deformation is experienced with increasing load (stiffness) and resistance to crack development and growth (fracture toughness). Aside from fracture toughness, all these parameters for a structure may vary according to size and shape. Consequently, it is important to distinguish between the intrinsic and extrinsic factors that influence bone mechanical performance so that causal pathways can be determined.

### 2.2.1 Material Properties of Bone

Adaptation of bone form is dependent upon genetic and environmental factors, meaning that there is wide variation in bone size and geometry in humans. Natural differences such as gender and age have long been known to change bone strength and size [36, 37]. With a number of other influencing characteristics personal to each individual (ethnicity, disease history, lifestyle etc) and the anisotropy of bone material, it is difficult to define mechanical properties for bone. Nevertheless, experimental data has been useful to provide expected bone mechanical behaviour and ranges of values for point of reference. Typical bone stress-strain relationship is displayed graphically in figure 2.6, where increased loading produces a linear region until a yield point is reached. This is followed by an extended plastic deformation region caused by multiple fracture mechanisms at different hierarchy levels [38] leading to eventual failure.

Regarding material values, Wirtz *et al* in 2000 [40] conducted a meta-analysis of 300 studies on human femora to inform material properties for use in finite element models found that cortical bone has a Young's modulus of approximately 7.5-17.5 GPa in the axial direction (aligned with osteons) and 4-7 GPa in the transverse direction, with variation due to mineral density. Trabecular bone was found to have a Young's modulus to

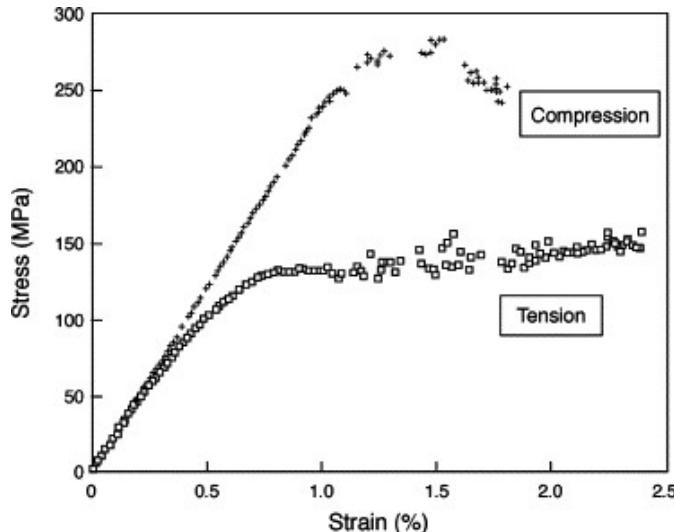


Figure 2.6: Stress-Strain Curves of Cortical Bone. Experimental stress-strain curves of human cortical bone under tensile and compressive stresses. Reprinted from *Acta Biomaterialia*, Vol 2, Issue 1; C. Mercer, M.Y. He, R. Wang and A.G. Evans, Mechanisms governing the inelastic deformation of cortical bone and application to trabecular bone, pages 59-68, Copyright 2006, with permission from Elsevier [39].

be approximately 0.05-1 GPa in the axial direction and 0.05-0.6 GPa in the transverse direction (again the ranges were found to be dependent on mineral density). Additionally, the tensile strength of cortical bone was found to be around 150 MPa, whereas for trabecular bone this was around 3-15 MPa.

Studies delving into the mechanical properties of bone are numerous and more recently obtained values generally agree with the Wirtz *et al* meta-study. For example, beam samples (180 specimens from 5 femur samples) machined from femur midshaft cortical bone from humans yielded an average Young's modulus value of 18.6 GPa (range between 14-22.8 GPa) from three-point bending tests [41].

Nanoindentation studies of individual trabecular spicules by Norman *et al* [42] produced moduli in the range of 12-20 GPa, with variation depending on the location within each spicule (the inner regions of the spicule was found to be higher in comparison to the outer regions). Nanoindentation of cortical bone osteons [43] showed an alternating pattern of 24 GPa and 27 GPa (average values) in the radial direction as a result of the lamellae structure, with interstitial bone regions much stiffer with a modulus of 30-32

GPa. In the same study, differences between interstitial bone and osteonal bone stiffness was found to correlate with calcium mineral content, with interstitial bone around 5-10% higher in calcium content.

These scale-dependent differences found in modulus values highlight the effect higher order structure has on bone. At the material level testable by nanoindentation, the highest stiffness values are measured, which are noticeably lower in the macroscopic experiments. This is most clearly seen in trabecular bone, where at the organ level the sponge-like morphology results in a low modulus even though the material itself is much stiffer. As described later, these compromises do lead to a higher fracture toughness and is further evidence of how bone balances a number of functional considerations.

Studies into the fracture toughness of bone have reported varying values due to differences between sample source, size and testing method. For example, values of 4.05 - 4.32 MPa $\sqrt{m}$  (from sample thicknesses of 3 mm and 2 mm respectively) from compact tension tests of machined human tibia samples [44]. It is generally accepted that the range of fracture toughness of bone is around 3-10 MPa $\sqrt{m}$  [45].

As a matter of interest, the position of cortical and trabecular bone, collagen and hydroxyapatite within a material property chart can be viewed in context with other familiar engineering materials in figure 2.7. Useful points to garner from the chart are the positions of hydroxyapatite (HA) and collagen and how cortical bone successfully merges desirable properties from these two components together, with strength greater or comparable to many woods, polymers and metal alloys.

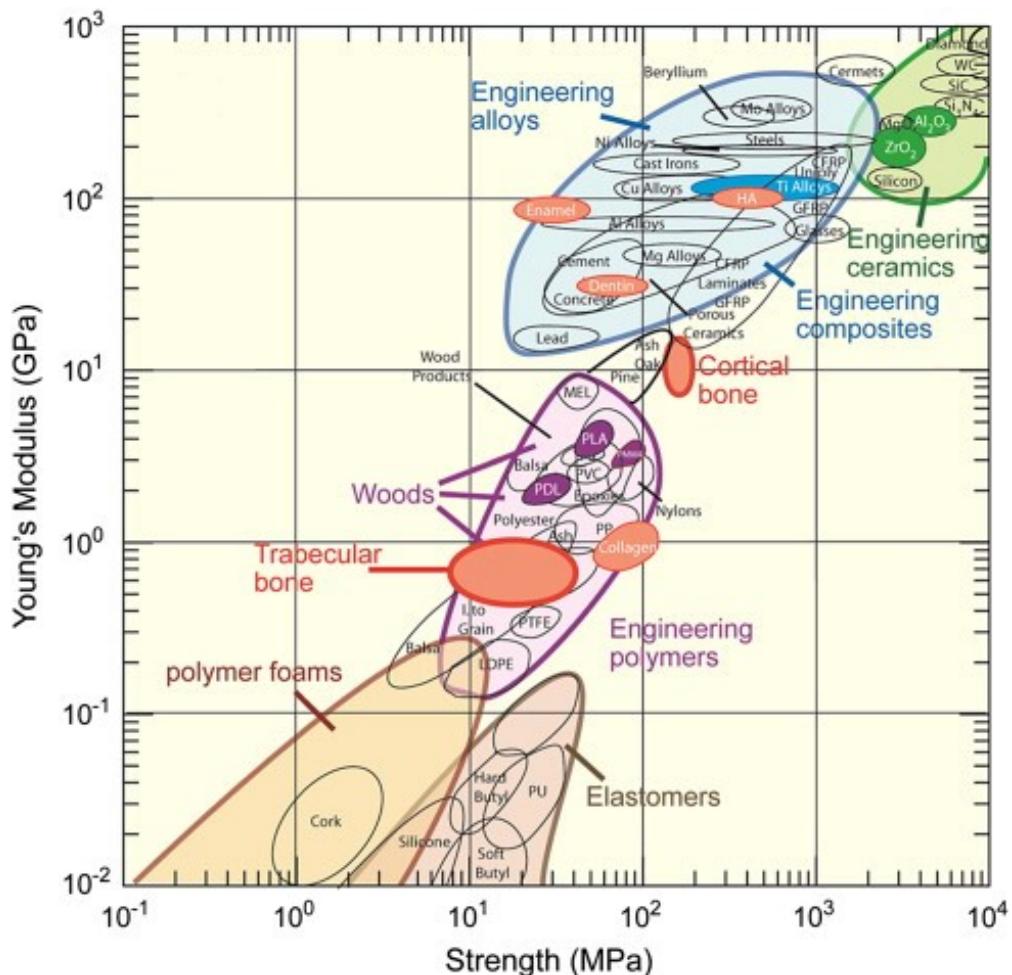


Figure 2.7: Bone in the Material Property Chart. The chart above plots materials depending on Young's modulus and Strength. The position of cortical bone shows it is stronger and stiffer than many woods and is comparable to some common engineering polymers, composites and alloys. Trabecular, in contrast, bone has properties similar to woods. Reprinted from Materials Science and Engineering: C, Vol 31, Issue 7; Q. Fu, E. Siaz, M.N. Rahaman and A.P. Tomsia, Bioactive glass scaffolds for bone tissue engineering: state of the art and future perspectives, Pages 1245 - 1256, Copyright 2011, with permission from Elsevier [46].

### 2.2.2 Bone Quality: Structure, Material and Strength

Evaluation of the form and function of bone has distinguished a highly evolved and intricate structure, where nature has coupled architecture and material closely to mechanical requirements. Bone quality is a concept that describes the multitude of factors that can affect mechanical performance and encapsulates a number of bone parameters and interrelations throughout the bone hierarchy [27, 47].

The bone quality term, however, remains undefined despite significant clinical interest. Proposals have suggested the use of bone quality to describe all factors that contribute to bone strength and fracture resistance (hence an umbrella term that incorporates mass, structural and material indices), although in general it is more common for bone quality to refer to all factors outside of bone mass (such as BMD measurements) that influence bone strength [48]. The latter definition is adopted within this thesis, that is: **bone quality describes the structural and material/compositional factors that affect bone strength and fracture resistance.**

As described, bone quality factors may be divided into two areas, structural and material, where structure describes the differences in bone form and morphology and material details the effects of the chemical form and mineralisation [27, 49, 50]. Figure 2.8 displays some key factors associated with structure and material and how these feed into bone quality and, along with bone mass, contribute towards strength.

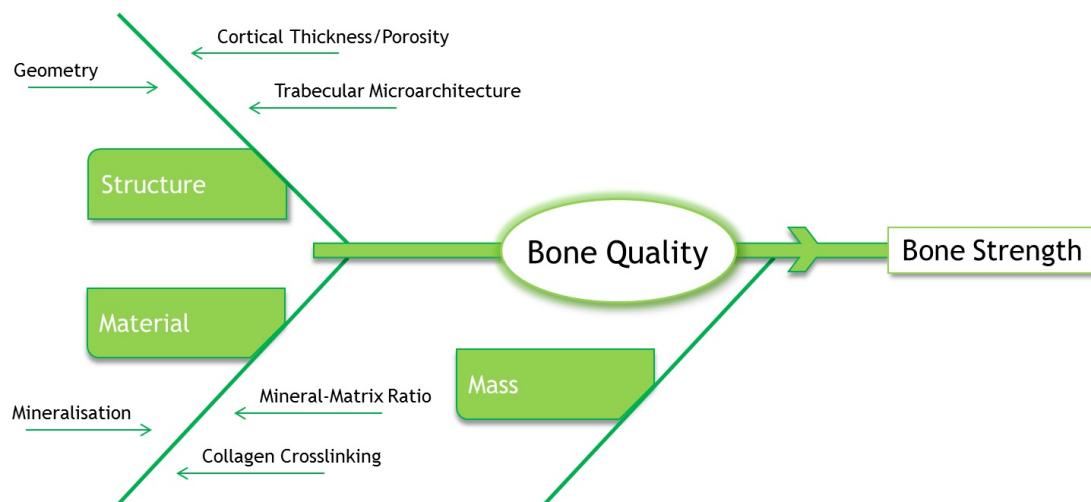


Figure 2.8: Bone Quality. Bone quality describes the multitude of structural and material factors, and any interactions between factors, that contribute to the strength of bone.

At the macroscopic level, and temporarily disregarding the anisotropic and heterogeneous material properties, many bones in the appendicular skeleton are broadly circular cross-sections and act as levers and load bearing structures. The arrangement of long bones promotes their ability within these roles, the tube-like construction of the diaphysis provides lightness whilst also shifting mass away from the neutral axis to provide

stiffness and greater resistance to bending moments [51]. Logically, a thicker cortical shell will provide a shaft with greater resistance to bending and this is often seen in cases of regular use, such as with exercise. The epiphyses of long bones are located at or near joint areas, such that the housed trabecular form is positioned in regions of increased compression to provide greater tolerance to localised areas of high pressure occurrence. Clearly, this macroscopic composite structure of bone (dense cortical and spongy cancellous phases) is advantageous as it allows bone to resist a variety of different forces.

On the level of mineralised collagen, this composite nature is able to exhibit both stiffness (resulting from the carbonated hydroxyapatite phase) and toughness (due to the collagen). Utilising the stiffness and toughness of these two constituents is hugely influential towards the mechanical strength of bone, and hence resistance to fracture [26]. It has long been demonstrated that the degree of mineralisation within the collagen network can affect the strength of bone overall, with increased mineral density increasing tensile modulus significantly beyond values of solely pure collagen fibrils for example [52]. The rings of extrafibrillar crystals around collagen fibrils and bundles have also been found to increase stiffness [31].

Study into this shows that, upon loading, the mineral crystals within mineralised collagen assume around four times the amount of stress experienced by collagen. Nair *et al* [52] explored the mechanism of this and found that with the presence of mineral crystals, collagen mechanical behaviour is modified. Pure collagen fibrils when in tension experience high degrees of deformation at the gap regions, whereas analysis of mineralised collagen fibrils show that HA locally increases the stiffness at gap regions, resulting in deformation mainly at overlap zones and hence an overall increase in fibril stiffness. Consequently, highly mineralised collagen have a stiffness four times greater than that of unmineralised collagen. Additionally, Nair *et al* postulate that salt bridges present between HA and collagen allow for effective load transfer and energy dissipation, likely by tolerable deformation of the organic phase.

This degree of bonding between the minerals and the organic regions may also change mechanical properties, with strong interfacial properties giving rise to better strength

and stiffness whilst weaker interfaces imparting a chain sliding effect resulting in better toughness. Evidently, differences in state at the tissue level of arrangement can impact upon the whole scale mechanical performance [4, 53, 54].

### 2.2.3 Fracture Mechanisms of Bone

Fracture toughness is a highly important material property that accounts for the inherent ability to resist initiation and development of cracks before failure. Experimentally derived strength values are beneficial in describing failure criteria of bone but fracture toughness values can accommodate the presence of pre-existing flaws and the mechanical behaviour in response. This is extremely pertinent in bone as small flaws within the material are likely to be present due to wear and natural remodelling activity. As fracture toughness is an inherent material property, values are independent of bone size and geometry and thus has been argued to be a more useful bone health metric than strength (particularly when considering the number of crack resistance methods derived from structure, described later in this section) [55].

The evolutionary compromise of stiffness and lightness found in bone has necessitated damage and crack presence from everyday stress. This is negated somewhat by self-repair capacity of bone by constant replacement of old material but it is reasonable to assume cracks can develop, hence emphasising fracture mechanics as a relevant bone property in maintaining overall integrity. Elucidating how crack propagation occurs and the mechanisms within bone that oppose this, it may be possible to reduce the risk of fracture [33].

Conceptually, applying stress to a structure increase the internal elastic energy within. In association with this, when surpassing a critical level, this may result in a crack within the material as a method to dissipate this energy. When this critical level is reached at the tip of the crack, new surfaces are able to form as a result and crack advancement occurs. Fracture toughness mechanisms therefore increase resistance to fracture by promoting tolerance of the energy at the crack tip, or to delocalise the increase in energy [45]. The fracture toughness value ( $K_c$ , units  $\text{Pa}\sqrt{\text{m}}$ ) describes the material resistance

to propagation of cracks.

The hierarchical structure of bone provides a multi-layer response to fracture resistance and essentially presents mechanisms on each level to oppose crack growth by dissipating energy that would otherwise contribute to the advancement of the crack. As such, the bone quality concept is closely linked to the hierarchical structure of bone [56]. As such, understanding the underpinning concepts of bone fracture toughness and how the hierarchical levels interrelate with each other is of great importance [45].

At the nano-scale level, as mentioned in section 2.2.2, the basic bone component of mineralised collagen fibrils increase in strength and stiffness with higher mineral density. The high strength that HA crystals are able to impart prevents these stress levels from causing failure of material around cracks, with high stiffness reducing the amount of elastic strain energy so crack propagation is reduced [33]. Due to remodelling activity (section 2.3.1), mineralisation of bone matrix is often locally varied and heterogeneous [26]. This heterogeneity has been demonstrated to promote energy dissipation by diffuse deformation over a larger area when compared to simulations of homogeneous materials [57], hence reducing crack growth energy.

In addition to the bone mineral phase, the organic collagen phase is also an important factor in influencing bone fracture toughness [26]. The collagen molecules absorb fracture energy through stretching and unwinding of the constituent hydrogen bonds that hold the molecule together. Scaling up, the amount of cross-linking between the collagen molecules and cohesion between the organic and inorganic components affect the total energy that can be absorbed through slip occurrences between bonded entities [27, 45].

Further to collagen and mineral crystals, non-collagenous proteins (NCPs) have been demonstrated to contribute to fracture behaviour of bone [58]. For example, osteopontin (OPN) has been shown to promote energy dissipation when stressed by providing sacrificial bonds and forming aggregate networks in pulling tests [59]. In relation to these findings, OPN deficiency has been related to decreased bone fracture toughness (independent of any changes in bone mass), reducing bone competence in opposing

crack propagation [60]. Additionally, osteocalcin (OCN) and OPN interactions at the nanoscale have been observed to diffuse strain energy when bone is loaded [61]. Clearly, the components of bone matrix contribute to the inherent fracture opposition present within bone material.

Within the fibrils and fibril arrays, it is believed that further sliding between collagen chains occur, with previously mentioned NCPs present to provide sacrificial bonds that absorb further crack growth energy (figure 2.9 illustrates the location of these elements). The sliding results in plastic deformation of the local bone material around a crack tip advance that slows and possibly halts crack propagation [45]. Within cortical bone, osteons appear to have an influential role within the fracture mechanisms of bone, with osteon structures opposing crack propagation paths. Indeed, the alternating presence of higher and lower regions with osteons seem to serve the purpose in halting crack growth [43]. Surviving bone lamellae and fibres are also suspected to have a crack bridging effect, reducing the stresses seen at crack tips, and assist in wound healing by keeping the two fracture components close together [27, 62].

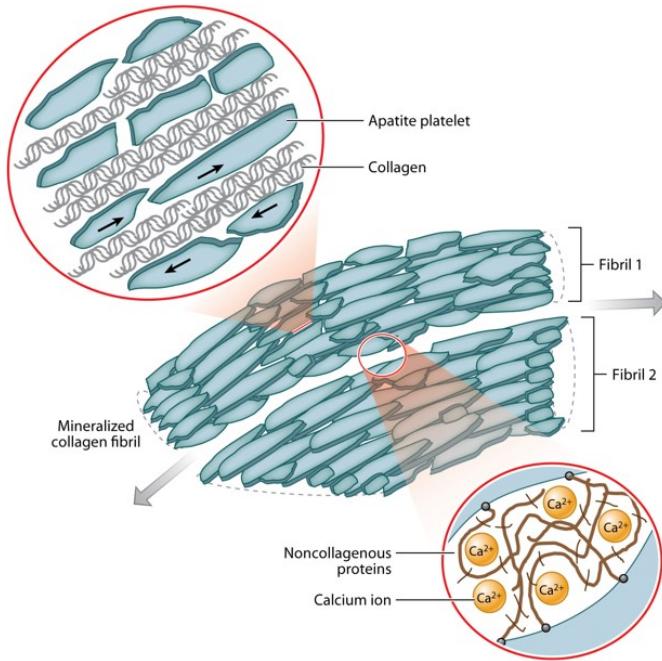


Figure 2.9: Non-Collagenous Proteins and Collagen. As an energy dissipation mechanism, non-collagenous proteins are found between collagen fibrils to act as sacrificial bonds to reduce crack growth. Image from Annual Review of Materials Research, 2011, R. Wang and H.S. Gupta, Deformation and Fracture Mechanisms of Bone and Nacre [63].

Microcracks within bone provide another method to reduce the impact of cracks by diffusing the energy present over a larger area and diverting crack direction. The mechanisms within bone appear designed to inhibit crack growth rather than initiation, an adaptation perhaps to the constant presence of cracks within the material due to how bone is formed and maintained at a cellular level as well as having to include natural stress concentration points such as Haversian canals and canaliculi [45, 63].

Each of these parameters that affect mechanics (geometry, mass and material) are influenced by initial bone formation and shaped through remodelling processes. As such, it is key to understand the biological procedures that determine physical properties of bone, using the hierarchical structure as a link between bone cellular biology and mechanical performance.

## 2.3 Cellular Bone Environment

It has been demonstrated that bone is a highly complicated, hierarchical organ whereby the mechanical performance is influenced by structural arrangement and material properties derived from constituent components at the nano- and micro-scale. Both these aspects are modulated by bone cellular activity and as such an understanding of this environment is necessary to determine the basis of bone mechanical competence in health and disease.

Osteogenesis describes the process by which bone is formed through cellular activity and involves highly inter-dependent and regulatory processes involving biochemical and molecular level events [5, 18, 64]. The primary cells in osteogenesis and their basic roles are [65]:

**Osteoblasts** are directed to secrete proteins and enzymes to first form an organic matrix (osteoid) and then use this as a base for mineralisation,

**osteoclasts** are multi-nuclear cells that are directed to areas where bone tissue must be resorbed and,

**osteocytes** are found in between lamellae and are thought to monitor bone health by signalling areas for repair and removal and regulation of bone mineralisation [66].

These three cell types interact together both in bone modelling, where bone is shaped and mass is increased (most associated with growth), and bone remodelling, where bone is replaced with newer tissue such that there is minimal change in mass (and so associated with the mature skeleton). Therefore, remodelling differs to modelling in that resorption and formation are coupled together. It is apparent then that these mechanisms are important in the development and maintenance of bone quality and strength.

### 2.3.1 Bone Modelling and Remodelling

#### Modelling

Bone modelling refers primarily to skeletal development and bone mass gain during childhood and early adulthood in humans. In modelling, bone is formed and is not coupled to resorption as with bone remodelling. During bone modelling, the major organic component of bone, type I collagen, is secreted in basic form (procollagen) by osteoblasts. The procollagen is then modified through enzymatic activity into tropocollagen, with both of the procollagen and tropocollagen monomers around 1-2 nm in diameter [25]. The tropocollagen molecules self-assemble through local interactions into fibril structures that can range from 30-100 nm in diameter [67] and begin to mineralise. Alkaline phosphatase is an early marker of osteogenesis and is thought to provide local availability of inorganic phosphate through enzymatic action to promote mineralisation [68]. Both phosphate and calcium are required as they form aggregates that act as a base for hydroxyapatite nucleation [69]. After maturation of the mineral phase, bone modelling is complete.

#### Remodelling

Once the skeletal form has been established, maintenance of the bone tissue predominates over growth. Remodelling and maintenance is essential for systemic mineral homoeostasis and to prevent degradation in bone mechanics by removing micro-cracks and defects [65] and so also has an influential role in susceptibility to bone disease. In healthy individuals remodelling is necessary, as within older tissue minerals continue to crystallise, resulting in more brittle material that has lower fracture toughness. Again in healthy adults, rates of bone deposition and resorption are roughly equal so that skeletal mass is maintained.

The remodelling process periodically replaces old bone with new and is achieved through osteoblasts and osteoclasts called basic multicellular units (BMUs). Hence, remodelling is localised [70] and an area a BMU operates within is covered by a canopy of cells [71], details of which remain broadly unknown but is thought to be constructed from

osteoblast lining cells and osteomacs (macrophages resident on the bone surface) [72]. The different stages of bone remodelling are displayed in figure 2.10.

The starting activation stage of remodelling is prompted by cues from multiple origins. Biochemical signalling from osteocytes, that are sensitive to local strain conditions within the bone matrix through dendritic processes, can initiate the migration of osteoclast precursor cells to the indicated area. Although the precise process remains to be determined, strain on bone is thought to drive interstitial fluid flow and induce shear stress that is sensed by osteocytes and consequently leads to osteocyte activation of osteoblasts and osteoclasts [73, 74]. Similar signals are also transmitted when osteocyte cell death occurs as a result of bone damage, again recruiting osteoclasts to the local area [66, 75]. Apoptosis also results in the cessation of osteocyte TGF- $\beta$  secretion, removing osteoclastgenesis inhibition. Resident osteoblast cells can also promote osteoclast progenitor recruitment, differentiation and activation by production and release of factors such as receptor activator of nuclear factor  $\kappa$ B ligand (RANKL). An example of such a case is when parathyroid hormone (PTH) is released systemically and binds to receptors located on osteoblasts or cross-talk and signalling occurs between osteocytes and osteoblasts, stimulating these events [71].

Osteoclast progenitor cells are derived from a haematopoietic lineage and are recruited to the bone matrix where differentiation and proliferation is coordinated within the presence of stromal cells and immature osteoblasts [71]. Expression of RANKL and colony-stimulating factor-1 (CSF-1) is necessary for osteoclastgenesis, with these two factors causing proliferation and expression of osteoclastic genes (such as TRAP and CTRK) [76]. Such is the importance of the RANK-RANKL signalling pathway that regulation is an effective method for halting or slowing osteoclast action. Biologically, this is achieved by osteoblast release of osteoprotegerin (OPG), which binds to RANKL and blocks interaction with osteoclast RANK receptor [77, 78]. Clinically, this has been successfully exploited through the use of Denosumab [79], an antibody able to bind to RANKL and treat patients with a high risk of bone fracture by reducing osteoclast activity and bone loss. RANK-RANKL-OPG interaction is shown in figure 2.11.

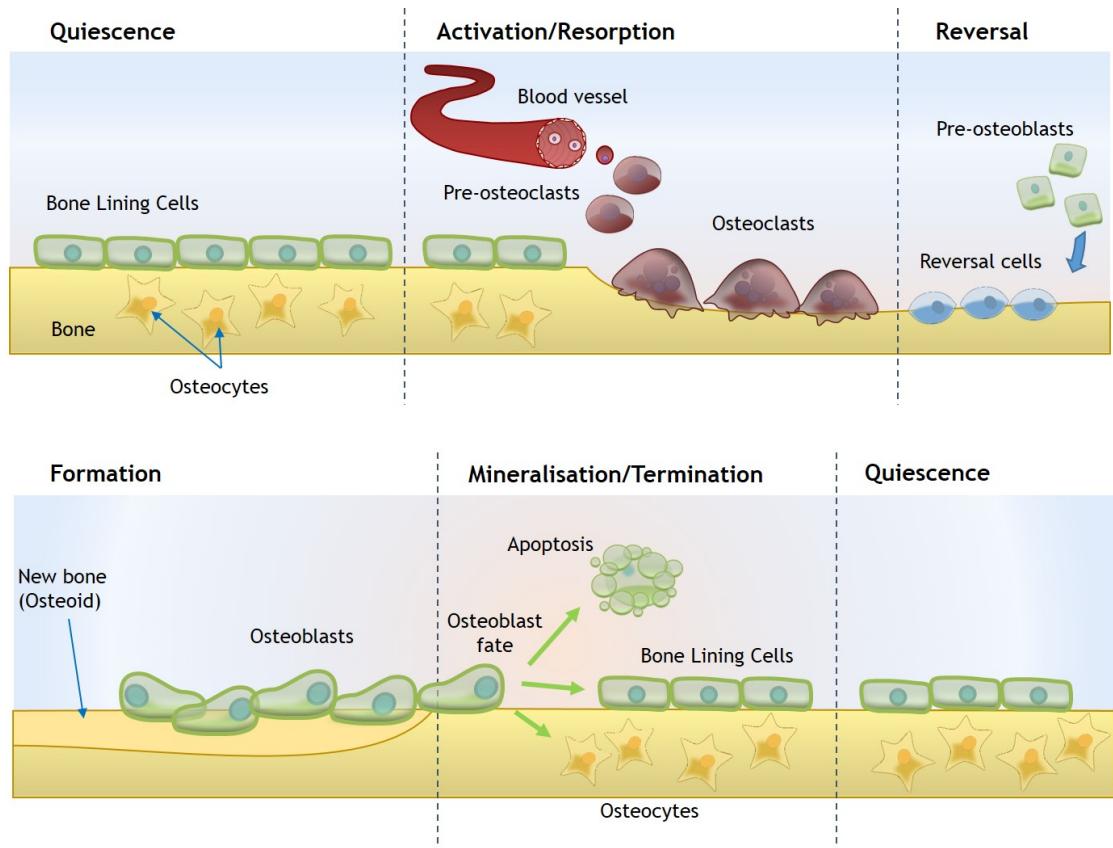


Figure 2.10: Bone Remodelling. The different stages of remodelling are presented in the illustration from recruitment of osteoclasts by osteocytes to begin resorption, the transition between bone resorption and formation (reversal), formation of osteoid bone following osteoblast recruitment and then eventual mineralisation to form new bone.

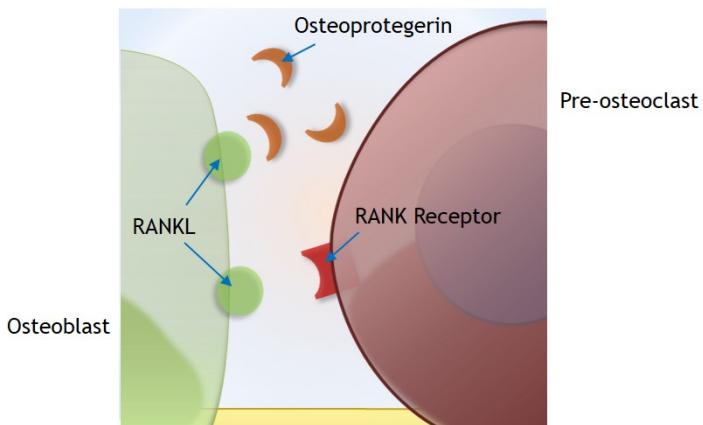


Figure 2.11: Osteoclast Differentiation: RANK-RANKL-OPG. RANKL binds to the RANK receptor protein on pre-osteoclasts to induce differentiation. The RANKL protein can be bound within the cell membrane or secreted. OPG is released to bind to RANKL to inhibit osteoclast differentiation, hence preventing RANK action in promoting the expression of osteoclast related genes such as TRAP.

The resorption phase is started by release of matrix metalloproteinases (MMPs) from osteoblasts to begin removal of osteoid tissue. The mature multinucleated osteoclast cells can then bind onto the bone surface through exposed extracellular matrix proteins, such as osteopontin [71]. Release of hydrogen ions from osteoclasts enables the dissolution of the mineral phase of bone within this acidic environment, which is accompanied by release of collagenases and proteinases to break down the organic components [80]. The mechanism of bone resorption involves osteoclasts attaching to the bone matrix and releasing hydrolytic enzymes that break down the organic matrix and enables the release of calcium that can be sent into the vascular system [17].

As osteoclasts resorb bone, growth factors (such as IGF-1, TGF- $\beta$  and FGF) are released to initiate osteoprogenitor cell migration and function. The growth factors also suppress osteoclast activity. In addition, proteins are secreted by osteoclasts when the resorption phase has been completed to act as sites for osteoblast attachment [80]. During the reversal phase remnant organic matrix is removed and the bone surface is prepared for subsequent formation. This action is performed by little understood mononuclear reversal cells proposed as precursors from either a phagocyte or osteoblast heritage. Recent study by Andersen *et al* [81] have shown these cells to express markers more characteristic of the osteoblastic lineage, such as RUNX2, and reversal cells found in an arrested state correlate with lower bone volumes in absence of bone formation. It seems likely that reversal cells and the reversal phase is highly important in the cellular transition from bone resorption to formation.

The formation phase begins with the migration of osteoblast progenitor cells towards bone resorption areas (a number of candidate mechanisms have been proposed as necessary for the coupling of osteoclast resorption to osteoblast formation that are not described here but details can be found in reference [71]). After undergoing differentiation, osteoblasts deposit collagen I in addition to other proteins (for example osteopontin, osteocalcin and matrix Gla protein) to form osteoid. Mineralisation is then supported through release of factors such as alkaline phosphatase [71, 80]. Bone mineralisation begins by the supply of salts (calcium and phosphate), originally in non-crystallised form. Once the levels of calcium and phosphate surpass a certain threshold, crystals of hydroxyapatite begin to form, with nucleation sites provided by non-collagenous proteins

as mentioned in section 2.1.2.

Once new bone has been formed, remodelling is terminated. It is thought osteocytes are influential in halting the remodelling process and return the local bone area to a quiescent state though the precise mechanism is not fully known. After mineralisation, osteoblast fate is directed towards apoptosis, conversion to bone lining cell phenotype or differentiation into osteocytes (when entombed by newly deposited osteoid) [71].

During development, metabolic focus is placed on the growth of the skeleton, whilst later in life more emphasis is on maintenance. In both cases cell activity is of great importance to bone generation and resorption. Consequently, modification of cell function will likely result in changes to bone material properties or structure and hence affect mechanical performance due to changes in development or maintenance. It is currently believed that BMU processes are the primary manner in maintaining bone integrity [33].

Osteogenesis or ossification occurs during multiple stages during the life course, such as skeletal development during foetal life, skeletal growth during puberty and maintenance of the skeleton in adult life through bone remodelling. Therefore, the beginnings of osteogenesis can be traced back to the initiation of stem cell differentiation towards an osteogenic lineage [82].

### 2.3.2 Skeletal Stem Cells

Stem cells are characterised by their ability to self-renew and differentiate into a number of different cell types possible when stimulated appropriately. These two defining aspects are achieved from mitosis, where one of the daughter cells remains within the stem cell line whilst the remaining daughter cell undergoes differentiation into a tissue cell [83, 84].

Embryonic stem (ES) cells are able to differentiate into any cell of the body under appropriate conditions and termed as pluripotent. In adulthood however, the stem cells within adult tissue can only differentiate into a limited number of cell types and are

described as multipotent [83]. The most common types of adult stem cells used in research include hematopoietic and skeletal stem cells (also referred to as mesenchymal stem cells), relatively easily obtained from tissue samples such as bone marrow [84]. Research interest into stem cells has primarily been driven by the potential for therapeutic application, replacing damaged parts of the body with cells and tissues generated from the patients themselves [85, 86].

Recently, it has been displayed that it is possible to return somatic cells back to a pluripotent state through forced expression of Oct 3/4, Sox2, c-Myc and Klf4 [87]. Takahashi *et al* demonstrated these induced pluripotent stem cells (iPS) display the same characteristic traits of ES cells, potentially offering cell-based treatments with patient specific histocompatibility match. Much work has been conducted, and is still required, to profile the characteristics of iPS for therapeutic benefits with the suggestion that iPS cells retain 'epigenetic memory' (section 2.4.3) of previous somatic states, in certain cases reducing differentiation efficiency when compared to ES cell counterparts [88].

Skeletal stem cells (SSCs, also referred to as mesenchymal stem cells, MSCs) are a specific group of multipotent stem cells that have the potential to form all of the stromal lineage; bone, fat and cartilage (figure 2.12). Culture of bone marrow cells have established that the cells adhere rapidly to tissue culture plastic [89] and assume an appearance similar to fibroblasts (figure 2.13). Establishment of these cells results in distinct colonies that are derived from a single cell, termed colony-forming unit fibroblastic (CFU-F). Study into cell surfaces have indicated that bone marrow cells expressing osteogenic markers such as STRO-1, MCAM or CD105 have increased clonogenicity, so that these cells have the ability to grow regardless of cell sample density [85, 90].

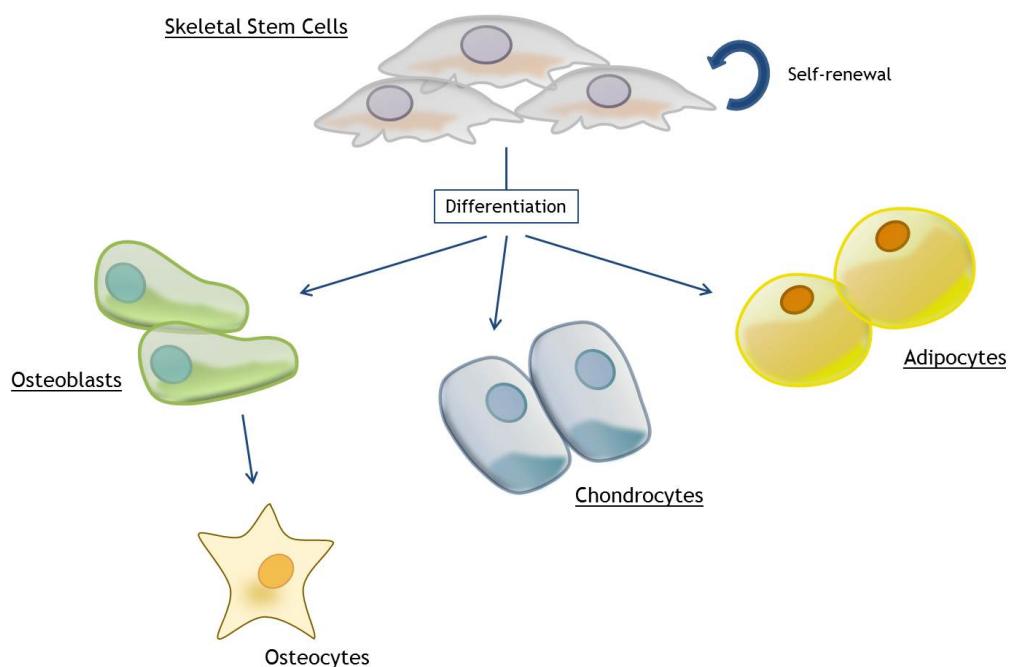
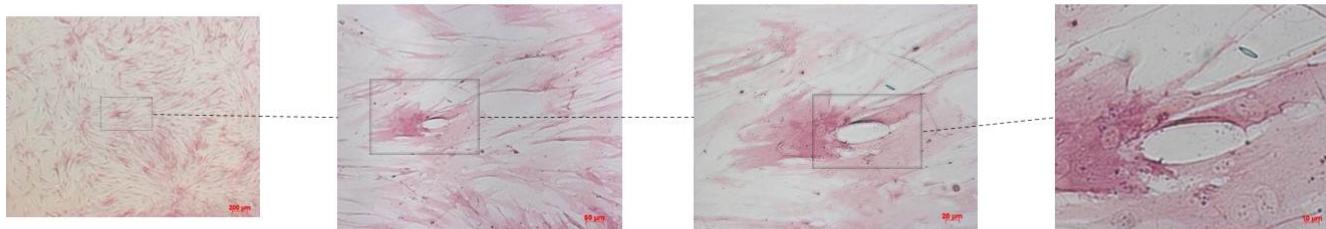


Figure 2.12: Skeletal Stem Cell Lineage Diagram. The figure illustrates the self-renewal capacity of SSCs in addition to the differentiation pathways that SSCs can commit to.

Day 21 M55 Human Bone Marrow Stem Cells (Basal Conditions), Alkaline Phosphatase Staining on Plates and Imaged at x2.5, x10, x20 and x40 Magnification



Day 21 M55 Human Bone Marrow Stem Cells (Osteogenic Conditions), Alkaline Phosphatase Staining on Plates and Imaged at x2.5, x5, x10 and x20 Magnification

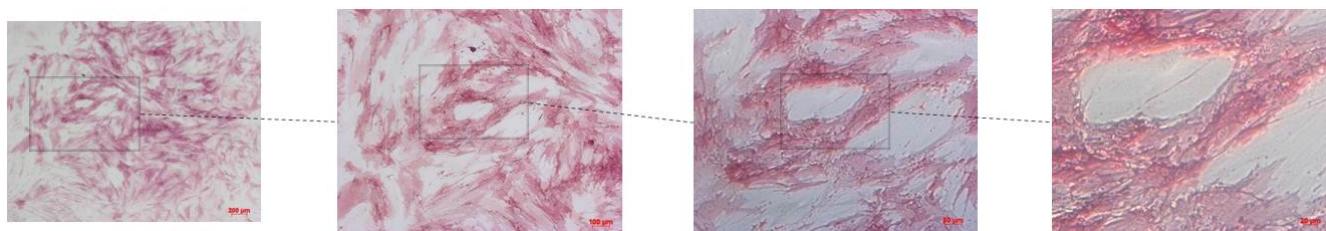


Figure 2.13: Cultured Human Bone Marrow Cells. Light microscope images at different magnifications of 21 day old cultured cells derived from human bone marrow in basal and osteogenic media and stained for alkaline phosphatase expression.

### 2.3.3 Bone Formation: Osteoblast Differentiation, Gene Expression and Growth Factors

Differentiation of skeletal stem cells, through to progenitor cells and to osteoblasts are catalysed by factors such as insulin-like growth factor 1 (IGF-1) and transforming growth factor- $\beta$  (TGF- $\beta$ ) [12, 91]. *In-vitro* studies of these growth factors have found that promotion of osteoblast phenotype marker expression, such as alkaline phosphatase, type 1 collagen and osteocalcin, occurs. In particular, TGF- $\beta$  influences cell phenotype by targeting the promoter region of *RUNX2* (also known as *CBFA1*), a key transcription factor required for osteoblastic differentiation [92]. After differentiation and proliferation, osteoblasts begin the process of generating new bone tissue by laying down osteoid [80], as previously mentioned.

Intramembranous and endochondral ossification are the processes whereby bones are formed and calcified. In both intramembranous and endochondral ossification, pre-existing mesenchymal tissue is initially constituted and eventually converted into bone. During intramembranous ossification a proportion of mesenchymal cells proliferate and differentiate into osteoblasts, which secrete collagen to begin matrix formation that may subsequently be mineralised. Differentiation of mesenchymal cells involves the release of bone morphogenetic proteins (BMP), which switch on the *RUNX2* gene, prompting SSCs to differentiate along the osteoblast lineage [93].

A diagrammatic overview of endochondral ossification can be viewed in figure 2.14 and is achieved through a number of stages. i) The first stage of endochondral ossification involves the **condensation and differentiation** of mesenchymal cells into cartilage cells to form the initial bone model. This is achieved through release of the factors Pax1 and Scleraxis to activate the appropriate gene in the mesenchymal cells. Expression of the *SOX9* gene also has been found to fulfill an important role for chondrocyte transformation, with production of necessary transcription factors. ii) **Proliferation** of the chondrocytes is then stimulated by release of growth hormone (GH) and insulin-like growth factor-1 (IGF-1). iii) Chondrocyte **hypertrophy** is then influenced by thyroid hormone, allowing for longitudinal bone growth to take place. Cells that have undergone

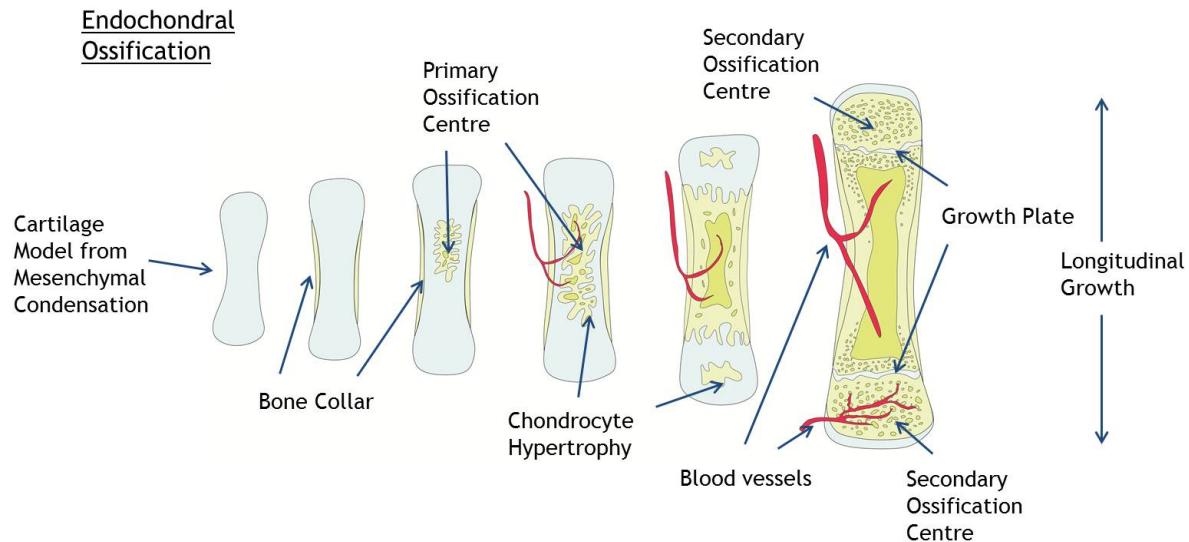


Figure 2.14: Endochondral Ossification. An overview of endochondral ossification detailing the formation of the cartilaginous template and subsequent ossification and growth, image courtesy of Emma Budd, Bone and Joint Research Group, University of Southampton, 2015.

hypertrophy express the matrix metallopeptidase 13 gene (*MMP13*) to produce collagenases, such that calcification can occur through the breaking down of the cartilaginous skeletal template and attraction of blood vessels and osteogenic cells. Longitudinal growth in early to pubescent life remains possible if the epiphyseal plates continue to maintain proliferating cells [93, 94].

## 2.4 Skeletal Health and Bone Diseases

Classically, changes to bone structure and remodelling are directed by Wolff's law [95], whereby bone form is adapted to the prevailing strain conditions. If bone tissue is exposed to high levels of strain, the remodelling rate is altered such that additional bone mass is established in high-strain regions. Equally, low-strain states (from, for example, sedentary lifestyles) experience an increase in remodelling to remove excess bone, resulting in thinner structures [33].

In humans, a steady accumulation of bone mass through childhood and early adulthood is experienced, with peak bone mass presenting at around 30 years of age. After this stage, bone mass decreases due to a higher bone turnover rate [96] and the typical pattern for bone mass level throughout life is illustrated in figure 2.15. As such, bone properties deteriorate with age. The higher remodelling rate, and consequently the increased density of osteons as a result, causes an accumulation of micro-cracks so that the degradation in overall bone mechanics surpasses the usefulness in their role of diffusing crack energy away. In addition to less bone, there is a decline in cross-linking at the collagen level, such that there is a reduction in fracture toughness. Initially, the first signs of bone quality deterioration are seen at trabecular bone sites because of the higher surface area of the structure is subject to increased remodelling [45].

Importantly, there is a natural decrease in bone mechanical competence that occurs naturally with age. Problems may occur if additional factors, such as environmental or nutritional, results in increased degradation of bone at later life or a significant reduction in the level of peak bone mass attained (in such a case, even normal rates of decline at old age may result in significantly weakened bone than is expected). One of the most prevalent bone diseases globally is osteoporosis, whereby fracture risk is increased due to greater fragility.

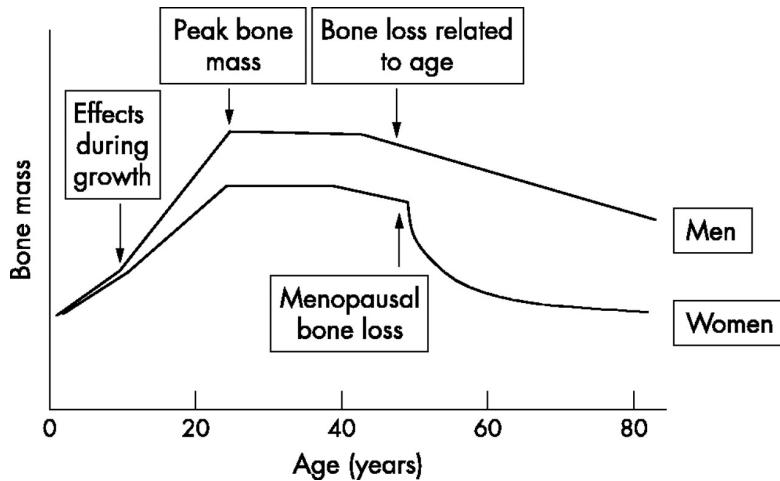


Figure 2.15: Effect of Age on Bone Mass. The above graph shows typical changes in bone mass through life. Up until adulthood bone is constantly being deposited, increasing mass until middle age where a plateau is reached. Later in life there is a gradual decline, more pronounced in females due to the menopause and dramatic changes on hormonal balance. Also apparent from the graph is that generally women have a lower bone mass than men. From Dogan and Posaci, 2002 [96]. Reproduced from: Monitoring Hormone Replacement Therapy by Biochemical Markers of Bone Metabolism in Menopausal Women, E. Dogan and C. Posaci, 78(78) 727-731, 2002, with permission from BMJ Publishing Group Ltd.

#### 2.4.1 Osteoporosis

Osteoporosis is a degenerative disease that affects the bones of the skeleton and currently there are two widely accepted definitions:

- The World Health Organisation (WHO) describes osteoporosis as a *disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk* [97].
- The National Institutes of Health (NIH) describes osteoporosis as a *skeletal disorder characterised by compromised bone strength predisposing to an increased risk of fracture* [98].

What is apparent from these two definitions is that osteoporosis impacts upon a multitude of bone features, all of which can increase the likelihood of bone fractures as a consequence (frequently to the hip, wrist and vertebrae [99]). Osteoporosis is a relatively common condition in developed countries; in the UK, 1 in 2 women and 1 in 5

men over fifty are predicted to suffer a fracture as a result of osteoporosis according to the International Osteoporosis Foundation [100]. This translates to an estimated 300,000 fractures per year at an economic cost of £2.3 billion for treatment [101] and also confirming osteoporosis to be the most prevalent bone disease.

Demographically, the ageing trend of populations [102] will likely cause a rise in the financial burden of treating conditions caused by osteoporosis. In addition to the primary cost of treatment, elderly patients who survive fractures often have some degree of permanent impairment and require further supportive therapy and care or assistance for everyday tasks [99]. Additionally, association with a temporary elevation in morbidity risk post-operation (up to a period of 90 days after surgery) has necessitated further work into reducing the impact of osteoporosis as well as improving clinical practice [103–105].

The multifactorial nature of bone strength and how osteoporosis affects each of these factors has resulted in much controversy over clinical diagnosis. Currently the gold standard is to measure bone mass using dual energy x-ray absorptiometry (DEXA) to provide a score that can be categorised against standards established by Kanis *et al* [97] in the 1994 WHO position report, whereby a threshold value is defined for clinical cases of osteoporosis (2.5 standard deviations below the mean bone mineral density (BMD) or content (BMC) measurement for an average premenopausal woman).

Category	Diagnostic Standard
Normal	Bone mineral density or content that is not greater than 1 s.d. below the young adult mean value
Osteopenia (low bone mass)	Bone mineral density or content that is between 1 s.d. and 2.5 s.d. below the young adult mean values. In these cases it is recommended that prevention of further bone loss be applied for most effect.
Osteoporosis	Bone mineral density or content that is below 2.5 s.d. below the young adult mean value.
Severe osteoporosis	Bone mineral density or content that is below 2.5 s.d. the young adult mean value and additionally a presence of one or more fragility fractures.

Table 2.1: Osteoporosis BMD Categories. Information from Kanis *et al* [97]

However, debate has arisen over the suitability of using BMD as the definition of osteoporosis, as this parameter is a risk factor of osteoporosis rather than a direct measure of the disease or bone strength itself. In contrast the NIH definition of osteoporosis incorporates reduction of bone strength (and so increase in fracture risk), therefore reflecting the role of bone mass and bone quality as contributors towards fracture likelihood. As mentioned in section 2.2.2, bone quality encompasses factors that affect bone strength independently of mass, such as peak bone mass and microarchitecture (figure 2.16) and the merits of using bone quality in addition to BMD measurements to increase the efficacy of osteoporosis diagnosis have often been recommended [47].

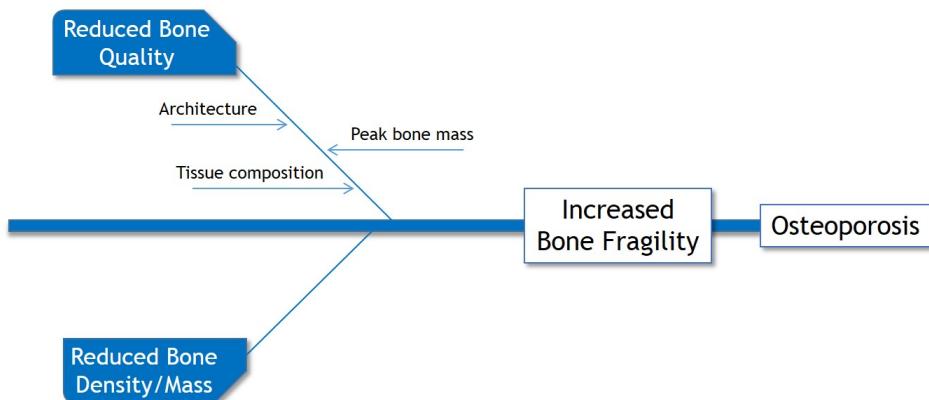


Figure 2.16: Fracture and Bone Quality.

However, there are a number of concerns with implementing bone quality to measure fracture risk and diagnose osteoporosis. Despite the potential to augment clinically established indicators of bone strength, such as BMD, currently there is no method available to produce a measurable bone quality score, nor criteria defining a healthy or disease level of bone quality [106]. As such, much research is being invested towards developing a clinically viable method to measure bone quality [50].

### UK Standards: Diagnosis and Treatment of Osteoporosis

Guidance in the UK for diagnosing and treating osteoporosis is recommended by the National Osteoporosis Guideline Group (NOGG). As of 2014, there is no systematic approach to screening for osteoporosis and instead a case-finding strategy is used when an

existing fragility fracture or strong risk factors are presented. Prevention of osteoporosis is effected by lifestyle guidance, such as ensuring adequate dietary calcium and vitamin D, exercise and assessment of risk factors for falls [107].

Fracture risk is usually assessed in postmenopausal women and men over 50 years of age. Previous fragility fracture usually results in administration of treatment. If other risk factors are presented in the absence of fracture then fracture probability is determined through FRAX, an algorithm that accounts for a number of risk factors, physical attributes and life choices to produce a score predicting fracture in the following ten years. In accordance with the WHO recommendations, diagnosis is based around the scanning of the femoral neck using DXA to assess BMD.

Within the UK, the National Institute for Health and Care Excellence (NICE) currently recommend supplementation of calcium and vitamin D to ensure normal levels in patients with high risk to osteoporotic fractures. For prevention of secondary fractures (i.e. after an osteoporosis-related fracture has already been suffered), bisphosphonates, selective oestrogen receptor modulators, strontium ranelate and parathyroid hormone (PTH) may be prescribed [108, 109]. Bisphosphonates (alendronate, risedronate and etidronate) interfere with osteoclast function (often by inducing apoptosis) so that bone resorption is inhibited [110], selective oestrogen receptor modulators (raloxifene) can be prescribed to women promote the beneficial action of oestrogen by decreasing bone turnover [111], strontium ranelate acts by promoting bone formation and decreasing bone resorption [112] and PTH is used to stimulate osteoblast activity and hence bone growth [113].

It is apparent that osteoporosis therapeutic strategy, particularly drug treatment, has currently been targeted towards increasing bone mass and reducing the rate of bone loss. This is primarily achieved through readjusting the remodelling imbalance that is often present in later life. Regarding the NIH consideration of bone quality however, research efforts have been directed towards more prophylactic measures and ensuring healthy skeletal development. For example, peak bone mass can influence fracture risk

in later life and subsequently factors that affect peak bone mass may be linked eventually towards increased osteoporosis risk in later life. As such, there is great interest in uncovering new risk factors of osteoporosis.

#### 2.4.2 Novel Risk Factors of Osteoporosis

Links between programming of skeletal growth during foetal and early life provide evidence that suggests susceptibility to osteoporosis in later life can be influenced by *in utero* conditions. Adaptations to basal hormone levels and to the way the hypothalamic-pituitary-adrenal axis functions could affect later levels of bone mineral content, reducing bone strength and increasing the risk of osteoporotic fractures [1]. What causes these changes has attracted interest and precisely how key nutrients for bone formation and maintenance function have directed research in the area.

The remodelling process differs between cortical and trabecular bone due to the morphological arrangements, with BMUs resorbing and depositing bone through canal passages that form new osteons in cortical bone, whereas remodelling in trabecular bone occurs at the surfaces. As such, remodelling at trabecular sites is more active due to the greater surface area, causing trabecular bone to be at particular risk of degradation if higher remodelling is present.

As trabecular bone volume diminishes, activity is concentrated on cortical bone, which then becomes more porous. Consequently, bone material away from the surface begins to experience more stress and accumulates microdamage. This region of bone is often older than the surface with a higher mineralisation and is less populous of osteocytes, resulting in an increased sensitivity to damage and fracture. High remodelling rates may result in an increase in risk of fracture as older and more established bone is replaced with newer, less mineralised bone with lower strength. High bone resorption will also cause temporary gaps within bone tissue, which can act as a stress raiser [63] or as a crack initiation site. The remodelling has also been found to affect the cross-linking of collagen, changing the material properties [27].

Differences in collagen cross-linking without appreciable changes in overall collagen content have been found within osteoporotic bone. This may be due to the remodelling process and thus could mark changes in osteoblast phenotype and cell signalling processes related to the matrix collagens [114]. It is apparent that fracture risk can be determined by the molecular mechanisms responsible for bone mineral density or through epigenetic and cellular mechanisms through osteocyte density and remodelling. This then requires a wider strategy to treat fracture cases due to differences in origin [27], with emphasis on research to identify high-impacting factors on early life in relation to adult life bone quality.

Though a useful, and well established, clinical assessment tool, BMD is still not able to fully predict fracture risk. It is likely that there are other contributory factors towards fracture, highlighting how additional data regarding bone quality may aid in characterising risk [115]. Cases where bone strength has remained unchanged despite treatment to increase bone density has challenged the conventional correlation between density and strength, with incorporation of bone quality necessary to provide a more accurate indication of fracture risk [116]. Consequently, much work has been conducted to infer important biological mechanisms that influence risk to disease, particularly into genetic and epigenetic influences.

### **2.4.3 Bone Health and Mechanics: Epigenetics**

A number of factors alter bone mechanical properties and hence reduce quality of life and increase the risk to bone diseases. A summary of this can be viewed in figure 2.17.

Within mammals, the environmental status can be communicated to the foetus by the mother through the placenta or lactation. Maternal challenges, such as diabetes, and pathologic disruptions caused by toxins can be translated across to the baby, causing an adaptive epigenetic response [6]. One of the most studied causes of epigenetic changes is nutritional stresses, which can come from both excessive and deficient diets [117].

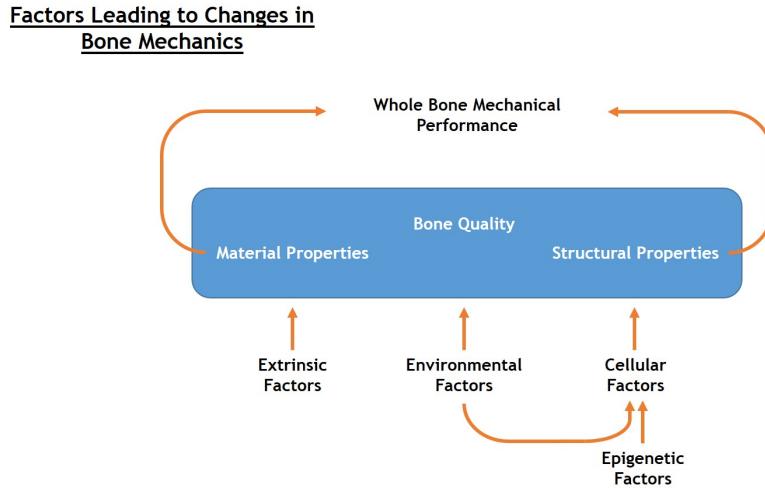


Figure 2.17: Factors Leading to Changes in Bone Mechanics. The figure demonstrates how events may eventually affect the mechanical properties of bone.

Epigenetics refers to changes in the gene expression without any alteration to the underlying DNA sequence. The entire genome is contained within each cell of the human body but epigenetic mechanisms allow for cell differentiation through the activation or inactivation of certain genes. Epigenetic changes can be induced from environmental factors and it is possible that adverse conditions may alter the epigenome such that inhibition of normal tissue function occurs [83, 118]. Evidence of epigenetic actions is found in monozygotic twin studies, where one twin has been found to be more prone to diseases due to a difference in environmental exposure [6]. There is emerging evidence for a role of epigenetics in normal and abnormal skeletal development.

### Epigenetic Mechanisms

Conditions during foetal and early neonatal life can have lasting effects throughout the life course. These changes may be manifested in a number of ways, such as differing gene expressions, reduction in cell populations or modified hormone release. As a consequence, it is possible that this results in changes to stem cell function, tissue structure or metabolic activity [119].

Characteristics of stem cells and subsequent differentiation into specific cell types are dependent on multiple factors, such as transcription, epigenetics and post-transcriptional

regulators such as microRNAs [120]. Different stem cells within the human body have been found to be epigenetically preprogrammed through methylation sites to selectively differentiate into a limited number of cell lineages [85]. Events such as methylation of DNA sites, histone modifications and chromatin remodelling all contribute to silencing of genes within stem cells. Such changes are also maintained through to progeny cells, maintaining tissue specific cells [121].

Therefore, the function and differentiation pathway of stem cells are influenced through epigenetic programming into a particular phenotype [122] and any changes can lead to an increased susceptibility to diseases such as cancer. Epigenetic control of expression is linked to how the DNA structure is packed, with the first level of packing involving histone proteins. Eight of these proteins cluster together to form a nucleosome, which the DNA molecule wraps around (figure 2.18) due to differences in electrical charges (the amino acids lysine and arginine in histones are positively charged and the phosphate groups in the DNA are negatively charged) [83].

DNA methylation is a key mechanism underlying gene expression, where methyl groups bind to CpG (cytosine and guanine nucleotides bound by a phosphate link) regions of the DNA structure through the recruitment of various DNA methyl transferases (DNMTs) proteins [124], such that transcription is prevented (figure 2.18). The methyl groups can also attach to the tails of the histone proteins to alter chromatin structure [83].

The effect of epigenetic operation on chromatin structure can be divided into three different groups. Constitutive heterochromatin regions are regions where the methyl groups attach to histone tails, condensing the chromatin structure and restricting transcription. This has the effect of gene repression as DNMTs are used as areas for methyl-CpG-binding proteins (MBPs) to bind onto, which in turn recruit histone deacetylases (HDACs), causing silencing of the gene through histone deacetylation (figure 2.18) [124]. This may be further facilitated through DNA methylation and reduced acetylation of histones [125].

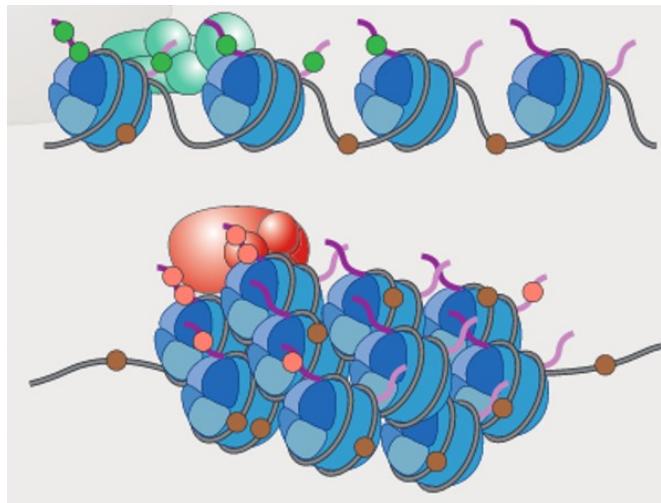


Figure 2.18: Epigenetic Mechanisms. The diagram above represents states of transcription active (top) and silent (bottom) genes. The blue spheres depict nucleosomes with DNA wrapped around each. Brown circles are methylated CpG sites and are few in the transcription active gene, resulting in a more open chromatin structure. Histone acetyl transferases (green protein structure) facilitate in opening the DNA structure. In contrast, multiple methylated sites are present in the silent gene, causing the closed chromatin structure and inhibiting transcription. The red protein represents histone deacetylases that also prevent transcription. Adapted by permission from Macmillan Publishers Ltd: Nature, A.P. Feinberg, Phenotypic plasticity and the epigenetics of human disease, 447(7143): 433-440 , copyright 2007. [123]

Genes are most accessible in euchromatin regions, where hyperacetylation of the histones opens up the chromatin. In contrast to methylation, histone acetylation involves the attachment of an acetyl group through the action of histone acetyl transferases (HATs) (figure 2.18), neutralising the positive charge so that the DNA structure opens up, allowing increased access to underlying genes and putting them into a state of activation [83, 124, 125].

Finally, facultative heterochromatin are areas which are similar to constitutive heterochromatin, with DNA methylation and histone modifications in the form of methylation and low acetylation. However, the condensed structure is not present in these regions, meaning that epigenetic silencing occurs through the prevention of transcription [125].

Inadvertent activation of genes during differentiation can occur with structural changes to the chromatin due to problems with methylation and acetylation. Multipotency, one

of the primary traits of stem cells, is thought to be due to how stem cells are able to maintain the majority of CpG regions in an unmethylated state. Differentiation of adult stem cells into required tissue cells indicates that some aspects of the epigenetic programming can be altered. Research has shown how methylation can be reversed to encourage specialisation into different cell types, changing the cell lineage pathway [86]. Whilst reversal of deleterious epigenetic modifications is of medical interest, of equal importance is how the changes occur in the first instance to enable preventative strategies to be employed.

#### 2.4.4 Diet and Skeletal Changes

Nutrition challenge during *in utero* life as a result of insufficient maternal diet has been shown to affect the developmental pathway. The scale of these changes differ depending on the time of altered nutrition availability, with rapid growth phases most at risk [119]. The lack of essential nutrients can cause growth retardation of specific organs, such as calcium for the skeleton, or a redistribution of certain diet elements occurs so that overall growth is slowed. For example, diversion of nutrients to adipose tissue can result as a consequence of low nutrient intake [117].

The Barker hypothesis was formulated on epidemiological data demonstrating that there is a link between poor growth rates during early life and status of disease in later life, most notably the correlation to low birth weight and disorders such as stroke, coronary heart disease and hypertension. This indicates that foetal life has influence over events a number of decades later, where risk of different diseases increasing as a result of when growth challenge occurs [126]. However, it has emerged that more subtle changes may also occur *in utero*. Potentially, histone acetylation and methylation changes that are not immediately apparent exist within a person, with no expression of these changes until the onset of adulthood disease [127].

Offspring growth during late pregnancy has been shown to be an indicator of skeletal properties at birth, whilst the growth trajectory during early gestation can be predictive of bone properties at early childhood (four years). Maternal lifestyle and diet influences offspring BMD in adulthood [128] and this is stressed through the plasticity of skeletal development in this period of life [2]. One of the most striking aspects of epigenetic modifications is that of a transgenerational effect [122, 129].

Deviations from normal diets during pregnancy have been shown to modify the development of the skeleton in offspring, such that structure is changed. Studies into high fat maternal diets during pregnancy have shown an increase in cross sectional area at the midshaft region of the femur within both control and high fat offspring [130]. The authors proposed that this occurred as a solution to the increase in mass, and hence skeletal load. Crucially though, the derivation of the extra bone material differed, with

the control maternal diet offspring increasing the spacing of trabecular bone whilst the high fat maternal diet offspring shortened femur length to provide the additional bone material at the midshaft, maintaining bone volume.

An explanation of this may be that the foetal mice subjected to a high fat environment assume an adaptive response to conserve bone mass, with a predicted unbalanced diet after birth. The reduction in femur length was similarly evidenced in male offspring from the same maternal and postnatal diets, with changes to the structure of the trabecular bone in the form of less connected struts. At a cell level, inspection of the distal femur showed a higher number and size of adipocytes [130], highlighting an adaptive response to altered diet conditions. High protein diet studies have also yielded differences, with lower alkaline phosphatase activity at seven days old [3]. Adaptation of bone morphology and cellular tissue composition during sensitive periods of development may have consequences later in life.

Protein and energy malnutrition during pregnancy has been identified as having a linear growth retardation effect, with a reduction in basal metabolism. Adequate intake of micronutrients such as calcium, zinc and vitamin D have important roles in skeletal development, most likely related to ossification processes. Deficiency of necessary nutrients during pregnancy is hypothesised to cause programming of IGF-1, hypothalamo-pituitary-adrenal (HPA) and parathyroid hormone(PTH)-vitamin D hormonal axes [8].

Low protein intake *in utero* has been shown to have an impact on bone biochemistry, affecting the process by which mesenchymal stem cell activity is regulated. Low levels of growth hormone and high levels of osteocalcin, indicating premature osteoblast activity, at four weeks old are thought to be some of the major alterations as a result of low protein. This then results in changes from normal bone development, which again can persist through to adult life [131, 132].

Any changes affecting the normal mechanical properties may increase the risk of bone diseases, particularly osteoporosis in later life, which heavily reduces the mechanical

competence of bone.

#### 2.4.5 Vitamin D

Low vitamin D intake status is a cause of the bone deformity condition Rickets, highlighting the importance of vitamin D in bone metabolism and the influence vitamin D has on systemic calcium levels within the body. Indeed, the presence of the active form of vitamin D, 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}_3$ ), facilitates in the absorption of phosphorus and calcium from the intestine, both of which are required for mineralisation [16]. Associating this with skeletal development, vitamin D insufficiency has been linked with reduced bone mineral content and bone size due to a reduced rate of mineral accrual [133].

Many cells within the human body have been found to have vitamin D receptors (VDRs), with  $1,25(\text{OH})_2\text{D}_3$  able to influence growth and differentiation. This suggests that vitamin D may have a pivotal role in the developmental stages of life, with the prevalence of the receptor leading to links between deficiency and likelihood of a range of diseases such as cancer, diabetes and hypertension. Studies into the function of VDRs have indicated suppression and induction of gene transcription, demonstrating the impact on cellular function and sensitivity of certain genes to vitamin D availability [16, 134].

Regarding skeletal development and maintenance,  $1,25(\text{OH})_2\text{D}_3$  and calcium are required together to ensure appropriate development of the growth plates through possible interaction with chondrocytes, whilst the deposition and resorption of bone from the remodelling units is heavily reliant on  $1,25(\text{OH})_2\text{D}_3$  and VDRs, modifying the population numbers of osteoblasts and osteoclasts through cell production changes [135]. This then has the effect of reducing the maximum level of calcium deposition in the skeleton [134], directly affecting the mechanical stiffness.

## 2.5 Perspective and Research Hypothesis

It has been estimated that around 1 billion people worldwide have insufficient vitamin D levels, inferring that the number of cases of vitamin D inadequacy during pregnancy are high. With evidence from multiple researchers that vitamin D status can enact phenotypic changes [136], there is a requirement to understand the exact consequences of deficiency on bone mechanical properties. Vitamin D deficiency during pregnancy has been correlated with low bone mineral content of children at nine years of age when compared to control populations. In addition, splaying of the femur has been observed, highlighting how the molecular make-up and eventual bone morphology is changed. Vitamin D is thought to be critical for intestinal calcium intake and with the effect noticed from early gestation, this indicates that skeleton development can be altered from an early time point [136, 137].

It is hypothesised that vitamin D deficiency in early life causes changes at molecular level, modifying bone cell behaviour. This then is reflected through changes in material and structural properties of bone and hence impacts upon mechanical performance. As discussed in section 2.4.4, low protein status *in utero* has also been demonstrated to adapt offspring bone development after birth. Recent interest has highlighted the possibility of intergenerational effects, whereby the effect of an environmental insult is transmitted to subsequent generations [138–140]. The work conducted therefore will be designed to answer the following hypotheses;

*“Altered maternal nutrition can adapt the long term epigenetic status of skeletal genes in the foetus, resulting in altered skeleton development and susceptibility to disease”*

And specifically;

*“Maternal low protein status during pregnancy induces a detrimental effect on second generation offspring skeletal health within female rats, observable through bone gene expression, bone micromechanical behaviour, bone fracture toughness and whole bone mechanics.”*

and,

*“Vitamin D deficiency in utero causes adaptations in epigenetic control of osteogenic gene expression in the offspring, which is manifested through changes in skeletal structure, adversely affecting the physical performance of bone increasing the risk of bone disease in late life.”*

To test these hypotheses, a range of techniques will be employed that will have the capability of obtaining information on gene expression, through real-time polymerase chain reaction, bone morphology, through high resolution imaging and bone mechanics, through strength and fracture toughness testing.

To answer whether nutrition during pregnancy has a role in eventual bone structure and functional performance, it is necessary to understand and quantify these material and mechanical properties mentioned. There exists multiple mechanisms at which deformation of bone can occur at different scales, with a fully integrated model of all events needed to explain how fracture happens. The action of the bone cells and the pathways that dictate their function are likely to have an effect on the bone material properties [35] as well. This is most clearly evident in the processes of deposition of protein to begin bone formation by osteoblasts and resorption of existing bone through osteoclasts. As such, the actions of these cells have an influence over the mechanics of the bone structures.

## Chapter 3

# Methodology



### 3.1 Methods

Bone tissue and bone health, due to the biological and physical components, can be characterised through multiple perspectives and hence a wide range of experimental approaches can be utilised. For a comprehensive analysis, application of a number of these techniques are necessary to interrogate bone. Such an approach would inform upon contributory factors towards healthy bone and importantly on the mechanistic differences between healthy and diseased states and possibly the origins of these changes.

The bone quality concept (section 2.2.2) highlights the structural and material factors that can attenuate the mechanical properties of bone and hence fracture risk. As stated in section 2.4.1, the current clinical method to analyse fracture risk is dual-energy x-ray absorptiometry (DXA) and algorithm scores derived from patient lifestyle [141]. However, both these approaches are surrogate measures of bone strength and are an indication of fracture risk rather than a direct measure [47] and a number of methods have been utilised to assess bone quality [50]. Within this thesis, several structural and material aspects of bone quality were measured, in addition to biological and mechanical investigation, to inform upon candidate factors for predicting mechanical performance of bone collected from laboratory rats. These factors, within this project, were categorised into three areas; biological, compositional/structural and material/mechanical, with each requiring different methods of assessment.

#### Murine Samples

Due to the practical difficulty and ethical constraints of tracking the effects of environmental assaults on skeletal development, animal models have been widely used for investigation as an alternative, to study different issues within bone health [3, 132]. The use of murine models in osteoporosis research is common, with similar responses in terms of pathology found between rats and humans. Using an appropriate animal model allows for a reliable, reproducible and comparative representation of bone development

that can deliver information on how skeleton growth trajectory is modified through different nutrition and environmental factors [142].

### 3.1.1 *in utero* Vitamin D Model

To study the effects of *in utero* vitamin D on future offspring skeletal development, Sprague-Dawley rat dams were raised in conditions restricting dietary and subcutaneous synthesis of vitamin D, a model describe by Eyles *et al* [143].

All animals from the vitamin D deficient model were kindly provided by Professor John McGrath of the Queensland Centre for Mental Health Research, Australia. All housing and behavioural assessment procedures were performed with approval from the University of Queensland Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia at the University of Queensland Breeding Colony, St Lucia, Brisbane, Australia. Rats were pair-housed in Macrolon cages with sanichip bedding and wire lids in a temperature controlled room (20 °C - 22 °C), on 12 hour light-dark cycles (lights on at 06.00). Food and water were provided *ad libitum*.

Four week old virgin females were assigned to either control or vitamin D deficient conditions and were maintained on these conditions for six weeks before conception and throughout gestation. During mating (at female age of 10 weeks), males with normal vitamin D levels were placed with females for a total of seven days.

Vitamin D deficiency was induced by housing under incandescent lighting to prevent subcutaneous synthesis of vitamin D (no UVB radiation in the vitamin D action spectrum, 290-315 nm) and fed a vitamin D deficient diet (AIN93G + AIN93-G mineral mix, AIN93-VX vitamin mix with 0 I.U. vitamin D3, Specialty Feeds, WA). Control females were fed a control diet (AIN93G + AIN93-G mineral mix, AIN93-VX vitamin mix with 1000 I.U. vitamin D3).

At birth, all dams and offspring were fed a standard diet (Rat and Mouse Cubes, Speciality Feeds). After weaning at three weeks of age, offspring were moved to separate housing under standard fluorescent lighting. Offspring were sacrificed at 21 and 140 days of age. Burne *et al* [144] utilising this model have reported neonatal vitamin D<sub>3</sub> levels of  $1.9 \pm 1.3$  ng/ml within rats from the vitamin D deficient conditions compared to  $27.4 \pm 9.6$  ng/ml measured in controls ( $n = 14$ ). Furthermore, Eyles *et al* [143] have demonstrated that, from two weeks after birth, vitamin D deficient offspring achieve vitamin D<sub>3</sub> levels comparable to controls. In addition, blood calcium, phosphorous and parathyroid hormone levels were also comparable to controls in adulthood.

### 3.1.2 Multigenerational Low Protein Model

Female Wistar rats were used to study the effect of maternal low protein diet during pregnancy on second generation female offspring, as described by Hoile *et al* [145].

Rats were raised in the University of Southampton Biomedical Research Facility under licence (number 70-6457) from the UK Home Office according to the Animals (Scientific Procedures) Act (1986).

An overview of the multigenerational study can be viewed in figure 3.1. Animals were housed in rooms maintained at 22 °C, with light and dark cycles of 12:12 hours ratio. Female rats (F0 generation) were maintained on standard chow for 14 days prior to being mated. Following this, mothers were assigned to control (protein sufficient) or protein restricted diets throughout gestation. Diet composition of control and protein restricted diets can be viewed in reference [145]. After birth of offspring (F1 generation), all mothers were fed AIN93G diet formulation during lactation (offspring were standardised to 8 pups in each litter, with preference towards females for subsequent breeding). After weaning at 28 days of age, all offspring were fed AIN93M diet formulation. For the F1 generation, mating occurred at 70 days of age and during gestation were fed the control, protein sufficient, diet. During lactation, mothers were again fed the AIN93G diet formulation. Offspring (F2 generation) were then fed the AIN93M diet formulation

and females were analysed at 70 days of age.

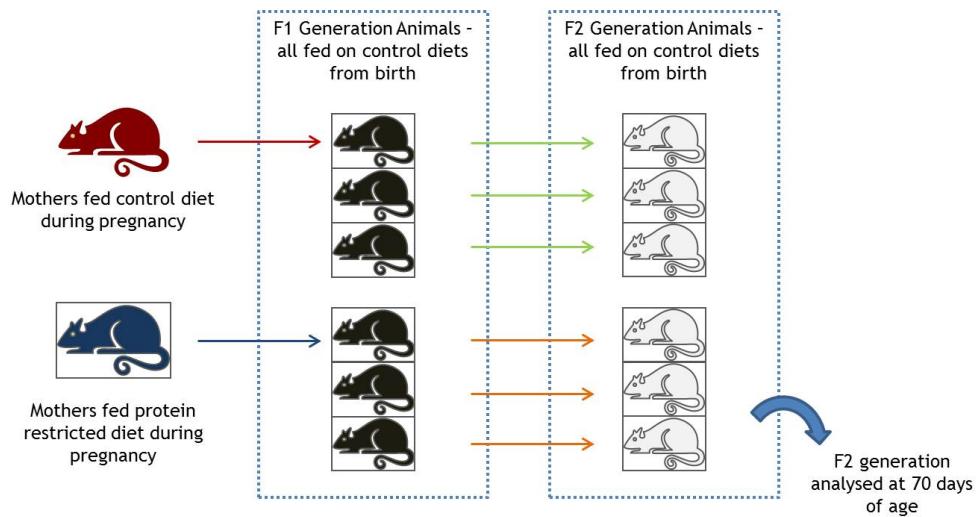


Figure 3.1: Multigenerational Low Protein Diet Experiment Design. A protein restricted diet was fed to grand-maternal dams to view the effects of dietary challenge on the F1 and F2 generations.

## 3.2 Biological Characterisation

### 3.2.1 Reverse Transcription Quantitative PCR

The complexity of the bone biology environment means assessment can be accomplished in numerous ways [146, 147]. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is a common molecular biology technique that is capable of quantifying gene expression within samples [148, 149] and hence can be utilised to examine many biological interests. As such, RT-qPCR is frequently employed to examine and compare gene expression between healthy and diseased states [150].

The principle of PCR is to amplify genes of interest within a starting sample of DNA, such that a measurable quantity is obtained and can be analysed [151]. Amplification can be conducted at the same rate within a number of samples so that the original inter-sample gene quantity differences present are maintained. With measurement of post-amplification gene quantity and knowing the amplifying rate, the original differences between samples can be calculated. Therefore, it is possible to compare gene expression differences in healthy and disease samples, for example.

Amplification of selected genes is achieved through a sequence of heating and cooling stages, which duplicates the amount of target gene transcripts after each cycle. Within a cycle, starting DNA double strands are separated and complementary DNA strands formed through the use of primer sequences to restore the double strand structure. Therefore, PCR occurs at an exponential rate. For the synthesis of complementary DNA strand, primers (small sequences of DNA designed to be complementary to the genes of interest, attaching at different sites along the gene) are required to bind onto separated strands before a polymerase enzyme constructs the remainder of the strand.

Stepwise, **denaturation** first occurs where samples are heated to 95 °C to break the sample DNA into single strands (this is also termed the melt phase). After this, **annealing** takes place where the temperature is then reduced to 50 °C to allow the primers to bind to the appropriate DNA locations. Finally, during the **extension** phase at 60 °C, the presence of a double strand (where the primer has attached onto a strand of starting

DNA) allows the polymerase to make a new complementary strand up to the site of the corresponding primer for the gene of interest. The amplification can be tracked through fluorescent markers that bind only to double strands, therefore measurement of light intensity at the end of each cycle indicates the quantity of DNA present. As mentioned previously, the fluorescent data along with the rate of amplification can be used to relate back to the starting DNA amount [152]. A simplified diagram demonstrating one RT-qPCR cycle can be seen in figure 3.2.

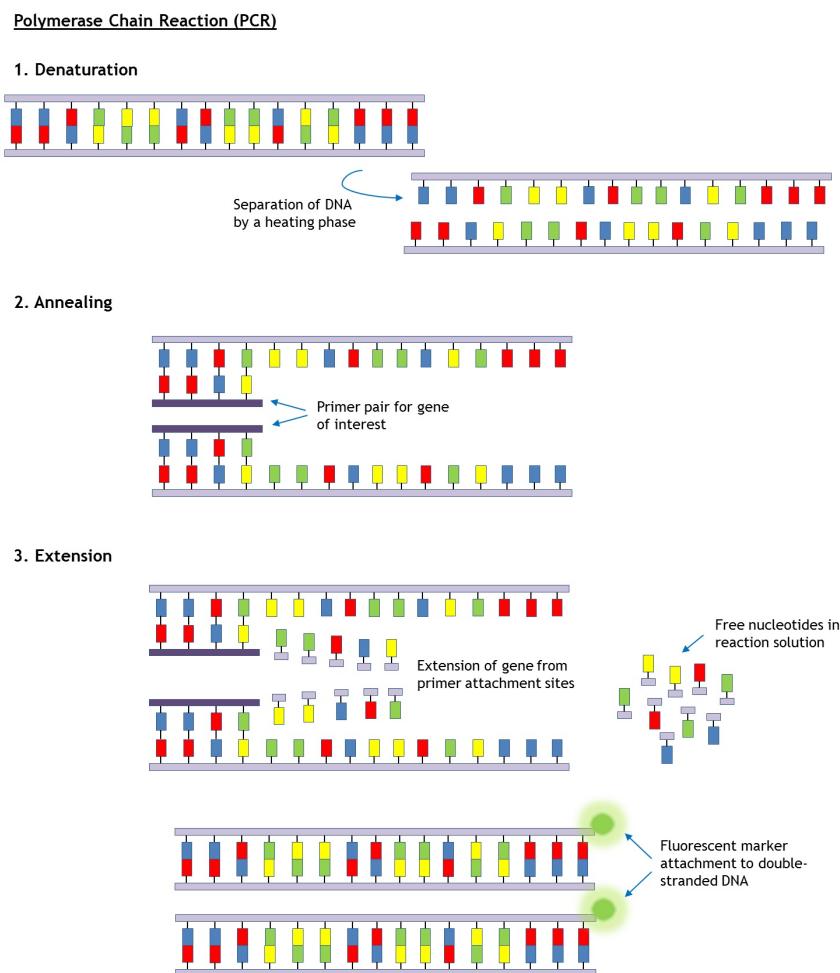


Figure 3.2: RT-qPCR. Process of one RT-qPCR cycle whereby starting DNA is separated and genes of interest are duplicated, thus doubling the starting amount at the beginning of the cycle.

### Osteoblastic Gene Expression Analysis

Rat femora were processed for molecular gene expression analysis within this thesis by RT-qPCR. Femora were excised from animals and soft tissue was removed from the bone. The proximal epiphyses (consisting of the femoral head and neck) were then removed and cut further into fragments using bone cutters (or a scalpel with younger, softer bones). Bone pieces were then washed with PBS to remove any marrow. The bone was then blot dried before 100 mg of bone fragments were added to 1 ml of TRIzol reagent (Life Technologies, Paisley, UK) and stored at -80°C until further processing.

For RNA extraction, TRIzol solutions were defrosted and extraction was performed according to manufacturer instructions. RNA sample purity was verified using absorbance at 260 nm on a NanoDrop 1000 Spectrophotometer (Labtech International Ltd, Uckfield, UK).

cDNA was generated from 500 ng RNA using a Superscript Vilo cDNA Synthesis kit (Life Technologies). The resultant 10 µl of cDNA suspension was further diluted with 70 µl of ultra pure water and stored at -20°C until needed for gene expression analysis by RT-qPCR.

cDNA was used for RT-qPCR analysis on an Applied Biosystems 7500 Real-Time PCR system with SYBR Green mastermix (Life Technologies). Target gene data was expressed in relation to a control gene and as  $2^{-\Delta Ct}$  values. Ribosomal 18s (*r18s*) and beta-actin ( $\beta$ -*actin*) were used as the control genes within this thesis. Primer sequences for housekeeping and target genes (*Runx2* - *Runx2*, alkaline phosphatase - *Alp*, collagen I - *Col1*, osteopontin - *Opn* and osteocalcin - *Ocn*) are shown in table 3.1.

A small validation study demonstrating the use of RT-qPCR to assess bone gene expression of cultured human bone marrow stem cells under different media can be viewed in Appendix A.

Gene	Forward Primer Sequence	Reverse Primer Sequence
<i>r18s</i>	5' - GCC GCG GTA ATT CCA GCT CCA - 3'	5' CCC GCC CGC TCC CAA GAT C 3'
$\beta$ - <i>actin</i>	5' - AGC CAT GTA CGT AGC CAT CCA - 3'	5' TCT CCG GAG TCC ATC ACA ATG C 3'
<i>Runx2</i>	5' GCC GGA ATG ATG AGA ACT A 3'	5' TTG GGG AGG ATT TGT GAA GA 3'
<i>Alp</i>	5' AGG CAG GAT TGA CCA CGG 3'	5' TGT AGT TCT GCT CAT GGA 3'
<i>Col1</i>	5' TGG CAA GAA CGG AGA TGA 3'	5' AGC TGT TCC AGG CAA TCC 3'
<i>Opn</i>	5' CTG CCA GCA CAC AAG CAG AC 3'	5' TCT GTG GCA TCG GGA TAC TG 3'
<i>Ocn</i>	5' CAG ACA CCA TGA GGA CCC TC 3'	5' GTC CAT TGT TGA GGT AGC GC 3'

Table 3.1: Primer sequences used for RT-qPCR testing of mRNA collected from rat bone collected at the proximal femur.

### 3.3 Structural and Compositional Characterisation

#### 3.3.1 $\mu$ CT Scanning

Modern advancements in imaging techniques have provided greater detail and information of bone at the nano- and micro- structure level [50, 153]. Micro-computed tomography ( $\mu$ CT) is an x-ray imaging technique capable of producing three dimensional digital reconstructions of small samples at high resolutions [115]. Consequently, information on small internal structures can be obtained non-destructively and as such  $\mu$ CT is a powerful tool to capture the complex morphology and microstructural details of bone. 3D reconstruction of bone structure has enabled accurate morphometric measurements of parameters such as trabecular thickness and separation, which have been demonstrated to be clinically important factors towards fracture risk and mechanical strength [115]. Modern scanners also have the resolution capability to distinguish between different levels of bone mineralisation [154], facilitating the study bone events at the cellular level.

$\mu$ CT scanners function by sending x-rays (originating from an x-ray tube) through a sample of interest (figure 3.3). These rays are then attenuated by the sample depending on the atomic composition of the penetrated regions, the degree of which is described by the linear attenuation coefficient. These attenuated x-rays form a projection of the sample that is captured by a detector behind the sample, which converts the x-rays to visible light. This procedure is performed over a number of projections at different angles of the sample to form a 3D representation of the object of interest from the collected linear attenuation coefficients through reconstruction algorithms that provide a three dimensional map of the attenuation coefficients [155].

One of the major advantages of  $\mu$ CT is that it is a non-destructive technique that can be used to assess patients or live samples, an important aspect when clinical evaluation and longitudinal studies are being considered. Use of both  $\mu$ CT measurements and BMD values has shown that it can be highly correlated with biomechanical properties of the imaged bone, providing a possible method to evaluate bone strength and fracture risk [156].

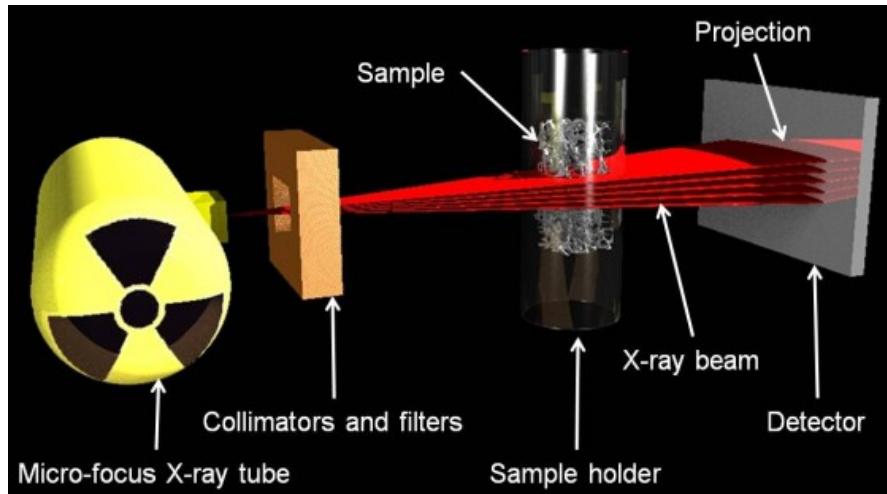


Figure 3.3: Working Principle of  $\mu$ CT. From a radiation source, x-ray beams are sent through the sample and the degree of attenuation is captured by a detector. Multiple scans are taken at different sample angles to recreate the sample digitally. Image from b-cube website, 2012 [155].

### $\mu$ CT Bone Architecture Analysis

Femora were  $\mu$ CT scanned by using a SkyScan 1176 *in vivo* scanner (Bruker microCT, Kontich, Belgium) at 18  $\mu$ m resolution (source voltage of 65 kV, current of 385  $\mu$ A, 0.5 mm Al filter and exposure time of 390 ms). Reconstruction was performed in NRecon software (version 1.6.4.6, Bruker) before microarchitectural analysis was conducted using CTAn software (version 1.11.9.3, Bruker). Cortical bone regions from the midshaft and trabecular bone regions from the distal femur were isolated out for further processing. The midshaft region was defined to be within  $\pm 5\%$  of the total femur length around the middle point of the femur. The distal femur was defined as the region between the middle of the femur and the beginning of the primary spongiosa. Biological landmarks rather than absolute distances were used to define bone regions to account for any differences in bone length between experimental and control conditions. The midshaft and distal femur regions are displayed in figure 3.4.

The bone from the cortical region of interest was segmented out within CTAn using a tasklist adapted from the Bruker technical notes (Appendix B). Once completed, bone was separated from non-bone regions using the Otsu threshold method and bone parameters were obtained. Bone from the trabecular region was segmented from cortical

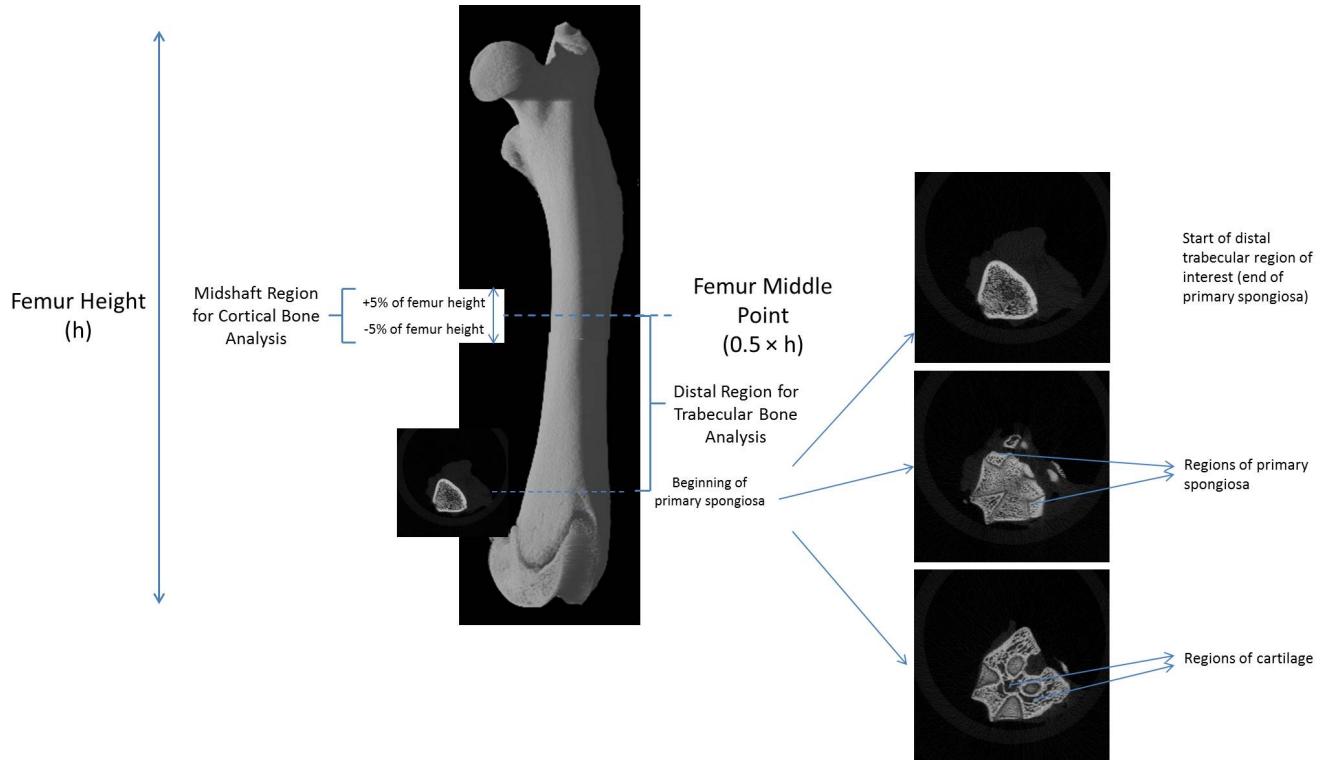


Figure 3.4: Cortical and Trabecular Bone Segmentation Regions. For  $\mu$ CT femur structure analysis, cortical bone regions were derived from the midshaft area, defined to be  $\pm 5\%$  of the total bone length around the midpoint. Trabecular bone regions were derived from the distal femur, this region defined as between the femur midpoint and the beginning of the primary spongiosa (defined manually).

bone in CTAn by manually tracing inside the endosteal surface at periodic intervals. The whole region of interest was obtained through an interpolation algorithm between manually defined slices as recommended by Bruker. Trabecular bone was then separated from remaining tissue using an adaptive threshold method before bone parameters were similarly obtained (CTAn tasklist can be viewed in Appendix B). Segmentation of bone from  $\mu$ CT scans for subsequent analysis is a highly important procedure that can impact upon output data and hence affect the reliability of analyses. Automatic thresholding is a technique that aims to separate object and background pixels without user input bias. However, inaccuracies can arise between samples from the automated process due to sample dependent differences in factors such as pixel intensity distribution [157], thus affecting the reliability of comparison between samples. Within this thesis, the global Otsu thresholding method was applied [158] and has been shown to accurately distinguish between images with bimodal pixel distribution [159], as is the case with bone scans where object and background elements are well defined (bone is a high density

region against low density background, such as air). Therefore the application of the Otsu method was deemed robust enough to use within the samples analysed and indeed review of the grayscale thresholds after segmentation in each scan confirms this (88 in all samples from both day 21 and day 140 rat cohorts).

Assessed parameters for both cortical and trabecular bone were chosen as recommended by the guidelines from Bouxsein *et al* (mean cross-sectional area, mean cortical bone area, mean bone area fraction and mean cortical bone thickness for cortical regions; bone volume to tissue volume percentage, trabecular number, trabecular thickness and trabecular separation for trabecular regions) [157]. Definitions of these parameters are;

**Mean cross-sectional area** (A), the mean cross-sectional of area enclosed by periosteum at the femoral midshaft.

**Mean cortical bone area** (Co.Ar.), the mean area of bone at the femoral midshaft.

**Mean bone area fraction** (Co.Fr.), the area percentage that is bone within the total area of the femur midshaft (A).

**Mean cortical bone thickness** (Co.Th.), the mean thickness of the cortical ring at the femur midshaft.

**Bone volume to tissue volume percentage** (BV/TV), proportion of distal trabecular volume that is made up of bone tissue.

**Trabecular number** (Tb.N.), number of traversals across trabecular structures per unit length.

**Trabecular thickness** (Tb.Th.), the average thickness of trabecular struts within the VOI.

**Trabecular separation** (Tb.Sp.), the thickness of spaces between trabecular struts.

Additionally, bone mineral density (BMD) measurements were obtained from trabecular bone regions and tissue mineral density (TMD) values were obtained from the cortical bone regions from the  $\mu$ CT data calibrated against hydroxyapatite phantoms of known density. BMD represents the amount of bone that is present in a volume of interest that has a mixture of tissues (and hence densities), such as with trabecular bone and marrow within the femur medullary cavity (figure 2.1, section 2.1). TMD refers to the density

of bone when the volume of interest includes only bone tissue, such as with the cortical bone in the midshaft femur.

## 3.4 Mechanical and Material Characterisation

Changes in bone mechanical properties may stem from a number of possible modifications, most notably in mass or architecture along the length scales [35, 160, 161] (reviewed in more detail in section 2.2). Consequently, it is advantageous to examine bone at different levels across the hierarchical structure and this is achieved within this project through micromechanical and whole bone mechanical evaluation. Additionally, fracture toughness is also assessed.

### 3.4.1 Reference Point Indentation

Reference point indentation (RPI) has been developed as a method to measure bone mechanical properties at the tissue level [162], particularly to evaluate the resistance of the bone material to crack growth and eventual fracture [163–165]. Critically, RPI offers the benefit of directly assessing bone material properties with limited sample preparation [166].

During RPI testing, a probe consisting of a reference and test probe (figure 3.5) is positioned onto the bone surface. A set pre-load is applied through the reference probe onto the surface such that the surface of the bone is identified. The inner test probe is then used to indent the bone at a defined load in; i) load, ii) hold and iii) unload phases during one cycle (figure 3.6). Multiple cycles are conducted at one site (figure 3.7) to obtain data on mechanical behaviour in response to repeated indentations. Each indentation distance (relative to the reference probe) is recorded and a typical test utilises ten load-unload cycles. Each cycle occurs at constant maximum applied force and frequency (both determined before testing and dependent on sample age, location, hardness etc) [166].

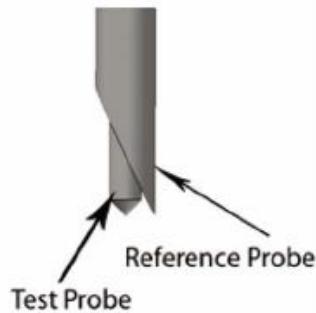


Figure 3.5: Microindentation Probe. The microindentation probe consists of a reference probe and a test probe. Figure from Hansma *et al* [162].

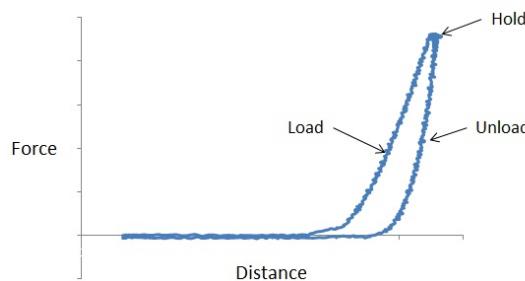


Figure 3.6: Example Indentation Curve. Force versus distance indentation curve with load, hold and unload regions during one cycle. Courtesy of Dr Tom Jenkins, Bioengineering Research Group, University of Southampton.

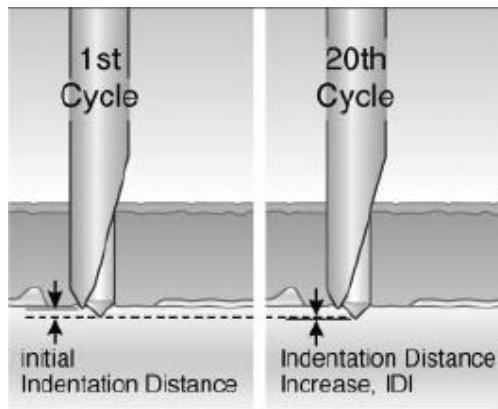


Figure 3.7: Microindentation Cycles. For each microindentation cycle, the reference probe is loaded on the bone surface and the testing probe is used to indent the bone. The indentation distance (in relation to the reference probe) is recorded. IDI is the difference between the first and last indentation distances. Figure from Diez-Perez *et al* [166].

The indentation measurements are used to calculate the total indentation distance ( $TID$ ), indentation distance increase ( $IDI$ ), creep indentation distance ( $CID$ ) and energy dissipated ( $ED$ ). Derivation of the these measurements can be viewed in figure

3.8. In hard tissue (such as bone), *TID* is the total distance that is measured after all indentation cycles have been complete, *IDI* is the difference between the indentation measured at first and last cycles, *CID* is the distance indented during the hold phase of an indentation cycle and the *ED* measurement is calculated from the area underneath each indentation curve and represents the non-recoverable response to indentation (likely to include contributions from material elasticity/plasticity, viscous behaviour [167] and cracking). Further details of these parameters can be found in the work by Diez-Perez *et al* [166]. Greater indentation distances have been implied to represent poorer resistance to fracture [166] and hence RPI offers the possibility of evaluating bone material properties and the degree to which bone samples are able to resist fracture.

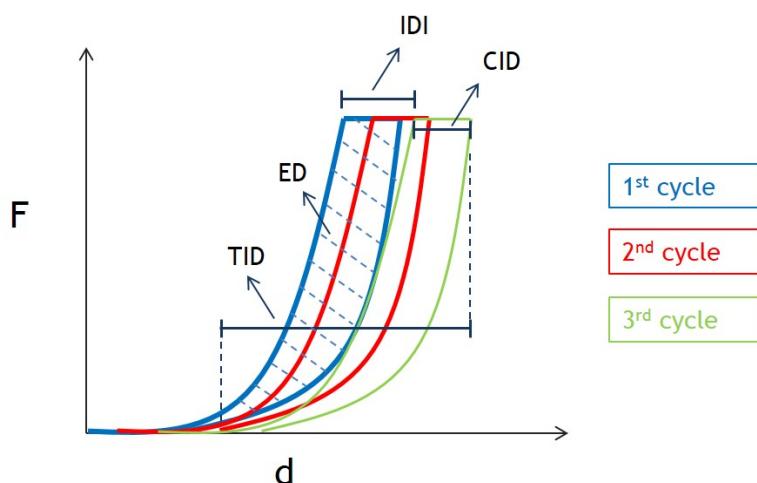


Figure 3.8: RPI Parameters. The diagram above displays the different measurements from a RPI test. Total indentation distance (*TID*) is the total distance indented after all indentation cycles are completed, indentation distance increase (*IDI*) is the distance difference between the first and last indentation cycles, creep indentation distance (*CID*) is the distance indented during the hold phase of a load cycle (*CID* is outlined for the third cycle in this example) and energy dissipated (*ED*) is derived from the area underneath an indentation curve (*ED* for the first cycle presented in this instance).

### Bone Micromechanics Analysis

Indentation was performed with a Biodent reference point indenter (Active Life Scientific, Santa Barbara, USA) at seven sites along the anterior surface (figure 3.9); the femoral head, femoral neck, three points along the midshaft, and twice at the distal

epiphysis (one at the top of the condyles and one between the condyles). Samples were rehydrated in PBS and excess soft tissue was removed from samples with a scalpel before testing. For each indentation, a maximum load of 5 N was applied for 10 cycles at 2 Hz, with defined preload of between 650 g to 700 g on the bone. Data was recorded using the built-in Bident software and measured variables were total indentation distance (TID), indentation depth increase (IDI), creep indentation distance (CID) and energy dissipated.

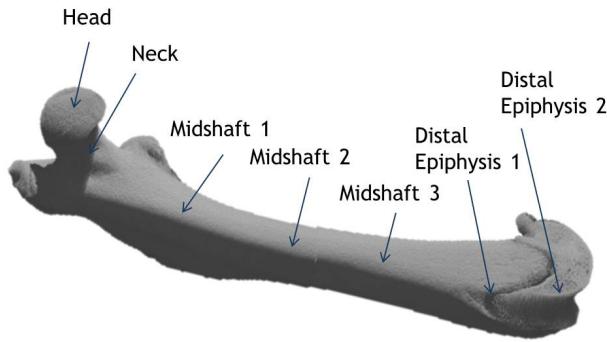


Figure 3.9: RPI Femur Locations. Diagram of indentation locations. Indents were performed at the femoral head, femoral neck, three points along the mid-shaft, at the top of the condyles and finally between the condyles.

### 3.4.2 Fracture Toughness Testing

The fracture toughness of bone is an important measurement that characterises how capable bone is of opposing crack initiation, crack growth and fracture. As such, fracture toughness describes an inherent property of bone material, independent of bone-matrix structure contributions [55]. The ASTM standard (E1820) for obtaining fracture toughness values involves loading a pre-cracked (notched) sample until either stable or unstable crack extension. During testing, load and displacement data is recorded at regular time intervals and linked to crack extension to calculate toughness. Usually, samples are cracked with a single-edge bend (SEB) or compact tension flaw (CT). In both cases the flaw is subjected to tensile forces to drive crack propagation; this is achieved through three-point bending of the specimen in SEB, whilst tensile forces are applied perpendicular to the crack in CT samples.

Within engineering materials, coupons are usually manufactured for mechanical testing to provide data. Due to the nature of bone development and particularly for small samples, it is necessary to adapt standard fracture toughness protocols to account for factors such as geometrical differences found naturally in bone. Methods have been described by Ritchie *et al* [55] for small animal bone testing and involve notching of long bones and placing specimens into a three point bend testing arrangement so that the notch is loaded in tension (similar to sample set-up in SEB testing, but with an

irregular tubular sample present by the long bone diaphyses). Subsequent fracture surfaces (figure 3.10) are then imaged through scanning electron microscopy (SEM) so that measurement of the crack growth regions is possible. The load (from the loading test) to drive a crack of known length (measured from the SEM imaging) can then be used to obtain a fracture toughness value,  $K_{init}$ , through equation 3.1.

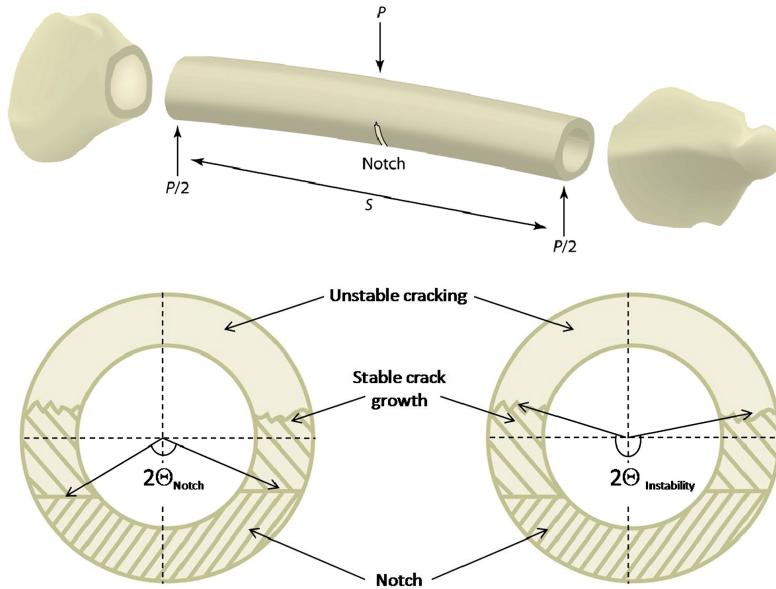


Figure 3.10: Fracture Toughness Testing. Loading configuration of bone during testing and diagram of surface topography after fracture toughness testing. The notched, stable and unstable crack growth regions are evidenced through different topography and the angles of these regions can be used to obtain a  $K_{init}$  value. Reprinted from Bone, 43(5), R.O. Ritchie *et al*, Measurement of the Toughness of Bone: A Tutorial with Special Reference to Small Animal Studies, 798-812, 2008, with permission from Elsevier [55].

$$K_{init} = F_b \frac{P_s S R_o}{\pi (R_o^4 - R_i^4)} \sqrt{\pi \theta_c R_m} \quad (3.1)$$

Where  $K_{init}$  is the toughness value for crack initiation,  $F_b$  is a geometry factor,  $P$  is the applied load,  $S$  is the length of the sample,  $R_o$  is the outer radius of the bone shaft,  $R_i$  is the inner radius of the bone shaft,  $\theta$  is the crack angle and  $R_m$  is the mean radius.

### Fracture Toughness Analysis

Fracture toughness assessment was performed based on recommendations on testing murine bones as described by Ritchie *et al* [55]. Briefly, the right femur was prepared for notched fracture toughness testing by first removing the remaining epiphysis (distal) using a low speed saw (Isomet, Buehler, Illinois, USA). The bone marrow within the femur shaft was removed using a jet of water and circumferential throughwall notches were then machined into the middle of the shaft on the anterior side, again by using a low speed saw. Notches were then sharpened using a scalpel blade whilst irrigated with 1  $\mu\text{m}$  diamond solution, with samples cleaned of the diamond solution through water bath sonification.

Mechanical testing conducted with the notch in tension (anterior side down) on a 10 mm span within a three point bending rig arrangement, with samples sub-merged in Hanks balanced salt solution (HBSS) (Life Technologies) to keep samples hydrated. Loading was applied at a rate of 0.001 mm  $\text{s}^{-1}$ , with force and displacement recorded, to induce crack propagation using an Electroforce 3200 mechanical tester (Bose, Minnesota, USA) until failure.

For analysis, fracture surfaces were dried and gold coated for imaging by scanning electron microscopy (SEM) (JSM-6500F, JEOL, Peabody, USA) to determine the angle of crack initiation (equivalent to the length of the starter notch) corresponding to the maximum load and initiation toughness. This angle was then used to calculate initiation toughness ( $K_{init}$ ) through equation 3.1 using the maximum load method of fracture toughness (due to difficulty in identifying the instability angle within some of the fracture surfaces) calculation for small animal bones.

### 3.4.3 Strength Testing

As bone deterioration occurs at both cortical and trabecular sites, it is of interest to examine the mechanical properties of the whole bone at the organ level [115]. A summary of strength testing has been published by Turner [168] but generally whole bone mechanics are traditionally investigated through tensile, compressive and bend testing. Within this review we will focus on bend testing due to the sample dimensions.

The force-displacement curves that result from three-point bend tests are able to inform upon values such as work to failure, strength and stiffness for tested samples (figure 3.11). With geometrical data, force-displacement curves can be used to derive stress-strain relationships [168] whereby material properties, such as Young's modulus, can be calculated.

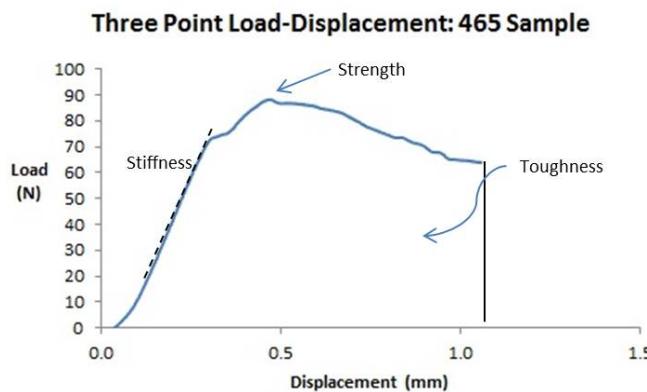


Figure 3.11: Three Point Bend Strength Testing. An example load-displacement graph obtained from three point bend testing.

### Three-Point Bending Analysis

The left femora were mechanically tested until failure within a three point bend arrangement using an electromechanical testing machine (Instron, High Wycombe, UK) to measure strength - different machines were used for fracture toughness and strength due to the higher loads necessary for fracture in the strength testing). Samples were placed on two supports of 10 mm span within a water bath filled with HBSS so samples stayed hydrated and loaded at  $0.01 \text{ mm s}^{-1}$  until fractured. Maximum loads were

recorded from experimental data.



## Chapter 4

# Multigenerational Effect of Maternal Low Protein and Multiscale Analysis of Bone Quality



## 4.1 Introduction

Bone fracture mechanisms are highly complex and a number of events and contributory factors are often involved [38, 63]. Prevention of fracture therefore is equally complicated and primarily this is a result of the intricate hierarchical nature of bone material. The bone quality framework [35, 47] is a concept that attempts to consider properties along the length scales that modulate overall bone strength and current research is invested into finding metrics that can be applied clinically to augment current measures in use. A number of techniques have been proposed to measure bone health [50] to achieve a comprehensive assessment of bone health.

Extending the multifactorial nature of bone quality (and therefore bone fracture risk), diseased bone states are often associated with numerous risk and causation factors [70, 169–172]. An emerging consideration is the developmental origins of health and disease (DOHaD) approach, positing that *in utero* experiences can shape risk to certain diseases in adult life [127]. de Assis *et al* [173] observed that this effect is present in multiple generations, whereby pregnant rats on high fat diets increased the risk of mammary tumourgenesis in both daughters and granddaughters. The candidate mechanism for this effect is thought to be primarily epigenetic in nature [174], where *in utero* experiences can modulate the epigenome and persists through to later life.

The complexity of bone diseases, such as osteoporosis, has led to the exploration of the role of DOHaD principles as a candidate risk factor [8]. Research by Lanham *et al* [131] has shown that maternal low protein diets modify the osteogenic environment of offspring during skeletal development with peak levels of alkaline phosphatase activity occurring four weeks earlier compared to controls as well as low protein background females having lower levels of IGF-1 at four weeks of age whilst osteocalcin levels were higher. Investigation into the effects later in life showed an altered bone structure at 75 weeks of age, with animals from a low protein background *in utero* presenting thinner, less dense trabecular bone within the femoral head and increased thickness of trabecular and cortical bone at the femoral neck and femur midshaft respectively [175].

This pilot study was conducted to examine the possibility of utilising of multiple techniques to produce bone health metrics from each murine sample (from a multigenerational low protein rat model).

### Objectives

With this approach, we i) investigate the effect of maternal low protein during pregnancy on second generation skeletal health, employing techniques to assess the genetic expression (RT-qPCR), micromechanics (reference point indentation), material properties (fracture toughness) and overall mechanical behaviour (three point bend testing); and

ii) investigate whether measured micromechanical and material properties can influence overall bone mechanics, as well as any genetic basis of these as viewed by gene expression data. It is predicted that this will begin the establishment of cause and effect that can then be used to predict important multiscale parameters that affect fracture toughness to inform treatment or preventative therapies.

## 4.2 Methods

### Animal Experiment Design

Females from a Wistar multigenerational low protein study were analysed. Maternal protein restriction was induced according to the method described by Hoile *et al* [145] and an overview of experimental design is illustrated in section 3.1.2, figure 3.1. Briefly, female rats were fed with either a control or protein restricted diet from conception until birth. Female offspring (F1 generation) were then weaned onto a control diet and mated. Subsequent offspring (F2 generation) were then also fed a control diet after weaning.

F2 female murine samples were assessed at 70 days of age, whereby animals were culled and stored in -80 °C until required for testing. Upon testing, hind limbs were rapidly thawed in a water bath and both femora and tibiae were retrieved and prepared for subsequent testing.

### Osteoblast Gene Expression Analysis

The right femur was processed for molecular analysis of osteoblast gene expression using RT-qPCR, as described in section 3.2.1. Target gene data was expressed in relation to a control gene and as  $2^{-\Delta Ct}$  values.  $\beta$ -actin was used as the gene control and *Runx2*, alkaline phosphatase (*Alp*) and osteocalcin (*Ocn*) were used for target genes.

### Bone Micromechanics Analysis

Indentation was performed on the left femur with a Biodent reference point indenter (Active Life Scientific, Santa Barbara, USA) at seven sites along the anterior surface; the femoral head, femoral neck, three points along the midshaft, and twice at the distal epiphysis (one at the top of the condyles and one between the condyles) (see section 3.4.1). Indentation data was recorded to produce indentation depth increase (IDI), average creep indentation distance (CIDavr) and last cycle creep indentation distance

(CIDL) measurements.

### Whole Bone Mechanics Analysis

The left tibiae were mechanically tested until failure within a three point bend arrangement (described in section 3.4.3) using an electromechanical testing machine (Electroforce 3200, Bose, Minnesota, USA) to measure strength. Samples were placed on two supports of 15 mm span within a water bath filled with HBSS so samples stayed hydrated and loaded at  $0.01 \text{ mm s}^{-1}$  until fractured. Maximum loads were recorded from experimental data.

### Fracture Toughness Analysis

Fracture toughness assessment was performed based on recommendations on testing murine bones as described by Ritchie *et al* (further detail in section 3.4.2). Briefly, the right tibiae were prepared for notched fracture toughness testing in a three-point bend testing arrangement. Loading was applied at a rate of  $0.001 \text{ mm s}^{-1}$ , with force and displacement recorded, to induce crack propagation using an Electroforce 3200 mechanical tester (Bose, Minnesota, USA) until failure. Fracture surfaces were then imaged through scanning electron microscopy (JSM-6500F, JEOL, Peabody, USA) and utilised for determining fracture toughness.

A subset of left tibiae was also indented prior to fracture toughness testing using the previously described method. Six indents were performed along the posterior, lateral and medial faces of the tibia midshaft.

### Statistical Analysis

Statistical analysis between the control and protein deficient groups was performed using unpaired t-tests, with Welch's correction to account for unequal standard deviation on data sets (Graphpad Prism, La Jolla, USA). All data sets were tested for Gaussian

distribution and significance determined at  $p < 0.05$ .

For the correlative study, gene expression (*Runx2*, *Alp* and *Ocn*), tibia RPI (IDI, average CID and last cycle CID), strength testing (maximum load) and fracture toughness ( $K_{init}$ ) data were analysed through univariate linear regression (Graphpad Prism, La Jolla, USA), with significance determined at  $p < 0.05$ .

## 4.3 Results

### 4.3.1 Effect of Maternal Low Protein on F2 Generation Bone Health

#### Gene Expression

Osteoblast RNA retrieved femoral head and neck was analysed for gene expression related to osteogenesis to assess the bone cellular environment. For the three genes investigated, no statistical differences were found between the control and protein deficient animals (figure 4.1). For all three genes interrogated, the control group displayed higher mean expression when compared to the animals from the low protein background.  $n = 5$  for each dietary group, one control sample was omitted from the analysis due to results being a magnitude larger than other samples tested.

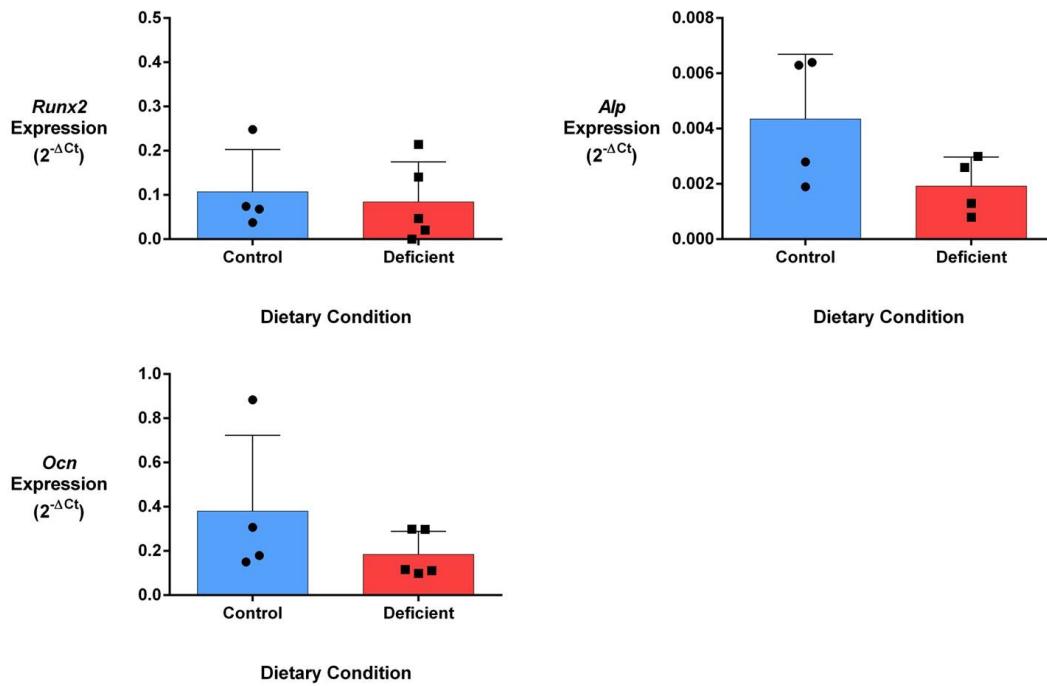


Figure 4.1: Multigenerational Low Protein F2 Gene Expression. Expression levels of *Runx2*, *Alp* and *Ocn* were assessed and no statistical differences were observed between the control and protein restricted background animals. *Runx2* and *Ocn* analysis:  $n = 4$  for control and  $n = 5$  for protein restricted group, *Alp* analysis:  $n = 4$  for control and  $n = 4$  for protein restricted group. Mean and standard deviation presented.

Reference Point (Micro) Indentation

Reference point indentation (RPI) experimentation and analysis was conducted in collaboration with Dr Louise Coutts, Bioengineering Research Group, University of Southampton. RPI was performed along the anterior surface of the femur bone length, with last cycle creep indentation distances recorded. Five of the seven locations along the femur (head, neck, diaphysis 2, diaphysis 3 and epiphysis 2) were found to result in higher mean indentation distances in the protein deficient cohort. However, none of the comparisons at each site between the control and experimental groups produced significant differences (figure 4.2).

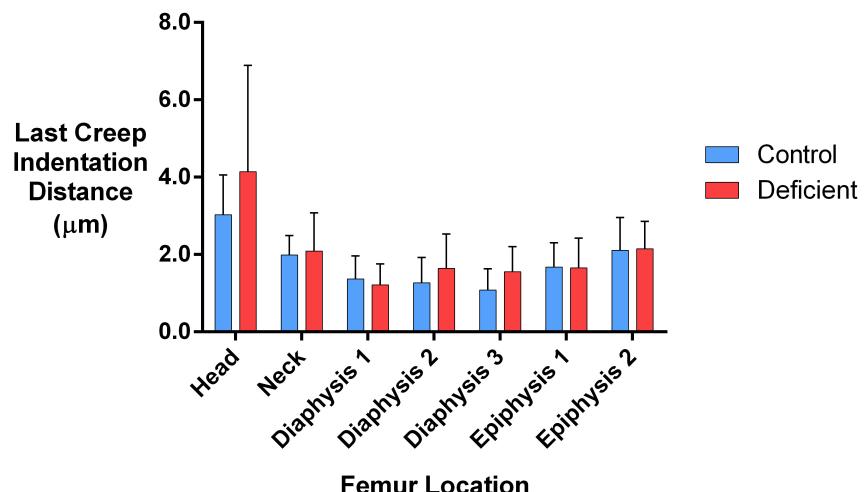


Figure 4.2: Multigenerational Low Protein F2 Mean Last Cycle Creep Indentation Distance: Femur. Creep distances along the femur recorded from the last hold phase of the RPI cyclical loading. No significant differences were observed at any site between the two dietary groups.  $n = 5$  for each dietary group, mean and standard deviation at each femur location presented.

Additionally, indentations tests were also carried out along tibiae, with six indents along the posterior, medial and lateral faces of the midshaft (18 indents per sample in total,  $n = 3$  for each dietary group, with one control sample lost during testing). Indentation distance increase (IDI), average creep indentation distance (CID) and last cycle creep indentation distance (CIDL) were compared between the two dietary groups. Little difference in mean values were observed between the two dietary groups and no statistical

changes were found (figure 4.3).

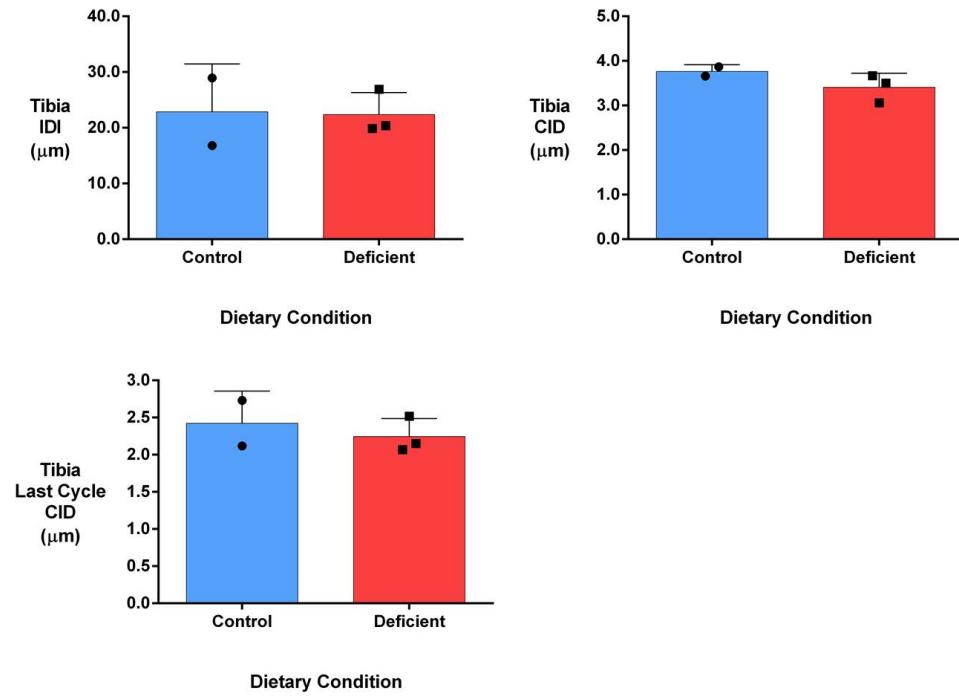


Figure 4.3: Multigenerational Low Protein F2 Reference Point Indentation: Tibia. Indentation measurements were conducted on tibia midshafts and indentation distance increase (IDI), average creep indentation distance (CID) and last cycle creep indentation distance (CIDL) were recorded for each sample at 18 different locations. A mean value for each sample was obtained from all the sites and all means were compared between the control and protein restricted background groups. No statistical differences were observed in the three different measures.  $n = 2$  for control and  $n = 3$  for protein restricted group, mean and standard deviation presented.

#### Tibia Maximum Load and Fracture Toughness

Mechanical testing was conducted on tibiae through three-point bend loading and fracture toughness testing (notched bend testing). Comparison of three-point bending (maximum load) and fracture toughness ( $k_{init}$ ) results yielded no statistical differences between the two dietary conditions (figures 4.4 and 4.5). Again, mean maximum load and fracture toughness was found to be higher in the control group. Due to experimental error during test set-up for maximum strength, one control sample was lost before data was collected.

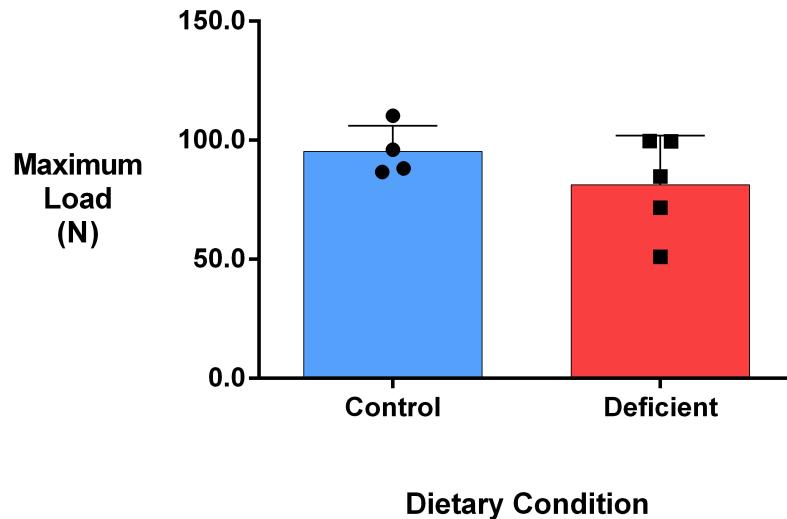


Figure 4.4: Multigenerational Low Protein F2 Tibia Maximum Load. Comparison of maximum loads recorded during three-point bend testing of tibiae showed no differences between the control and protein restricted background groups.  $n = 4$  for control and  $n = 5$  for protein restricted group, mean and standard deviation presented.

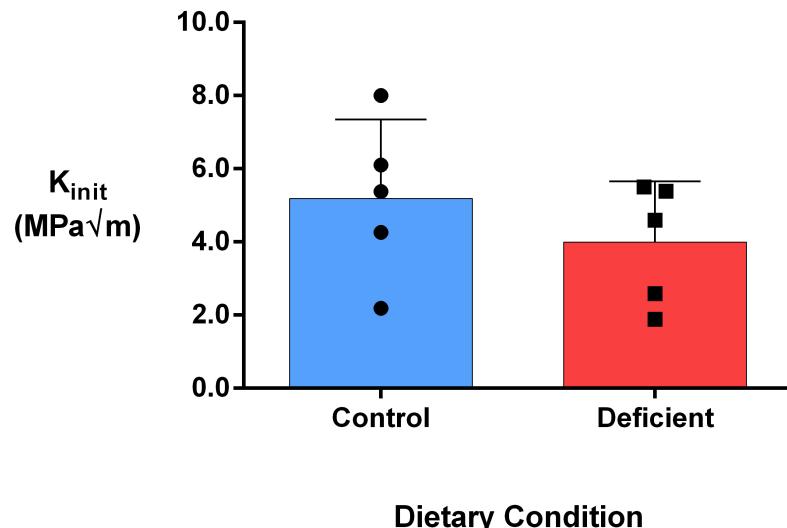


Figure 4.5: Multigenerational Low Protein F2 Tibia Fracture Toughness. Tibia fracture toughness was measured through notched bend testing. No differences were observed between the two dietary conditions.  $n = 5$  for each dietary condition, mean and standard deviation presented.

### 4.3.2 Multiscale Analysis of Bone Health Relationships

Gene expression (*Runx2*, *Alp* and *Ocn*), tibia RPI (IDI, average CID and last cycle CID), strength testing (maximum load) and fracture toughness ( $K_{init}$ ) data were analysed through univariate linear regression to investigate bone health relationships of individual parameters across the biological and physical interface and through different length scales.

Data was initially entered into a correlation grid and  $r$  values calculated between all variables (table 4.1).  $r$  values of  $r > |0.707|$  (resulting in  $r^2 > 0.5$ ) were then selected for additional statistical analysis. Using this criteria; *Runx2* and *Alp* expression, *Runx2* expression and average CID, *Alp* expression and average CID, *Ocn* expression and  $K_{init}$ , IDI and last cycle CID, IDI and  $K_{init}$ , and last cycle CID and  $K_{init}$  values were investigated further.

Correlation Grid								
	<i>Runx2</i>	<i>Alp</i>	<i>Ocn</i>	<i>IDI</i>	<i>Creep</i>	<i>Creep L</i>	<i>Mx Load</i>	<i>FracTou</i>
<i>Runx2</i>		0.988	0.229	0.683	0.956	-0.060	0.108	-0.061
<i>Alp</i>			0.122	0.700	0.996	-0.616	0.099	-0.331
<i>Ocn</i>				-0.379	0.394	0.620	0.659	0.764
<i>IDI</i>					0.498	-0.734	-0.446	-0.810
<i>Creep</i>						0.207	0.226	0.092
<i>Creep L</i>							0.623	0.952
<i>Mx Load</i>								0.690
<i>FracTou</i>								

Table 4.1: Multiscale Analysis Correlation Grid.  $r$  values between gene expression, tibia RPI, maximum loading and fracture toughness measurements were calculated for potential relationships. Values of  $r$  above 0.9 are highlighted in yellow.

Of these, *Runx2* expression and *Alp* expression ( $p < 0.01$ ), *Runx2* expression and average CID ( $p = 0.01$ ), *Alp* expression and average CID ( $p < 0.01$ ), and last cycle CID and  $K_{init}$  ( $p = 0.01$ ) were found to be significant and plots are displayed below.

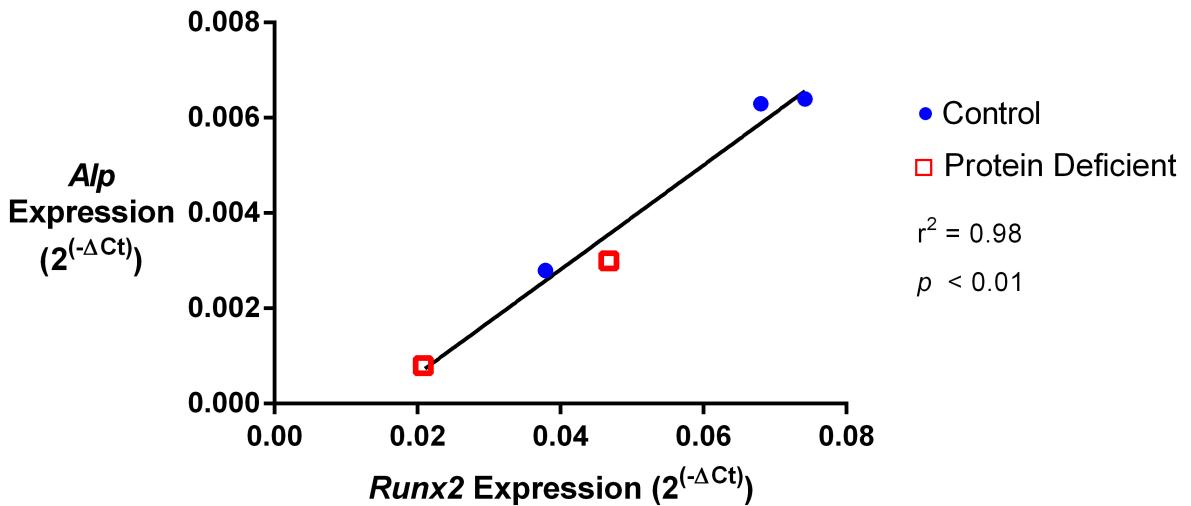


Figure 4.6: Multigenerational *Runx2* and *Alp* Expression Correlation. A positive correlation was observed between expression levels of *Runx2* and *Alp*,  $n = 5$ .

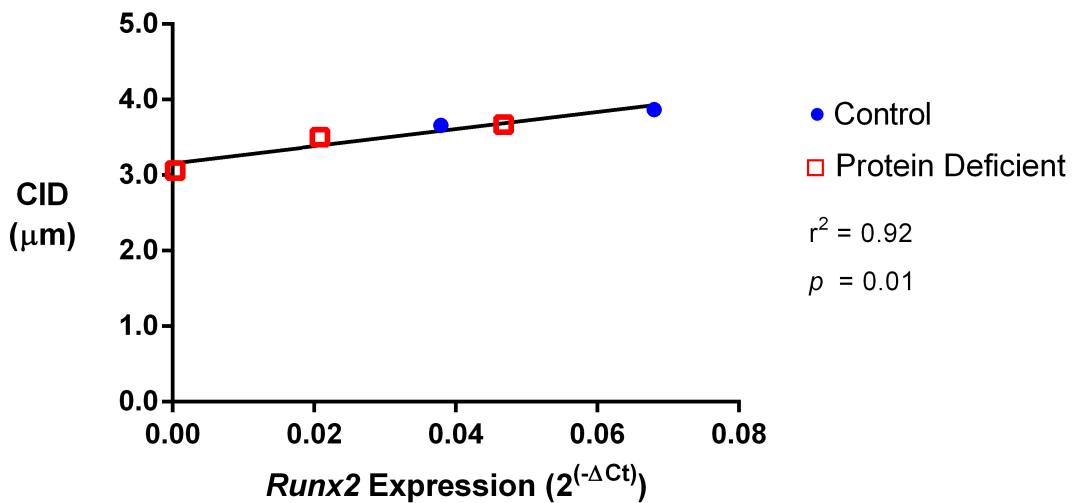


Figure 4.7: Multigenerational *Runx2* and Average Creep Indentation Distance Correlation. An increase in expression levels of *Runx2* and increased average CID was observed,  $n = 5$ .

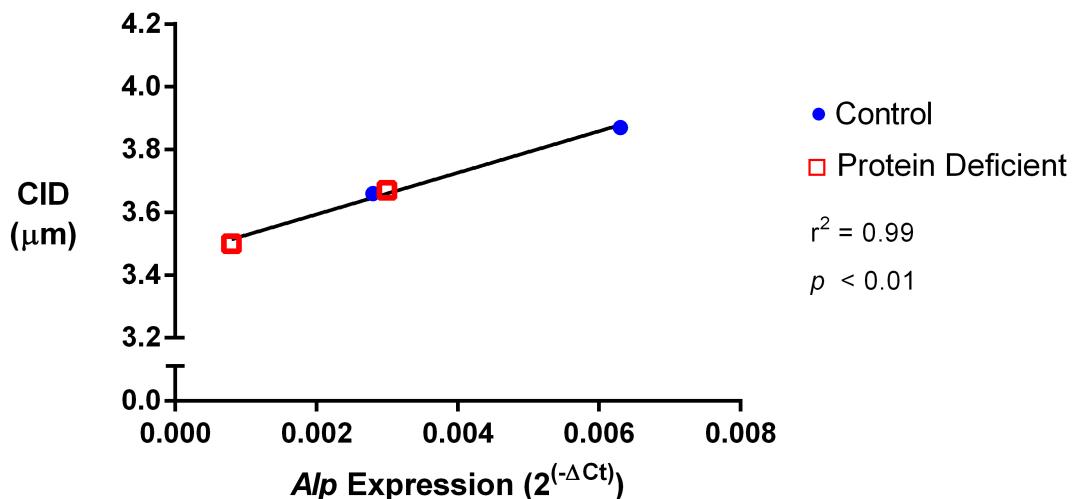


Figure 4.8: Multigenerational  $Alp$  and Average Creep Indentation Distance Correlation. Higher expression levels of  $Alp$  was positively associated with higher CID,  $n = 4$ .

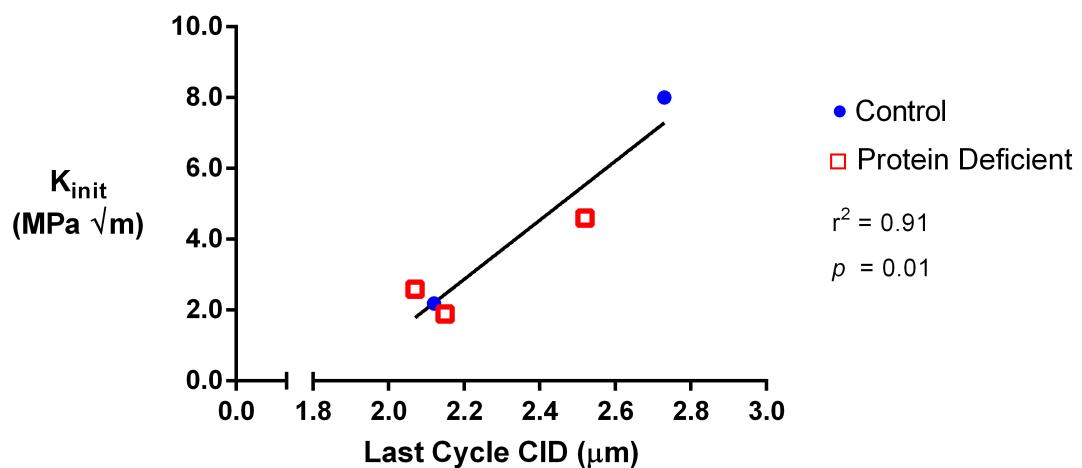


Figure 4.9: Multigenerational Last Cycle Creep Indentation Distance and Fracture Toughness Correlation. CID measurements of the last indentation cycle was found to be positively correlated with fracture toughness measurements,  $n = 5$ .

## 4.4 Discussion

### 4.4.1 Low Protein Multigenerational Study

This pilot study examined the effect of maternal low protein intake during pregnancy on skeletal development of second generation (F2) female offspring in rats. No statistical differences were found between low protein background and control F2 females at 70 days of age for any of the bone metrics measured (gene expression, micromechanics, fracture toughness and whole bone mechanics). Although not significant, rats from a low protein background exhibited lower mean osteogenic gene expression (*Runx2*, *Alp* and *Ocn*), greater indentation distance in micromechanics analysis, lower fracture toughness and lower maximum load during three-point bend testing. These results suggest that no change persists, or is incurred, to bone health in second generational offspring as a result of maternal low protein status, though consistent trends of low protein animals having reduced gene expression and mechanical competence may highlight an adaptive response.

Precedence for the effects of grand-maternal diet on adult health has been suggested by recent studies. King *et al* [176] found that diet induced obesity within mothers resulted in increased insulin levels, reduced birthweight and modifications in hepatic gene expression in the second generation, even though limited differences were displayed at the first generation. de Assis *et al* [173] also showed in rats that mothers exposed to high fat diets resulted in second generation offspring with a higher level of tumour incidence when compared to controls. Both studies hypothesise that adaptations are transmitted through the maternal lineage through suspected epigenetic mechanisms and germ cell development during first generation *in utero* life. To our knowledge, no similar study has been conducted into similar effects with low protein diets and bone health, though maternal low protein has been shown to modulate bone development.

Lanham *et al* [131, 175] found differences in overall growth trajectory of first generation offspring from an *in utero* low protein background, with the deficient animals having a higher birth weight but then lower weight at four weeks of age. Weight differences were then not observed for the remainder of the experiment (up to 47 weeks). Further differences at four weeks of age were also indicated in alkaline phosphatase activity (higher

in the deficient group), serum osteocalcin levels (higher in deficient) and female IGF-1 (lower in deficient).

However, these changes were not evidenced within the animals examined in this study. This may be due to the age (ten weeks) of the rats as Lanham *et al* found differences only within early life (four weeks). It is possible that similar growth trends have occurred in the cohort investigated here and any alterations to the bone environment have diminished with adulthood. Our data on mechanical and material properties of bone produced no differences, which agree with findings by Lanham *et al* on the same group of *in utero* low protein animals where modifications in femur structure were only seen in old age 75 weeks old [175].

Limitations of this study were the low number of samples analysed ( $n = 5$ ). As mentioned, trends were observed whereby F2 offspring from maternal low protein backgrounds in all of the bone measurements conducted. It is possible with larger cohorts that this may be a statistically significant difference. Additionally, existing reports on the effect of maternal low protein on offspring skeletal development indicate changes in early and late life and this was not possible to explore within the samples examined in this study.

In conclusion, gene expression, micromechanics, fracture toughness and whole bone mechanics were measured to interrogate multiple properties of bone. No statistical differences were observed in second generation female offspring from low protein grandmaternal diets during pregnancy.

#### 4.4.2 Multiscale Analysis of Bone Quality

Investigation into the relationships between different measures of bone health showed significant correlations between gene expression and micromechanics and micromechanics and fracture toughness. It is well established that bone mechanical competency is dictated by cellular level events due to matrix deposition and control of mineralisation

[171, 177] and the influence of a number of non-collagenous proteins within the matrix towards fracture mechanics has recently received interest as well [61, 178].

Correlations found within this study have shown levels of *Alp* expression increasing with higher levels of *Runx2* expression. *Runx2* is a major transcription factor important for osteoblast differentiation through the role it assumes in promoting expression of subsequent bone proteins [179]. *Alp* expression is required to stimulate local calcium viability and deposition, hence mineralisation, [180] so the enhanced co-expression levels of both *Runx2* and *Alp* indicates an improved osteogenic environment.

Average CID was found to increase with higher levels of both *Runx2* and *Alp* expression. A greater CID value indicates bone is less resistant to the constantly applied force phase of indentation and so suggests a weaker material. As discussed earlier, higher levels of these genes suggests an improved osteogenic cellular environment, so higher gene expression and increased CID averages is somewhat counter-intuitive. A possible explanation is that both *Runx2* and *Alp* are both early markers of osteoblast differentiation (it has been found that *Runx2* expression is not necessary to maintain mature osteoblast function, [179]), such that immature bone, rather than fully mineralised bone, is present. Sample age (70 days old) could also be another factor, where within rats skeletal development is still occurring [142]. Phases of bone modelling and appositional growth would therefore mean that the tissue indented (periosteal) will be more junior in comparison to bone radially closer to the centre of the bone midshaft.

Last cycle CID was also significantly correlated with fracture toughness measurements, suggesting increased micromechanical fragility is related to improved fracture toughness. Use of reference point indentation (RPI) as a predictor for bone fracture toughness remains inconclusive. RPI measures have been shown to be related to predict bone toughness as measured during three-point bend testing [163] but unable to predict fracture toughness measured through notched bend testing [181]. The positive relationship between last cycle CID and fracture toughness is unexpected but Beutal *et al* [182] have demonstrated that the indentation damage mechanism is distinct with each loading cycle and load amount, with later load cycles inducing more radial, rather than

depth, damage. The complexity of the damage mechanism necessitates further research and may explain the finding here. There was also a negative relationship found, albeit not statistically significant, between IDI and fracture toughness ( $r = -0.810$ ) that could imply reduced IDI, and so improved micromechanics, is related to higher overall fracture toughness and is more inherently understandable. Generally, though, no other RPI parameters other than last cycle creep, were statistically correlated with fracture toughness, supporting the findings from Carriero *et al* [181].

Another relationship we found was that *Ocn* expression is positively linked with fracture toughness ( $r = -0.764$ ), though again not statistically significant. *Ocn* has been found to be important for biomineralisation, as well as having a role in energy dissipation and bone response to load [61], so the relationship found here appears to reflect this importance.

One of the limitations in the data presented here are the low numbers of samples used for the correlative study ( $n = 5$ ), so further research is required to elucidate whether the relationships observed here hold in greater populations. In particular, the  $r$  values between IDI and fracture toughness and *Ocn* expression and fracture toughness may suggest relationships between different bone disciplines and potentially may be utilised to predict mechanical behaviour. We expect also that gene expression will be representative of overall bone health but there may still be differences in the cellular environment at the proximal femur analysed here and the tibia where the mechanical data was obtained from.

We have developed a systematic method in employing a number of testing procedures of bone health so that the biological, material and mechanical environments are analysed within the same biological sample. With appropriate storage of samples and experimenting strategy, it is possible also to include further methods (such as CT scanning before mechanical testing so long as bone is hydrated and undergoes minimal freeze-thaw cycles). Adopting this protocol of methods we have demonstrated how disease models of bone health can be characterised and have shown this through the maternal low protein effect in the second generation samples.

In conclusion, the results we obtained in this study display no effect of grand-maternal low protein on murine bone health at 70 days of age when compared to control samples. We have also utilised this data to explore gene expression relationships to bone quality parameters. The correlations found warrant further research to investigate whether bone health metrics at the cellular and nano- to micro-scale level, and if so which are most important, can be employed to predict fracture behaviour. Due to the low number of samples utilised, further study will be conducted (presented in chapter 5) to investigate whether bone outcomes are capable of predicting overall bone mechanics.



## Chapter 5

# Molecular Origins of Bone Mechanics

Work accepted for oral presentation at Patras, Greece, ESB 2013, poster presentation at Oxford, U.K. BRS 2013 and poster presentation at Genova, Italy, TERMIS 2014.

Fracture toughness testing and reference point indentation testing assisted by Dr Tom Jenkins, Bioengineering Research Group, University of Southampton.  $\mu$ CT scanning assisted by Dr Stuart Lanham, Bone and Joint Research Group, University of Southampton. RNA extraction and cDNA synthesis assisted by Kate White, Bone and Joint Research Group, University of Southampton.



## 5.1 Introduction

The bone quality concept characterises skeletal health through appreciation of multiple physical properties, such as structure and size, and is likely to be able to predict the risk of fracture with great accuracy. It has been acknowledged that current surrogate measures of bone strength, for example bone mineral content, cannot fully predict fracture risk [183]. A comprehensive assessment of bone quality parameters will therefore augment measures that are currently used as clinical assessment tools.

The key feature of bone quality is that it encapsulates material and structural contributions to the fracture toughness of bone in addition to the traditional consideration of bone size [27]. From examination of the mechanistic events that result in bone fracture, it will be possible to delineate contributory factors and to highlight important properties that are influential in crack growth resistance. Development and maintenance of these mass, material and structural properties are regulated by events at the cellular level. As such, in terms of understanding the origins of factors that influence bone mechanics, it is important to explore the link between cell molecular events and physical traits such as bone microarchitecture.

A number of studies have demonstrated that various proteins within the bone matrix contribute to bone strength, either directly or by controlling mineralisation [60, 61, 68, 184]. These processes, combined with the effect of bone hierarchical structure at larger length scales, essentially determine the strength of bone. Examples of this cellular, protein and mechanical cascade include the regulatory effect of the TGF- $\beta$  signalling pathway, whereby demonstrations have shown that, through SMAD3 knockout, downregulation of RUNX2 occurs [91]. This regulation of RUNX2 inhibits differentiation of progenitor cells along the osteogenic lineage, affecting the bone matrix material properties through a reduction in mineralisation. The direct function of non-collagenous proteins have also attracted attention. For example, osteopontin has been demonstrated to provide sacrificial bonds to dissipate crack growth energy and has a role in recoverable deformation [185].

However, repercussions of molecular to macroscopic level events and the intermediary mechanisms remain undetermined. This is partly due to the variety and complexity of exploring the multi-faceted proponents that coordinate to provide the fracture resistance found in bone. However, due to the organic component of bone matrix being closely dictated by cellular activity, we propose that bone mechanical competence factors, particularly at the micro-scale where protein function is immediately observable, can be predicted in part by gene expression levels.

The aim of this investigation was to examine bone biology through skeletal cell gene expression analysis and to determine whether this correlated to bone properties (material, structural and mechanical) at the micro- and macro-scale in healthy bone tissue. As such, this may inform indices of bone quality and if disease status is caused by specific deviations from biological function and form.

### Hypothesis

“Higher levels of osteogenic gene expression represent improved bone matrix and hence greater bone strength and fracture resistance at the organ level”.

### Objectives

Therefore, using femora from healthy 140 day old Sprague-Dawley rats, our objectives within this study were to measure in each sample;

- i) osteoblast gene expression through reverse transcription polymerase chain reaction (RT-PCR), cortical and trabecular bone morphology through micro computer tomography ( $\mu$ CT), micro-mechanical competency through reference point indentation (RPI), bone material properties through fracture toughness testing and mineral density evaluation and mechanical strength through three-point bend testing and,
- ii) to assess any correlations between gene expression and other measured bone parameters to determine whether any factors at the cellular and micro-level can be used to predict overall bone mechanical competency.

## 5.2 Methods

### Sample Preparation

The animals tested within this study were from a control group of rats used within a vitamin D deficiency model (described in detail in section 3.1.1). Offspring were harvested at 140 days of age and stored at -80°C until required for further processing. Prior to testing, hind limbs were rapidly thawed in a water bath and both femora were retrieved and prepared for subsequent experimentation.  $n = 8$  for each gender.

### Osteoblastic Gene Expression Analysis

Gene expression was measured using RNA obtained from the right proximal femora and was performed using RT-qPCR. Further details of the RT-qPCR procedure can be found in section 3.2.1. Expression of target genes were presented in relation to a housekeeping gene and as  $2^{-\Delta Ct}$  values. *r18s* was used as a housekeeping gene, *Runx2*, *Col1*, *Opn* and *Ocn* were used as target genes.

### $\mu$ CT Bone Architecture Analysis

The left femora were imaged by  $\mu$ CT using a SkyScan 1176 *in vivo* scanner (Bruker micro-CT, Kontich, Belgium) at 18  $\mu$ m resolution (source voltage of 65 kV, current of 385  $\mu$ A, 0.5 mm Al filter and exposure time of 390 ms). Reconstruction was performed using NRecon software (version 1.6.4.6, Bruker) before microarchitectural analysis was conducted using CTAn software (version 1.11.9.3, Bruker) at the cortical bone regions from the midshaft and trabecular bone regions from the distal epiphysis. Further details of this procedure and on parameters measured can be found in section 3.3.1.

### Mineral Density: $\mu$ CT

Bone mineral density (BMD) measurements were obtained from trabecular bone regions and tissue mineral density (TMD) values were obtained from the cortical bone regions from the  $\mu$ CT data calibrated against hydroxyapatite phantoms of known density. Further details of this procedure can be found in section 3.3.1.

### Bone Micromechanics Analysis

Indentation was performed with a Biodent reference point indenter (Active Life Scientific, Santa Barbara, USA) at seven sites along the anterior surface; the femoral head, femoral neck, three points along the midshaft, and twice at the distal epiphysis (one at the top of the condyles and one between the condyles). Data was recorded using the built-in Biodent software and measured variables were total indentation distance (TID), indentation depth increase (IDI), creep indentation distance (CID) and energy dissipation. Further details of this procedure can be found in section 3.4.1

### Three-Point Bending Analysis

The left femora were mechanically tested until failure using a three point bend arrangement and an electromechanical testing machine (Instron, High Wycombe, UK) to measure strength - different machines were used for fracture toughness and strength due to the higher loads necessary for fracture in the strength testing). Samples were placed on two supports of 10 mm span within a water bath filled with HBSS so samples stayed hydrated and loaded at  $0.01 \text{ mm s}^{-1}$  until fractured. Maximum loads were recorded from experimental data. Further details of this procedure can be found in section 3.4.3.

### Fracture Toughness Analysis

Right femora were measured for fracture toughness through notched (three-point) bend testing. Samples were placed on a 6 mm span with the anterior wall of each femur

notched and tested in tension. Loading was performed at  $0.001\text{ mm s}^{-1}$  on an Electro-force 3200 mechanical tester (Bose, Minnesota, USA), with load and displacement data recorded. Fracture surfaces were then imaged through scanning electron microscopy (JSM-6500F, JEOL, Peabody, USA) and utilised for determining fracture toughness. Further details of this procedure can be found in section 3.4.2.

### Statistical Analysis

Data was examined by gender. Statistical analysis was performed for univariate linear regression using Pearson correlation coefficient ( $r$ ) and coefficient of determination ( $r^2$ ) values between datasets (Graphpad Prism, La Jolla, USA). Significance was deemed when  $p < 0.05$  for deviation from zero.

### 5.3 Results

Right and left femora from eight male and eight female healthy rats (140 days of age) were excised and measured for gene expression (*Runx2*, *Col1*, *Opn* and *Ocn*), *BMD* (trabecular bone), *TMD* (cortical bone), bone architecture of cortical and trabecular regions, reference point indentation (RPI) micromechanics, fracture toughness and whole bone mechanics. These results were then processed for relationships between different bone metrics and by calculating Pearson correlation coefficients  $r$  (correlation grids can be viewed in tables C.1 and C.2, Appendix C). Relationships with  $r$  values greater than 0.7 were further analysed for coefficient of determination values ( $r^2$ ) and statistical significance.

#### Males: Proximal Femur

Gene expression and micromechanical parameters measured by RPI at the proximal femur (femoral head and femoral neck) yielded no strong relationships (table C.1, Appendix C) and were consequently not reviewed further for significant relationships.

#### Females: Proximal Femur

When investigating relationships between gene expression and micromechanics, the female cohort was found to produce a number of correlations at the femoral head. A significant increase in *Runx2* expression was associated with both greater total indentation distances ( $r^2 = 0.87$ ,  $p < 0.01$ , figure 5.1) and indentation distance increase ( $r^2 = 0.60$ ,  $p = 0.02$ , figure 5.2) measurements conducted on the femoral head.

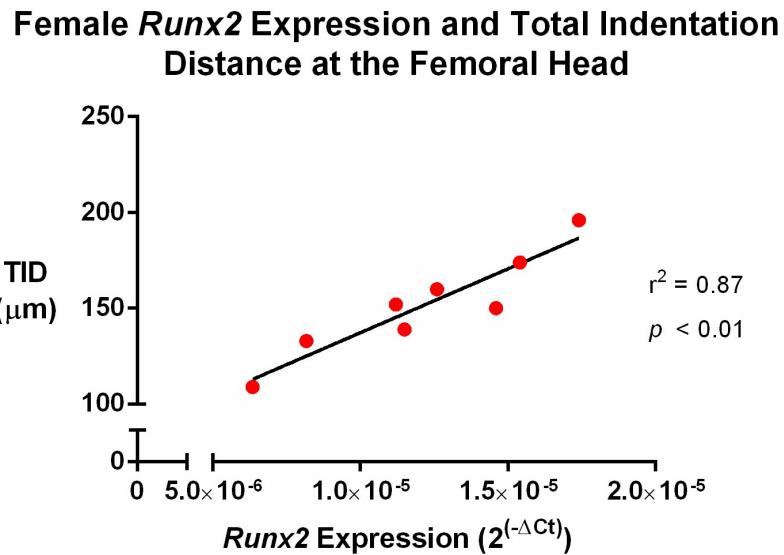


Figure 5.1: Female *Runx2* Expression and TID at the Femoral Head. A positive correlation was observed between *Runx2* expression levels and TID measured by RPI at the proximal femur.  $n = 8$  and line of best fit displayed.

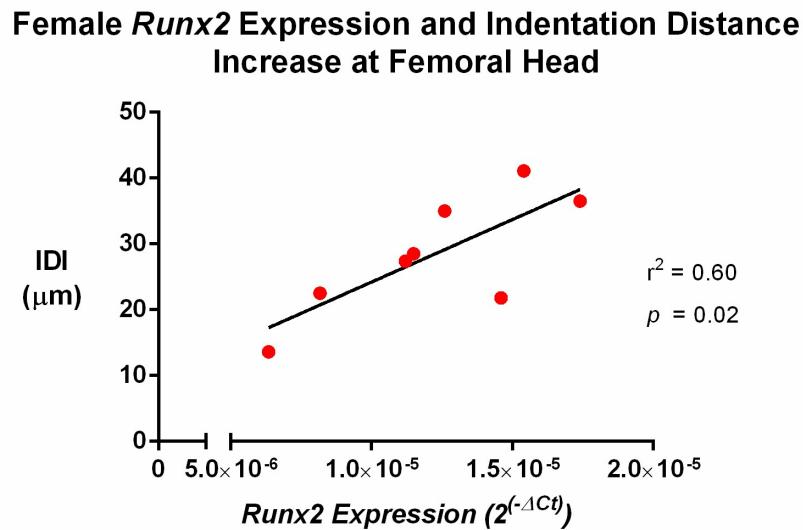


Figure 5.2: Female *Runx2* Expression and IDI at the Femoral Head. A positive correlation was found within female samples between *Runx2* expression levels and IDI measurements at the proximal femur.  $n = 8$  and line of best fit displayed.

Similarly, increased TID measurements at the femoral heads within the female samples were correlated with greater *Col1* expression ( $r^2 = 0.65$ ,  $p = 0.02$ , figure 5.3) and greater *Ocn* expression ( $r^2 = 0.57$ ,  $p = 0.03$ , figure 5.4).

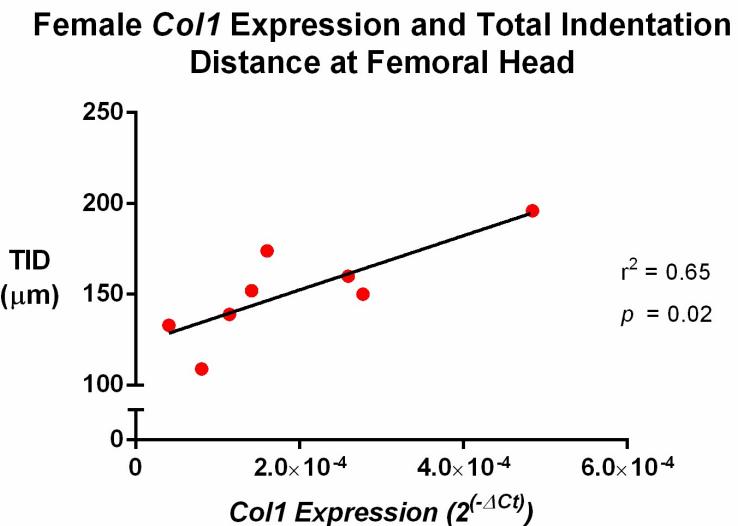


Figure 5.3: Female *Col1* Expression and TID at the Femoral Head. Increased *Col1* expression was found to be correlated to increased TID measurements at the femoral head within females.  $n = 8$  and line of best fit displayed.

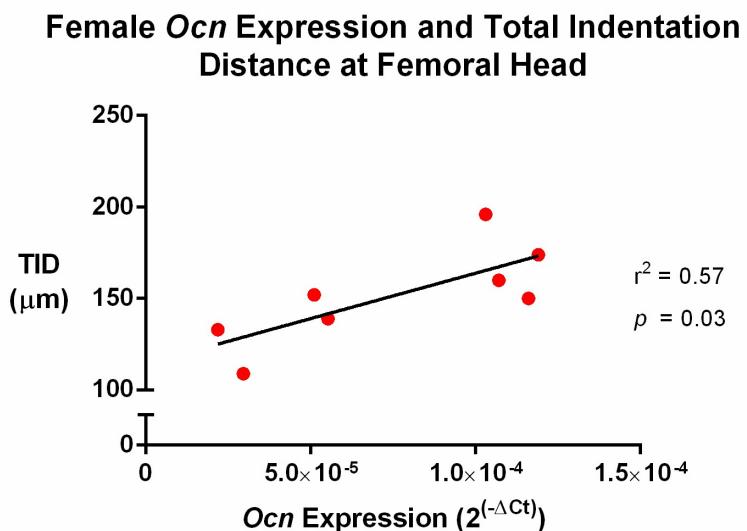


Figure 5.4: Female *Ocn* Expression and TID at the Femoral Head. Similar to *Col1*, *Ocn* was found to be positively correlated to TID measurements at the femoral head within females.  $n = 8$  and line of best fit displayed.

Males: Femur Midshaft

Data collected from male animals from the femoral midshaft was analysed to investigate relationships between gene expression and measurements of bone quality (material, structure and mechanics). Higher *Runx2* expression levels were found to be significantly inversely related to a number of other femur midshaft properties. This increase in *Runx2* expression was found to be correlated with a reduction in midshaft cortical thickness ( $r^2 = 0.74$ ,  $p = 0.01$ , figure 5.5), a decrease in fracture toughness  $k_{init}$  values measured with notched bend testing ( $r^2 = 0.83$ ,  $p = 0.01$ , figure 5.6) and maximum recorded load in three-point bend testing ( $r^2 = 0.69$ ,  $p = 0.02$ , figure 5.7).

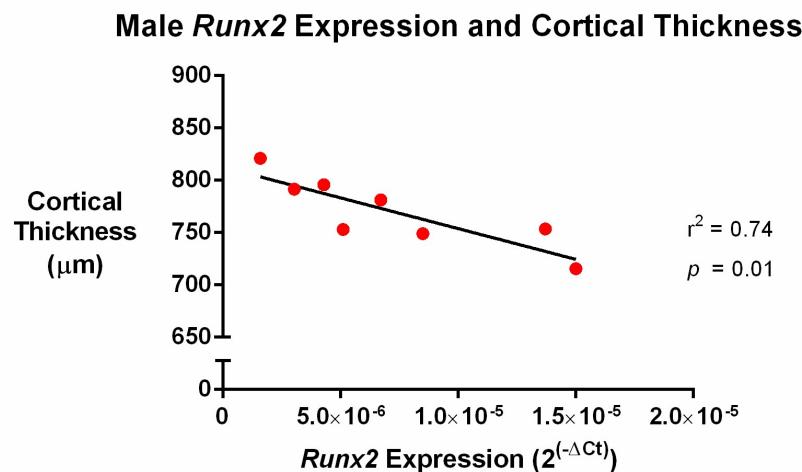


Figure 5.5: Male *Runx2* Expression and Cortical Thickness at the Femur Midshaft. Higher *Runx2* expression was found to be negatively correlated to cortical thickness at the femur midshaft of male rats.  $n = 8$  and line of best fit displayed.

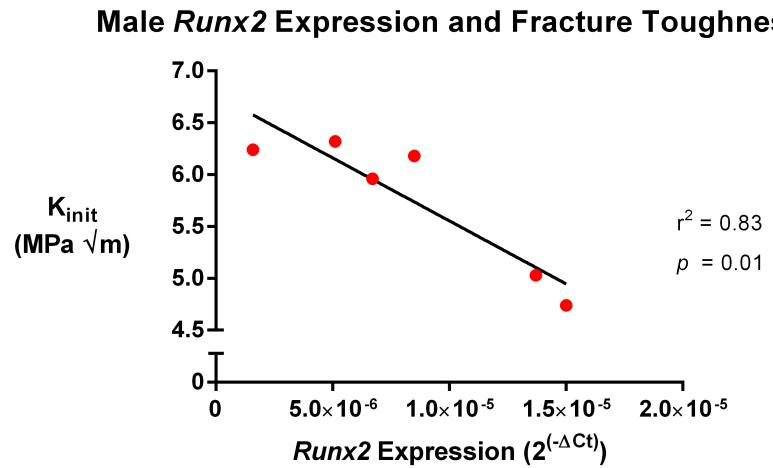


Figure 5.6: Male *Runx2* Expression and Fracture Toughness at the Femur Mid-shaft. A negative correlation between *Runx2* expression levels and fracture toughness measurements at the femur midshaft of male samples was observed.  $n = 6$  and line of best fit displayed.

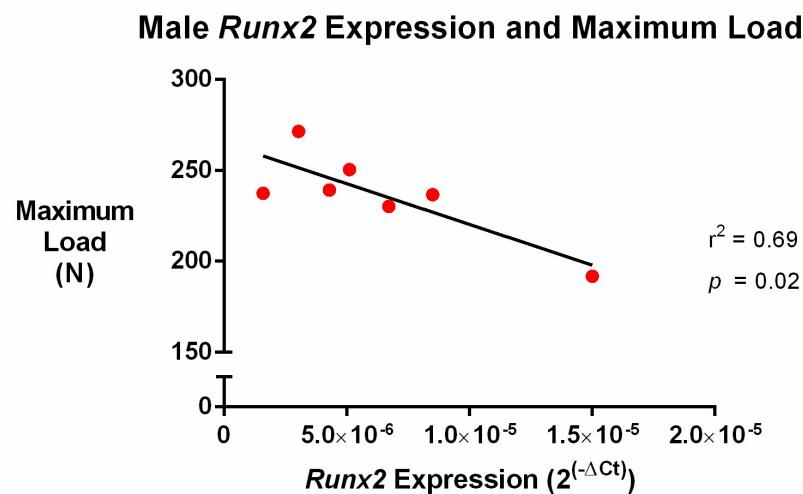


Figure 5.7: Male *Runx2* Expression and Maximum Load at the Femur Midshaft. A negative correlation was found between *Runx2* expression levels and maximum load during three-point bend testing of the femur midshaft was observed in male rats.  $n = 7$  and line of best fit displayed.

Females: Femur Midshaft

Investigation of osteoblast gene expression and relationships to measured femur midshaft properties also yielded a number of correlations within female rats. A positive correlation was observed with increased *Runx2* expression levels and cortical bone to total bone area ratio ( $r^2 = 0.67, p = 0.01$ , figure 5.8). Increased *Runx2* expression was also found to be indicative of increased femur midshaft cortical thickness ( $r^2 = 0.60, p = 0.02$ , figure 5.9). RPI measurements of bone at the femoral midshaft also showed that increased *CID* values were related to both increased *Runx2* ( $r^2 = 0.72, p < 0.01$ , figure 5.10) and increased *Col1* ( $r^2 = 0.55, p = 0.03$ , figure 5.10) expression.

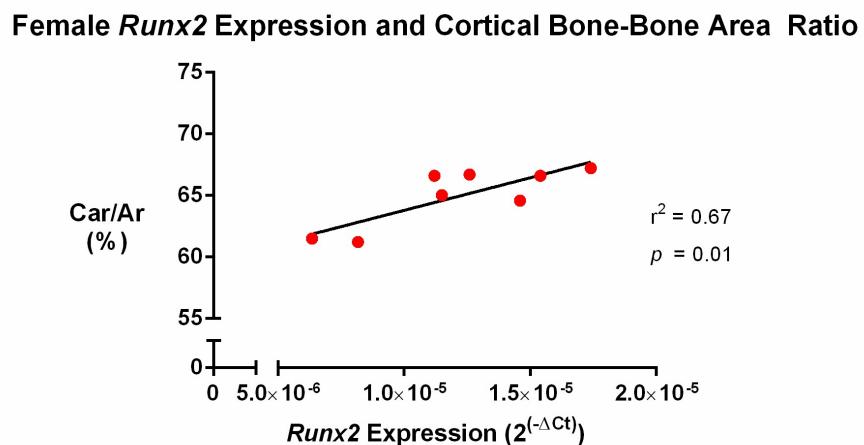


Figure 5.8: Female *Runx2* Expression and Cortical Bone to Area Ratio at the Femur Midshaft. A positive correlation was observed between *Runx2* expression and cortical bone to bone area ratio of the midshaft femur in females.  $n = 8$  and line of best fit displayed.

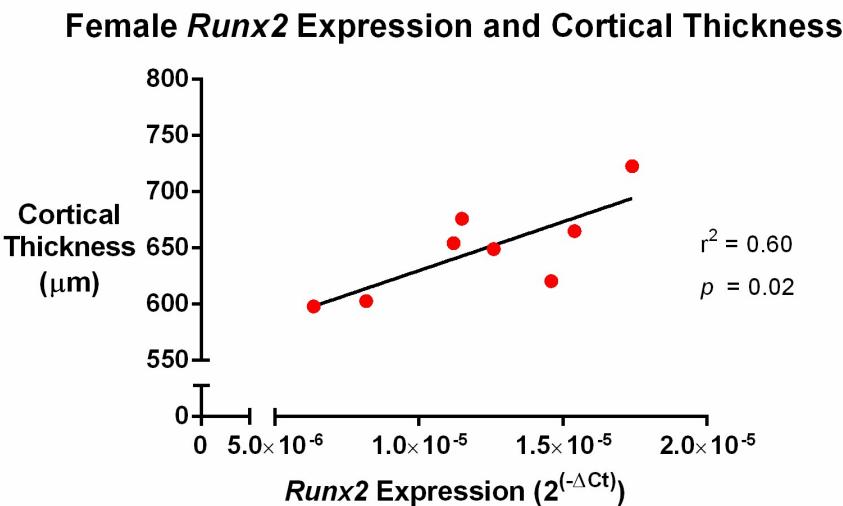


Figure 5.9: Female *Runx2* Expression and Cortical Thickness at the Femur Mid-shaft. Increased *Runx2* expression was correlated to increased cortical thickness at the femur midshaft in the female group.  $n = 8$  and line of best fit displayed.

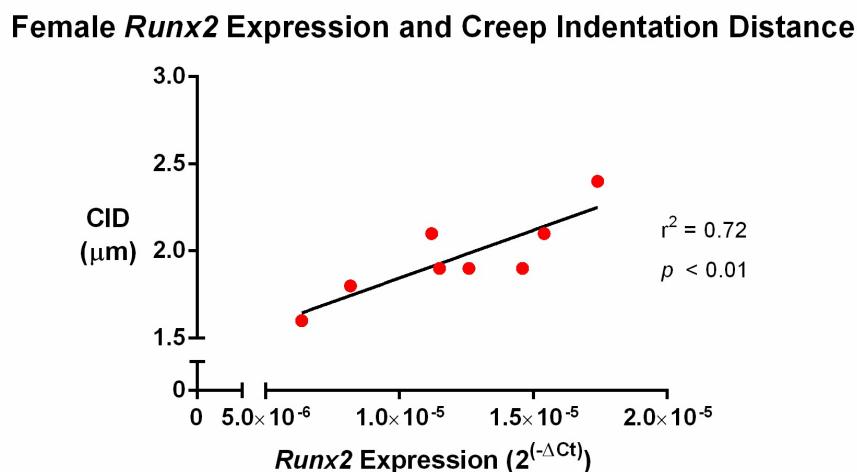


Figure 5.10: Female *Runx2* Expression and CID at the Femur Midshaft. *Runx2* levels were found to be positively associated with CID measurements at the femur midshaft of female samples.  $n = 8$  and line of best fit displayed.

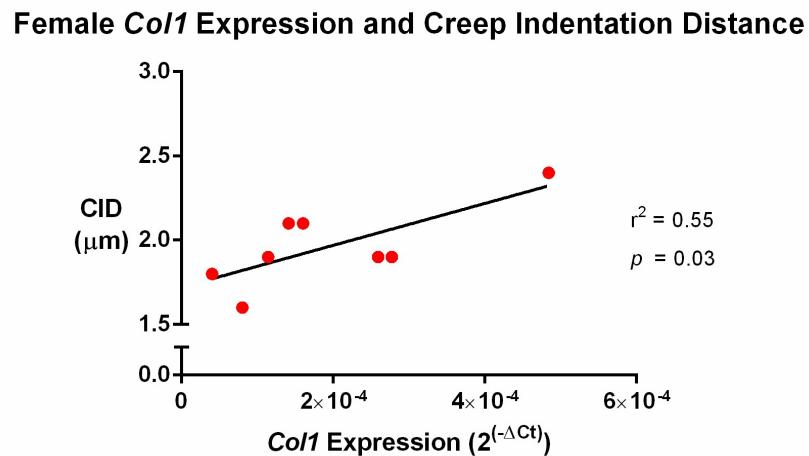


Figure 5.11: Female *Col1* Expression and CID at the Femur Midshaft. Similar to *Runx2*, *Col1* expression levels were positively correlated to midshaft CID measurements taken within females.  $n = 8$  and line of best fit displayed.

## 5.4 Discussion

Research within bone mechanics has seen scrutiny of various biological components of the bone environment and the consequent impact on mechanical competence. Important transcription factors [91] and proteins [60, 61, 186] involved with bone matrix development have been highlighted due to observable changes in bone mechanical behaviour when normal levels have been modulated. In many of these studies (such as in references [91], [186] and [60]), animal knockout models have been utilised to view the subsequent effect when these transcription factors and proteins are absent and present extreme case scenarios. In this present work, we adopted a different approach by analysing the bone environment within healthy, control animals (from a vitamin D deficiency study, chapter 7) and whether naturally occurring differences lead to alteration of bone quality metrics. Specifically, the expression of four genes (*Runx2*, *Col1*, *Opn* and *Ocn*) were investigated for relationships between micro-mechanical, micro-architectural, material and organ level mechanical properties. Higher expression of these genes was anticipated to indicate an improved osteogenic environment and therefore linked with enhanced bone mechanics.

At the femoral head, increased expression levels of *Runx2*, *Col1* and *Ocn* were all found to correlate with an increase in the measured total indentation distance (TID) in female rats. Additionally, an increase in *Runx2* also correlated to an increase in indentation distance increase (IDI) over the course of the indentation procedure. Similarly, greater levels of *Runx2* and *Col1* expression were linked to higher average creep indentation distances (CID) at the femur midshaft. Both IDI and TID measurements at the anterior surface of the tibia have been found to be higher in fracture patients (hip and vertebral) when compared to a control group of subjects with no prevalent fractures [166], with IDI measurements on rat femur, rat vertebrae and dog ribs negatively correlating with whole bone toughness [163]. Conceptually, higher indentation distances from RPI measurements indicate a material that is less resistant to applied force and hence fracture toughness is lower. The relationships found here imply higher expression levels of these osteogenic genes were associated with a reduction in the micro-mechanical competence of bone matrix at indentation sites.

*Runx2* is a well-known transcription factor during osteoblast differentiation, targeting and inducing the production of a number of important proteins for bone formation. Therefore, positive correlations to greater indentation depths were contrary to expectation. Interestingly, continued *Runx2* expression is not found in mature osteoblasts [187], so the higher levels of *Runx2* found in this study could be confirmation of an early bone formation period within naturally healthy variation. *Runx2* was also positively correlated with cortical bone to bone area ratio indicating higher expression is related to a greater areal quantity of cortical bone within the midshaft cross-section. This is supported by the increases in cortical thickness also observed at the midshaft, such that these relationships could represent an environment whereby recent bone formation has occurred (increasing the amount of bone). The increase in fragility seen with greater indentation distances (associated with reduced hardness) may show that higher *Runx2* expression is indicative of newly formed bone before mineralisation has been fully achieved.

A similar proposal can be adopted for the increased *Col1* levels and relations to TID at the femoral head and CID at the femur midshaft. Collagen is traditionally considered to provide the initial structural scaffold for bone that is subsequently matured by the addition of non-collagenous proteins (NCPs) and apatite crystals [184], but has also been implicated in improving material toughness [26, 188] and directing mineralisation by both initiation and growth of crystals independent of NCPs [189]. The findings here conflict with these functional roles of collagen. However, *Runx2* has been shown to positively regulate *Col1* expression specifically in osteoblast maturation [190], such that immature bone tissue, as discussed earlier, could still be related to *Col1* expression and hence the micro-mechanical results found here.

Less clear is the correlation found with increased *Ocn* expression and higher TID at the femoral head in females. *Ocn* is a late osteoblast-related gene and upregulation immediately precedes mineralisation, with further expression continued by higher levels of mineralisation [191]. Though the precise function of *Ocn* is unknown, *Ocn* has been suggested in controlling crystal nucleation and binding to the collagen fibrils, aiding mineralisation [192] as well as, through interaction with osteopontin, promoting the ability of bone to diffuse strain energy when loaded [61]. Therefore, it is uncertain why this

relationship was found though a caveat of these results is that mRNA extracted from the proximal femur may not be representative of measurements obtained at other sites along the femur.

Similar to the findings within the female group, male *Runx2* expression at the femur midshaft was found to be negatively correlated with cortical bone thickness, fracture toughness and maximum load during three-point bend testing. Again this indicates poorer bone structure and mechanical competence with higher levels of *Runx2* and we suspect again that this is suggestive of a junior bone environment. This seems to be the case within both genders and also agrees with the findings from chapter (4), where *Runx2* increases were also related to greater CID at the femur midshaft.

The data within this study implies a highly influential role for *Runx2* in predicting bone mechanical performance at both the micro- and macro-scale. Balooch *et al* [91] demonstrated that increased TGF- $\beta$  signalling decreased bone mass, mineralisation, fracture toughness and elastic modulus. The proposed mechanism of increased TGF- $\beta$  signalling targeting *Runx2* up-regulation [193] and preventing osteoblast maturation is supported by the results presented here, where *Runx2* expression is directly examined.

Links between gene expression and mechanics have been presented here. Not all genes analysed, osteopontin for example, yielded relationships to physical bone properties. This was somewhat unexpected as osteopontin has been shown to influence bone fracture toughness by aiding opposition to crack propagation [60], with the protein providing a multitude of sacrificial bonds that dissipate crack growth energy [194]. Recent findings by Poundarik *et al* [61] have presented osteocalcin and osteopontin acting together in protein complexes that again can dissipate energy. Therefore, multivariate regression analysis would be necessary for future study to take account of any collective contributions of bone proteins towards mechanics.

The value of assessing bone gene expression has been highlighted recently where RNA from femur trabecular bone was investigated. Rodrigues *et al* [150] found significantly lower levels of osteocalcin expression as well as lower osteocalcin to collagen I expression

ratio in hip fracture (osteoporotic) patients when compared to osteoarthritic patient controls. These lower levels were also found to be correlated with decreased trabecular bone strength measured through compression testing, demonstrating that gene expression could be utilised as a predictor for bone quality parameters.

Overall, the data in this study implies the possibility to apply osteogenic gene expression as a predictor for bone quality metrics, although trends were not as immediately expected. A multi-disciplinary approach was used to assess the biological origins of various physical bone parameters. The organic phase of bone has been evidenced to be highly important for fracture toughness, both in terms of collagen [195] and non-collagenous proteins [52]. Specifically, we found that *Runx2* expression is especially indicative of bone mechanical behaviour, as well as some evidence of *Col1* and *Ocn* expression also demonstrating relationships. It would be interesting to also directly measure the protein content in future studies to confirm that gene expression is representative of the bone matrix composition. We conclude that bone gene analysis is a potentially useful metric, together with other metrics, in understanding the derivation of bone quality and subsequent modulation, and hence upon further investigation may offer an analytical tool for bone fracture toughness and disease analysis.



## Chapter 6

# Effect of *in utero* Vitamin D Deficiency on Rodent Offspring Skeletal Health at 21 Days Old

Work accepted for poster presentation at Southampton, UK, TCES 2015 and poster presentation at Boston, U.S.A., TERMIS World Congress 2015.

FEA computer simulations were conducted in collaboration with Dharshini Sreenivasan and Dr Justin Fernandez of the Auckland Bioengineering Institute, New Zealand. Fracture toughness testing assisted by Dr Tom Jenkins, Bioengineering Research Group, University of Southampton.  $\mu$ CT scanning assisted by Dr Stuart Lanham, Bone and Joint Research Group, University of Southampton.



## 6.1 Introduction

The importance of vitamin D during pregnancy and impact upon offspring skeletal health into adulthood has been extensively examined but the topic remains contentious. Findings from numerous studies report conflicted results; either the benefit of vitamin D sufficiency towards offspring bone development or the lack of any influence at all [133, 196, 197]. In addition to this, no obvious mechanism has been identified that may explain the role *in utero* vitamin D can have on future offspring health.

Functionally, vitamin D is known to be important for calcium availability and hence mineralisation [198]. Additionally, vitamin D is required for expression of osteocalcin and osteopontin [199, 200], highly important proteins within the bone matrix that affect mineralisation and mechanical behaviour [60, 61, 185]. Clearly, vitamin D can affect bone health through the modulation of a number of different bone health facets, but less certain is whether these mechanisms extend towards an *in utero* influence towards adult bone health.

Evidence over the last three decades indicate that early life events can cause adaptation to future development thereby changing risk to chronic diseases, termed the developmental origins of health and disease (DOHaD), leading to an advocacy of a lifecourse approach to disease risk [201–203]. Although the precise mechanisms are not fully established, a likely candidate is through changes at the epigenomic level, facilitating persistent adaptations to alter gene expression status [174]. Examples of vitamin D interaction with epigenetic mechanisms are slowly emerging, but the exact processes involved are still to be uncovered. Vitamin D has been shown to modify both histone acetylation and DNA methylation through acting directly or in association with other proteins on histone acetyltransferases and methyltransferases [204].

The purpose of this chapter was to analyse various offspring bone outcomes at 21 days of age from a rat model of maternal vitamin D depletion during pregnancy. The results from a dietary controlled study such as this can determine how bone health is influenced solely by vitamin D status during *in utero* life. Therefore, the data may then be utilised

to further inform upon the controversial issue of whether vitamin D supplementation during pregnancy is beneficial for offspring future bone health.

### Hypothesis

The current programme of work examined the hypothesis that “vitamin D deficiency *in utero* causes a persistent, detrimental change in bone health throughout the lifecourse, regardless of an otherwise healthy environment”. Consequently, these changes will be observable in rat offspring during early life (21 days old).

### Objectives

Our objectives then were to measure any changes caused by *in utero* vitamin D deficiency by assessing;

- i) the bone biology environment through analysing gene expression of femur osteoblasts by reverse-transcription PCR (RT-qPCR),
- ii) the cortical bone structure at the femur midshaft using  $\mu$ CT scanning,
- iii) bone composition of the femur midshaft by mineral density measurements ( $\mu$ CT) and material properties by fracture toughness analysis, and
- iv) whole bone femur mechanics by three-point bend testing and finite element analysis (FEA) compression tests of the midshaft.

## 6.2 Methods

### Sample Preparation

21 day old male and female Sprague-Dawley rats were used for this experiment, with  $n = 8$  for each gender and maternal dietary condition (either control or vitamin D deficient, see section 3.1.1 for more detail). Animals were weighed and after sacrifice stored at  $-80^{\circ}\text{C}$  until required. Right and left femora were retrieved from each sample for bone health analysis.

### Gene Expression: RT-qPCR

Gene expression was measured through RNA obtained from the right proximal femora and was performed using RT-qPCR. Further details of the RT-qPCR procedure can be found in section 3.2.1. Expression of target genes were presented in relation to a house-keeping gene and as  $2^{-\Delta\text{Ct}}$  values. *r18s* was used as a housekeeping gene, *Runx2*, *Col1*, *Opn* and *Ocn* were used as target genes.

### Cortical Bone Morphology: $\mu\text{CT}$

Left side femora were imaged by CT using a SkyScan 1176 in vivo scanner (Bruker micro-CT, Kontich, Belgium) at  $18 \mu\text{m}$  resolution (source voltage of 65 kV, current of 385  $\mu\text{A}$ , 0.5 mm Al filter and exposure time of 390 ms). Reconstruction was performed in NRecon software (version 1.6.4.6, Bruker) before microarchitectural analysis was conducted using CTAn software (version 1.11.9.3, Bruker) at the midshaft region. Further details of this procedure can be found in section 3.3.1. Additional evaluation of the buckling ratio of midshaft structures was conducted by assuming a circular cross-section and deriving an outer radius ( $r_o$ ) from the average midshaft tissue area ( $A$ ) through equation 6.1. The buckling ratio was then obtained by dividing the radius by average thickness (*Co.Th*), (equation 6.2) [205].

$$r_o = \sqrt{\frac{A}{\pi}} \quad (6.1)$$

$$\text{Buckling ratio} = \frac{r_o}{Co.Th} \quad (6.2)$$

### Cortical Bone Mineral Density: $\mu$ CT

Bone mineral density of left side femur midshafts were recorded through calibration against hydroxyapatite phantoms of known density. Further details of this procedure can be found in section 3.3.1.

### Fracture Toughness Testing

Right side femora were measured for fracture toughness through notched (three-point) bend testing. Samples were placed on a 6 mm span with the anterior wall of each femur notched and tested in tension. Loading was performed at  $0.001 \text{ mm s}^{-1}$  on an Electro-force 3200 mechanical tester (Bose, Minnesota, USA), with load and displacement data recorded. Fracture surfaces were then imaged through scanning electron microscopy (JSM-6500F, JEOL, Peabody, USA) and utilised for determining fracture toughness. Further details of this procedure can be found in section 3.4.2.

### Bone Strength: Three-Point Bend Testing

Left side femora were tested for strength through three-point bending using an ElectroPuls E1000 mechanical tester (Instron, High Wycombe, UK). A span of 6 mm was used and femora were loaded anterior side down at a displacement rate of  $0.01 \text{ mm s}^{-1}$ . Load-displacement data were recorded and maximum load determined. Further details of this procedure can be found in section 3.4.3.

### FEA Estimation of Bone Strength

Compression load was applied to simulated models of femur midshafts (derived from a subset of male femora  $\mu$ CT topography scan data,  $n = 5$  for each diet) along the long axis in the distal direction (figure 6.1) in ABAQUS (Dassault Systèmes, RI, USA). The simulation procedure was conducted as described by Sreenivasan *et al* *Sreenivasan2013*. Stereolithography (STL) data was generated from  $\mu$ CT scans of the femoral midshafts (region as defined in section 3.3.1). STL files were then cleaned digitally by removal of artifacts, duplicate faces, duplicate vertices and unreferenced vertices in Meshlab (ISTI-CNR, Pisa, Italy). Model sizes were also reduced by 30% by decimation [206] to improve computational efficiency whilst maintaining accurate topological information. Cleaned STL files were then utilised to generate full finite element (FE) models for subsequent structural analysis, with hexahedral elements fitted in Hypermesh (Altair Engineering Inc., Troy, MI, USA).

Load simulations were conducted in ABAQUS, with 1 N steps applied incrementally up to a maximum of 15 N. Strain was restricted to the direction of loading and models were fixed on the bottom face of each midshaft (figure 6.1), material properties were defined to be isotropic and homogeneous, with Youngs modulus ( $E$ ) of 1 GPa and Poisson's ratio ( $\nu$ ) of 0.3. Mean Mises stresses were recorded for each iteration and failure force was obtained by force required to experience a pre-defined average von Mises stress to indicate failure.

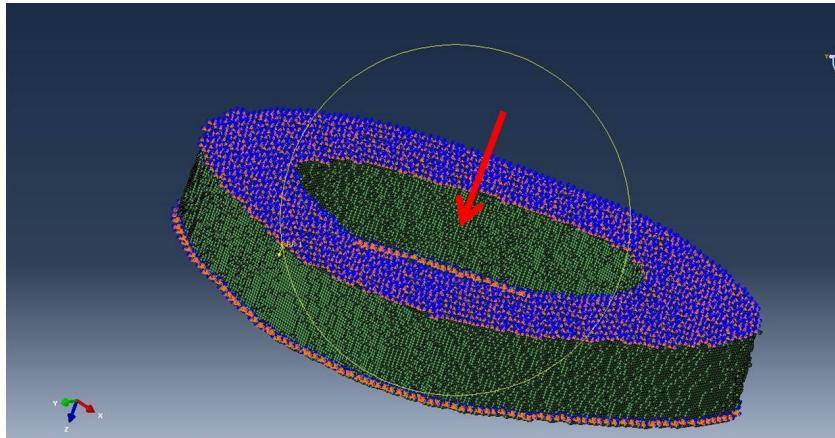


Figure 6.1: Vitamin D Day 21 Compressive Load Simulation. The image displays a FE model of a day 21 midshaft section to be computationally tested under compression. Direction of load is depicted by the red arrow.

### Statistical Analysis

It has been demonstrated that skeletal development and the role of vitamin D differs according to gender. As such, the experiment was split according to gender before analysis of control and vitamin D deplete samples was performed. Statistical analysis was performed using unpaired t-tests, with Welch's correction to account for unequal standard deviation on data sets (Graphpad Prism, La Jolla, USA). All data sets were tested for Gaussian distribution and significance determined at  $p < 0.05$ .

### 6.3 Results

*In utero* vitamin D depleted rats were assessed at 21 days of age for bone development. Gene expression, *TMD* and cortical bone architecture parameters at the femur mid-shaft, fracture toughness, whole bone mechanics were all measured. Tabulated data and *p* values for these measurements can be viewed in Appendix D.

#### Weight

There were no statistical differences found in males between the control and vitamin D deplete animals for measured weight. Similar to the male groups, no significant results for weight were observed between the control and vitamin D deplete females (figure 6.2).

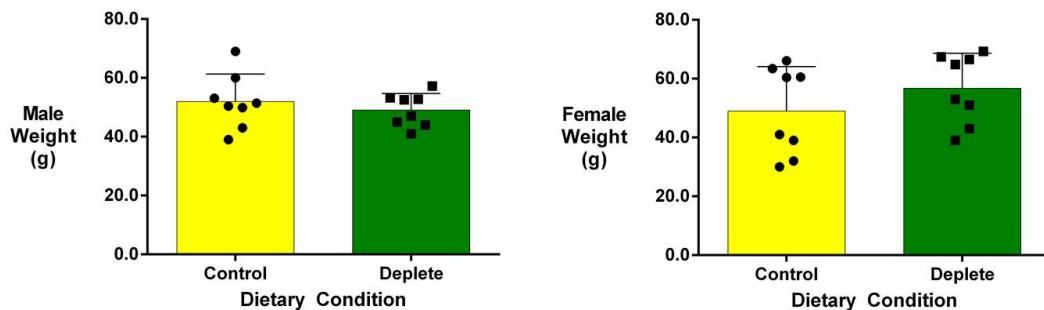


Figure 6.2: Vitamin D Day 21 Animal Weight. No differences in weight of animals was present at 21 days of age regardless of control or vitamin D deficient backgrounds.  $n = 8$  for each dietary condition, mean and standard deviation presented. Similar to males, females did not exhibit any changes in weight between control and vitamin D deficient backgrounds.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Male: Gene Expression

Gene expression was analysed from femur osteoblast RNA to evaluate bone cellular function. Comparison of osteogenic gene expression between the male control and vitamin D deplete groups found no statistically significant differences, though mean values were higher in the deplete group for *Runx2*, *Opn* and *Ocn*, whilst *Col1* expression was lower (figure 6.3).

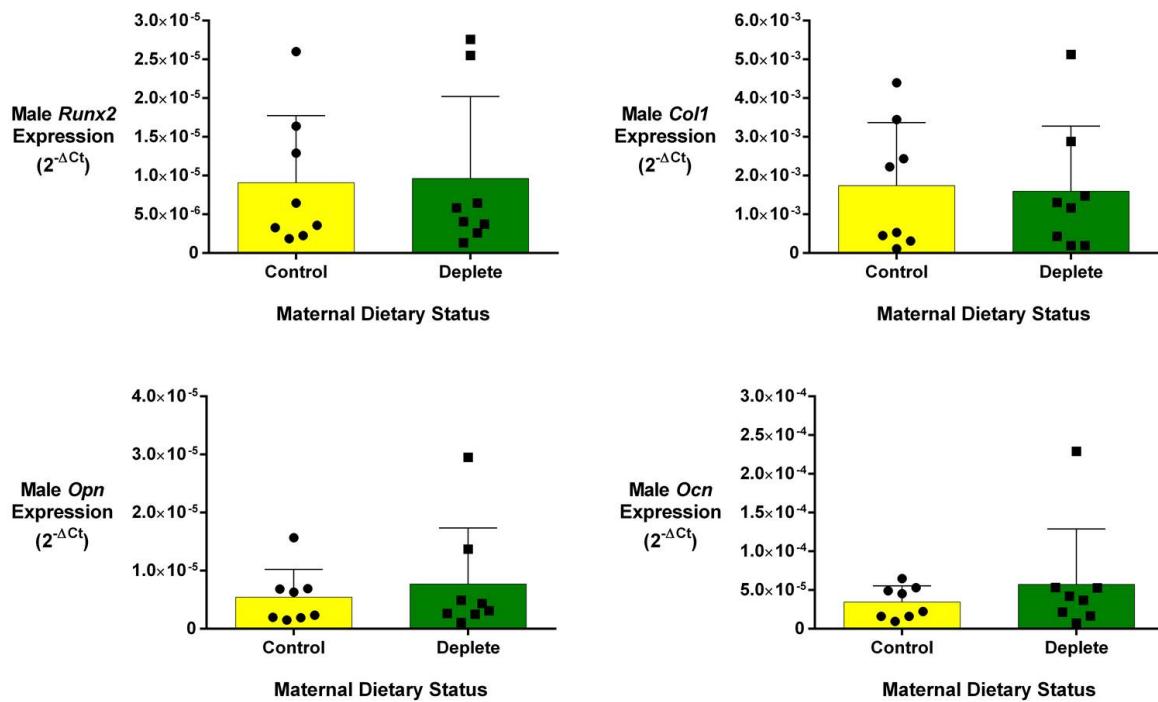


Figure 6.3: Vitamin D Day 21 Male Osteogenic Gene Expression. No osteogenic gene expression differences were found between the control and vitamin D deficient male groups at 21 days of age.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Female: Gene Expression

Again, there were no statistical differences found in females for *Runx2*, *Col1*, *Opn* and *Ocn* gene expression between the control and vitamin D deplete animals (figure 6.4).

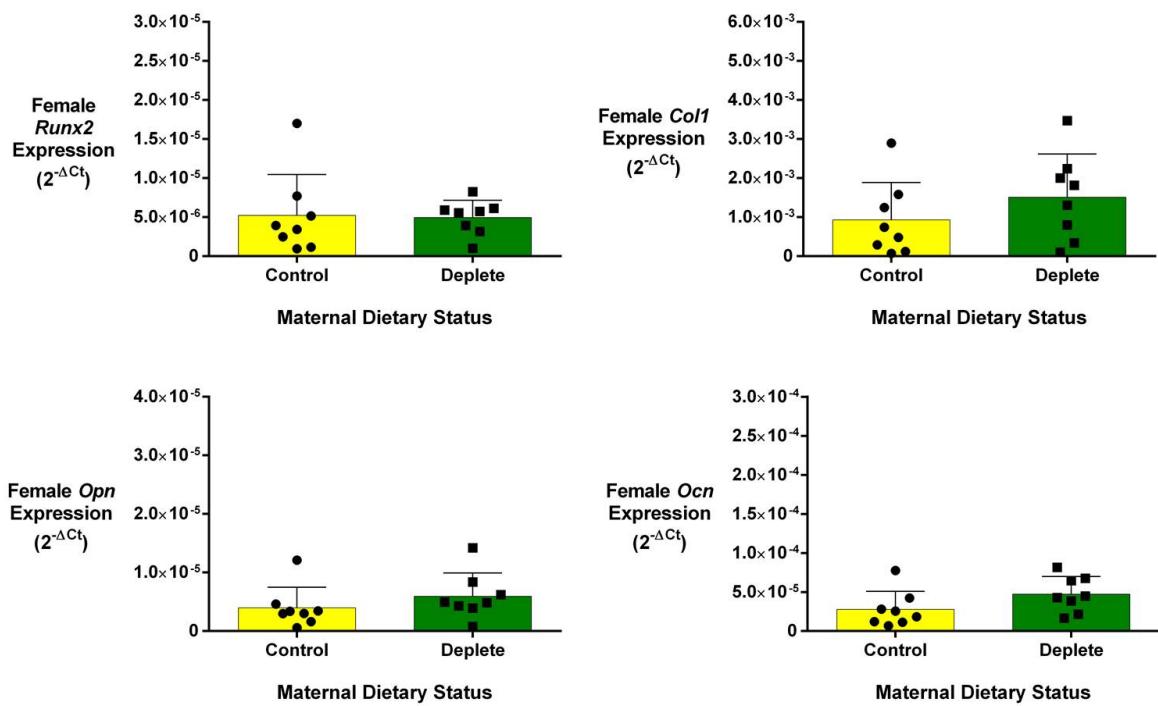


Figure 6.4: Vitamin D Day 21 Female Osteogenic Gene Expression. No differences in osteogenic gene expression were found in female offspring at 21 days of age when control and vitamin D deficient groups were compared.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Male: Cortical Bone Morphology

Cortical bone CT data was used to evaluate bone morphology development. Examination of male femoral midshaft morphology showed the mean cross-sectional area at this region to be significantly smaller in the vitamin D deplete animals when compared to controls ( $4.0$  vs  $4.7$   $\text{mm}^2$ , a  $14.9\%$  reduction,  $p = 0.04$ , figure 6.5). Interestingly, mean bone area, bone area fraction and cortical bone thickness measurements were also all reduced, although these did not reach significance ( $p = 0.06$ ,  $0.49$  and  $0.09$  respectively), showing that quantity of bone at the midshaft remained unchanged.

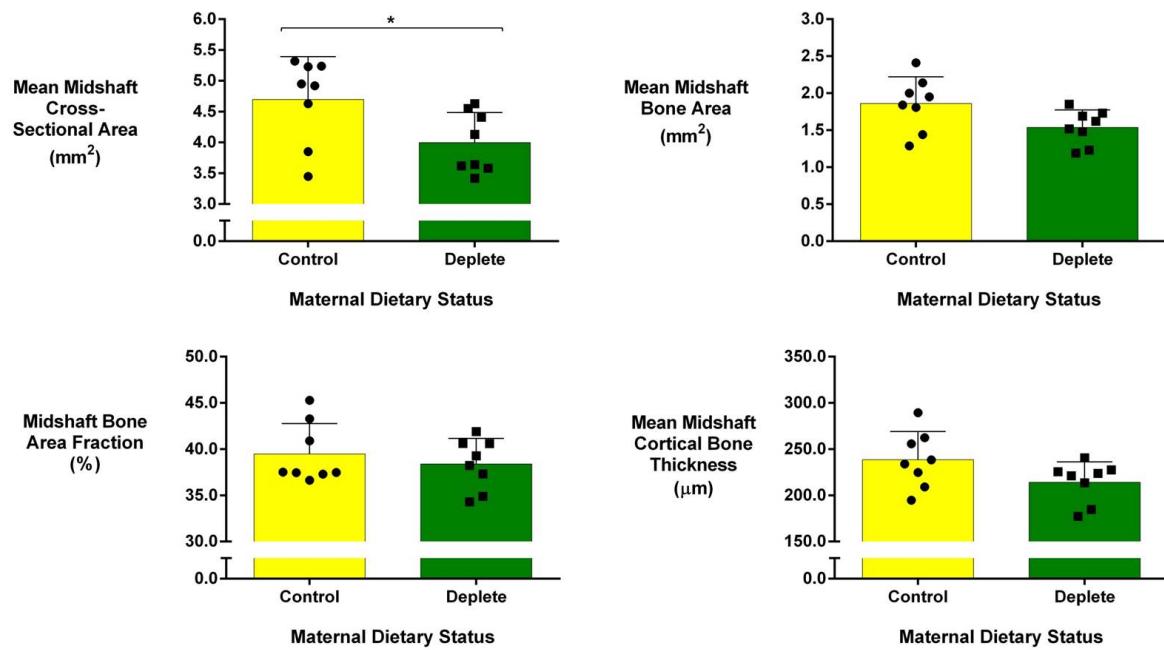


Figure 6.5: Architectural Analysis of Male Cortical Bone. Morphology of cortical bone at femur midshafts were assessed through  $\mu\text{CT}$  scanning and found that the tissue area was decreased in the vitamin D deficient rats ( $p = 0.04$ ). No other significant changes within morphology were recorded.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Female: Cortical Bone Morphology

For the female cohort, the mean femur midshaft morphology measurements were not found to be statistically different between the control or vitamin D depleted group for any of the four parameters of cross-sectional area, bone area, bone area fraction and cortical bone thickness (figure 6.6).

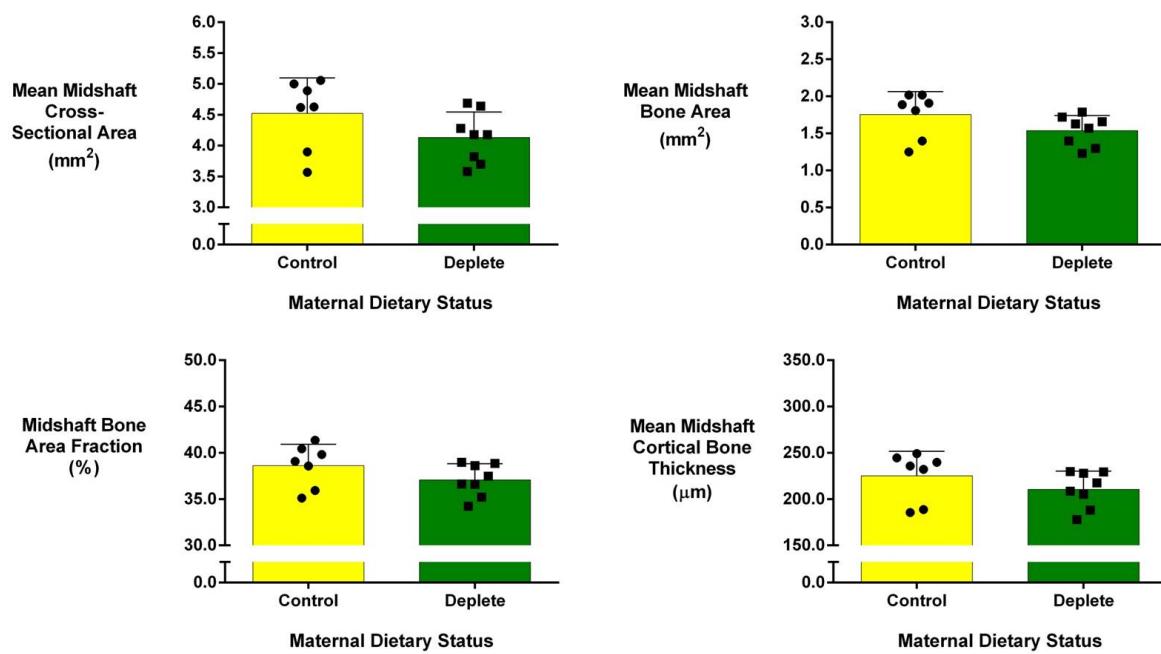


Figure 6.6: Architectural Analysis of Female Cortical Bone. Analysis of the femur midshaft morphology of females at 21 days of age showed no differences between the control and vitamin D deficient animals for any of the parameters measured.  $n = 7$  for the control condition and  $n = 8$  for the vitamin D deficient condition, mean and standard deviation presented.

### Buckling Ratio

Further investigation into the vitamin D deficient decrease of midshaft area was conducted by analysing the buckling ratio of each midshaft structure. The buckling ratio measures the likelihood of local buckling occurring as a result of low wall thickness (defined as ratios greater than 10). However, no differences were found between the control and vitamin D deficient groups for either gender (figure 6.7).

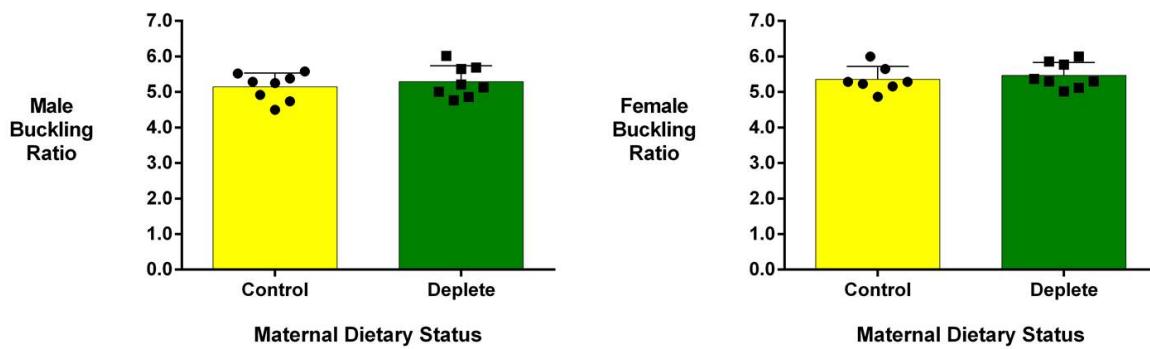


Figure 6.7: Femur Midshaft Buckling Ratio. No differences were observed in buckling ratio values between control and vitamin D deficient animals in either gender at 21 days of age, indicating no change in localised instability caused by cortical wall thinning. For males  $n = 8$  for each dietary condition, females  $n = 7$  for the control condition and  $n = 8$  for the vitamin D deficient condition, mean and standard deviation presented.

### Tissue Mineral Density

Tissue mineral density was measured at the femur midshaft to evaluate the material density within this region. There were no significant differences found between the control and vitamin D deplete groups in both male and female groups (figure 6.8).

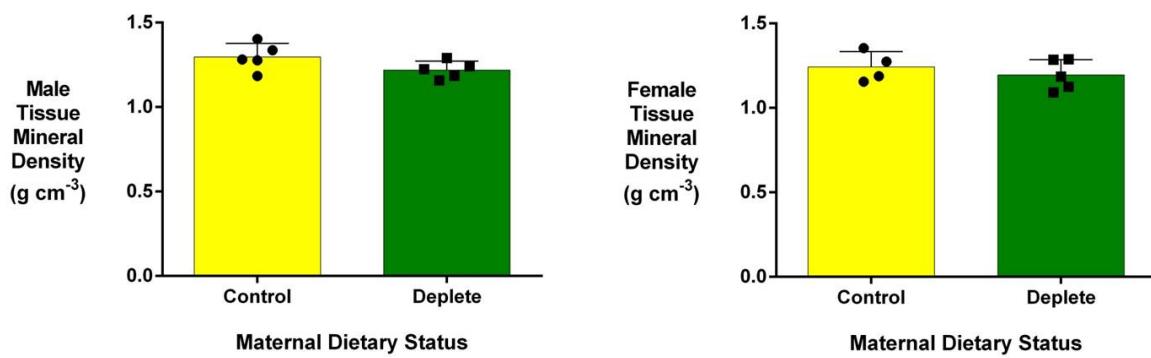


Figure 6.8: Femur Midshaft Cortical TMD. No differences were measured in bone mineral density at the femur midshaft region between control or vitamin D deficient groups, in either male or female cohorts. For males  $n = 5$  for each dietary condition, females  $n = 4$  for the control condition and  $n = 5$  for the vitamin D deficient condition, mean and standard deviation presented.

### Fracture Toughness

Crack initiation fracture toughness ( $K_{int}$ ) was measured at the femur midshaft region by notched bend testing to determine any changes in bone material resistance to fracture. Within the male group, there were no differences found between the control and vitamin D deplete groups. Similar to males, fracture toughness measurement yielded no differences between the control and deplete conditions (figure 6.9).

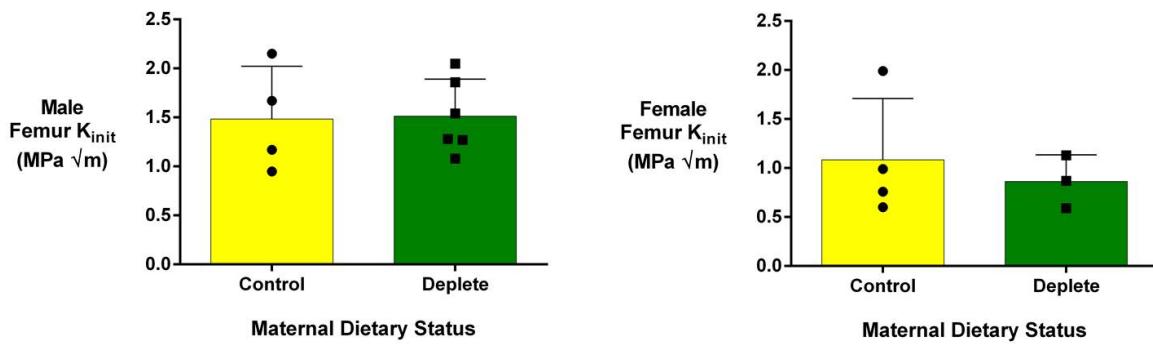


Figure 6.9: Fracture Toughness of Femur. Fracture toughness testing was conducted on notched femur midshaft samples and no differences were observed between control and vitamin D deficient animals in either male or female cohorts. For males  $n = 4$  for the control condition and  $n = 6$  for the vitamin D deficient condition, females  $n = 4$  for the control condition and  $n = 3$  for the vitamin D deficient condition. Mean and standard deviation presented.

### Bone Strength

Strength of samples was evaluated through three-point bend testing. The mean maximum load during three-point bend testing of the femur was found to be significantly lower in the male vitamin D deplete group compared to the control group (8.3 N v 11.4 N,  $p = 0.04$ ). In contrast to males, three-point bend testing of female femora yielded no statistically significant differences between the control or vitamin D deficient groups (figure 6.10).

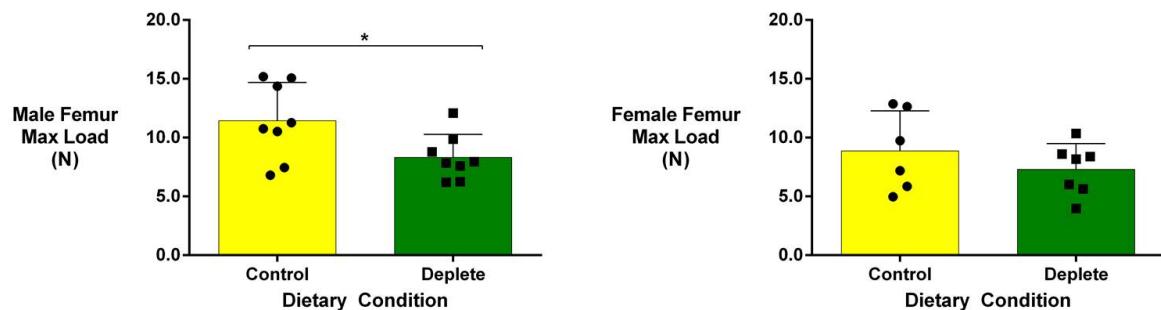


Figure 6.10: Three-Point Bend Tests of Femora. Three-point bend testing of whole femora found a reduction in maximum load in vitamin D deficient samples within the male cohort ( $p = 0.04$ ). No such differences were evidenced in the female group. For males  $n = 8$  for each dietary condition, females  $n = 6$  for the control condition and  $n = 7$  for the vitamin D deficient condition, mean and standard deviation presented.

#### Male: Bone Strength Simulation

Resulting from the differences found in bone midshaft size, compressive strength of femur midshafts were computationally evaluated in collaboration with Dharshini Sreenivasan and Dr Justin Fernandez of the Auckland Bioengineering Institute, New Zealand. Computational modelling of compression along the femur long axis on the subset of male femur midshaft regions predicted a lower necessary force to reach failure for the vitamin D deplete rats when compared to controls (7.0 N vs 5.2 N,  $p = 0.04$ , figure 6.11). Failure was defined by the average von Mises stress (a single resolved stress value derived from the three dimensional stress state of each finite element hexahedron) of the midshaft structure exceeding a pre-determined value. A screen capture of a resolved test can be viewed in figure 6.12.

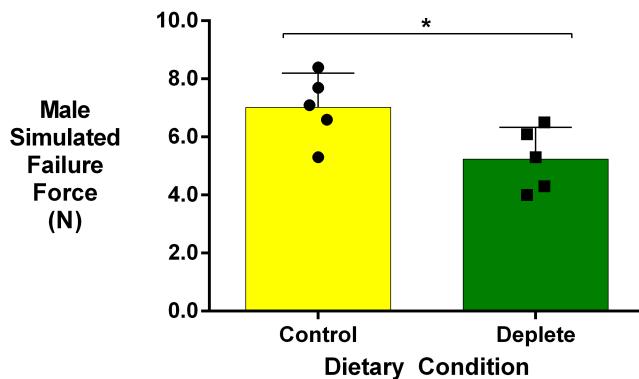


Figure 6.11: Femur Midshaft: Maximum Load Simulation. Computational analysis of a subset of male femur midshafts ( $n = 5$ ) predicted lower forces to failure in the vitamin D deficient group ( $p = 0.04$ ) when compression tests along the long axis were simulated.  $n = 5$  for each dietary condition, mean and standard deviation presented.

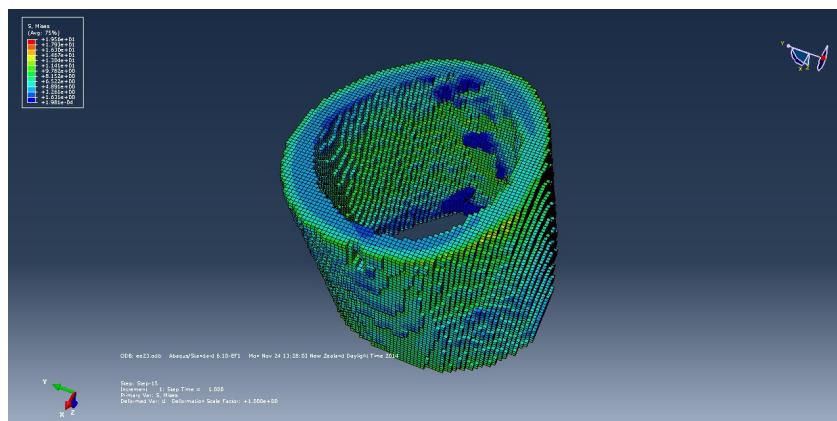


Figure 6.12: Resolved Load Simulation Test. An example of a completed mid-shaft femur computational compression test is depicted, with Von Mises stress calculated for each element.

## 6.4 Discussion

This study found mean cross-sectional area at the femur midshaft and femur maximum load to be lower in male rats from a vitamin D deficient background when compared to controls at 21 days of age. This was despite there being no observed significant differences in the quantity of bone within the femur midshaft. No further changes were evidenced in any of the other measures of bone health or in the female cohort. These results suggest that a deficiency of vitamin D during *in utero* life results in a reduction in cross-section of the femur and consequently a detrimental effect on bone loading mechanics. Interestingly, these outcomes were only observed in male offspring, suggesting vitamin D deficiency during pregnancy and the effects on offspring may be gender specific at 21 days of age.

Further investigation into the effect of these differences in femoral midshaft area on bone mechanics was conducted through the use of  $\mu$ CT scans from a subset of the male cohort ( $n = 5$  for each dietary condition) to generate finite element models for bone strength. Compression tests were simulated along the long axis of femoral midshaft sections, with the material properties of each sample identical ( $E = 1$  GPa,  $\nu = 0.3$  and isotropic material). Therefore, the material properties of the cortical bone models within this study were normalised such that the structural contribution towards bone compression strength was evaluated. The strength of each sample was measured using a Von Mises failure criteria, which was based on experimental compression tests conducted on 3D printed bone as described by Sreenivasan *et al* [206].

These simulated compression tests on femoral midshaft sections showed the vitamin D deficient group experienced lower applied force to reach mean failure stresses (defined prior to testing) within the midshaft structure, indicating the reduction in midshaft area within the vitamin D deficient male group as pronounced enough to cause a significant reduction in predicted bone strength. However, the mean tissue area measurements for this subset of femur midshafts analysed were found to be statistically different at 4.91 and  $3.92\text{ mm}^2$  for mean and deficient groups respectively ( $p = 0.03$ ), whilst mean bone area was not statistically different at 1.96 and  $1.49\text{ mm}^2$  for mean and deficient groups respectively ( $p = 0.054$ ). Consequently, it is unclear why the force (a function of area)

was found to be significantly different between the two dietary conditions whilst bone area was not. A likely explanation could be that bone area measurements were derived from the average midshaft area over the whole length of the bone sample through  $\mu$ CT scans, which may differ from the area where force was applied (solely on the top face of the bone sample) during the FE simulations. It is possible that the mean area of the top face of bone samples was statistically different between the dietary conditions. The importance of this link between structure and mechanics is emphasised by our experimental data on bone mineral density and fracture toughness of the femur midshafts, where no difference in either of these properties were found suggesting material properties were indeed unaffected by dietary backgrounds.

Currently, the effect of maternal vitamin D concentration on future bone health of offspring remains a contentious issue and the results from human observational studies have provided conflicting outcomes [207]. Part of this may stem from the difficulty of isolating and measuring the causal pathway between maternal vitamin D levels and offspring skeletal health. Examples of this include different serum level classifications for deficiency and sufficiency, different bones being assessed across studies and differences in follow-up protocol (due to experimental design or practicality) for offspring lifestyle factors that can affect skeletal development. As there are a multitude of factors outside of vitamin D that can change bone health, calls have been made for controlled, double blind studies to provide more clarity and the MAVIDOS trial in human patients is currently underway at the time of writing in an effort to remedy this [128, 208].

Therefore, in the context of existing studies into the role of maternal vitamin D on offspring bone health, the environment utilised in this experimental design is much more controlled, certainly more than is possible with human trials. The vitamin D deficiency has been confined to the duration of *in utero* life, with post-birth conditions consistent between control and vitamin D deficiency groups. In terms of vitamin D serum levels, normal levels are returned within the deficient case within 2-3 weeks post birth [143, 209]. As such, any differences in offspring bone development can be attributed to modifications of vitamin D status occurring in the period of *in utero* and the first two weeks of postnatal life.

During pregnancy, nutrient provision from the mother to the developing foetus is a major contributory factor towards *in utero* conditions. Mineral (specifically calcium) accrual within humans mostly occurs in the final trimester, hence the perception that adaptations within this phase may have most effect on adult peak bone mass and future osteoporosis risk [2]. Within rats, there is a similar process whereby 95% of *in utero* calcium transferal occurs in the last 4-5 days of gestation [210]. As vitamin D is closely associated with calcium levels and the high correlation between maternal and newborn infant plasma 25(OH)D levels [211, 212], vitamin D deficiency during *in utero* life has been proposed as a risk factor to impaired bone development and increased susceptibility to bone fracture in later life.

The results presented here indicate that depletion of vitamin D during *in utero* and immediate post-natal life resulted in a skeletal change observable up to at least 21 days of age in male offspring. Although serum vitamin D levels were not recorded within this experimental group, this model of maternal dietary vitamin D deficiency has been shown to deplete both maternal and offspring immediate post-natal vitamin D levels [213]. In addition, 25(OH)D levels of animals from the vitamin D deficient background have been confirmed to have returned to normal by weaning (21 days of age) [143], with normal levels of calcium and no evidence of rickets observed from birth (development and maturity trajectory have also been shown to be comparable to control samples). Therefore, the model here indicates that maternal vitamin D depletion has hindered the bone modelling phase within male offspring despite normal post-natal calcium levels (remodelling is considered to begin at 3 to 12 months of age depending on location and whether trabecular or cortical bone is observed [142]).

From the same rat model, Lanham *et al* [209] implicated bone structure was adapted due to *in utero* vitamin D depletion. A vitamin D deficient background was associated with impaired trabecular bone development (increased bone surface to bone volume ratio and reduced trabecular thickness of bone at the proximal femur) at 0 and 140 days of age. Additionally, the 0 day aged rats were evaluated to have lower cross-sectional moment of inertia measurements at the femur midshaft, suggesting impaired mechanical competence. Our findings from the day 21 animals showed similar trends, with observed reductions in femur midshaft size and mechanical loading capability in the vitamin D

deficient cohort. No differences in the buckling ratio were found suggesting a decrease in cross-sectional moment of area due to reduced area accounts for lower mechanical loading ability. Taking the results from both studies, this suggests vitamin D status is important during the course of femoral bone development independent of calcium levels.

In contrast, Lanham *et al* found bone differences were present in female offspring, but absent in males. This is in contrast to the study findings here where differences were only present in males. Unfortunately, the cohort we have examined here and the experimental outputs obtained do not allow us to further explore the origins of this difference, but Lanham *et al* postulate that gender specific responses may be in place to program development according to expected environmental challenges derived from foetal life experiences. Determination of this effect will require a more detailed analysis of gender specific components of bone development. Additionally, female midshaft differences were not reported by the authors at 140 days of age, so this may indicate male midshaft response to vitamin D deficiency manifests at a different (later) timepoint when compared to females.

Examination of mother-infant pairs by Javaid *et al* [133] demonstrated a relationship between lower maternal serum 25(OH)D at late pregnancy and lower child whole body bone mineral content, bone area and areal bone mineral density measurements at 9 years old. Further examination of umbilical cord blood calcium concentration was found to correlate with whole body bone mineral content of children, although the influence of vitamin D was more dominant. The authors conclude with the postulation that maternal vitamin D deficiency reduces calcium transport across the placenta, an effect on skeletal health that persisted, and was observable, up to 9 years of age. The results here support the effect of vitamin D (reducing the femur midshaft area in rats) but interestingly conflict with the assertion that changes are partly due to differences in calcium provision to the foetus.

The presence of bone changes found in this animal cohort and the emphasis of vitamin D during pregnancy by Javaid *et al* is in contrast to a number of findings regarding calcium status. Osteopenia in low birth weight human infants have been related to a

deficiency in calcium and phosphorus rather than vitamin D [214] along with endocrine maintenance of calcium and phosphate balance (both important mineral components of hydroxyapatite) being cited as more important in defining postnatal bone development [136]. However, calcium supplementation seems to only be important for foetal bone mineralisation in cases where maternal calcium levels are originally inadequate [215]. This may explain the differences in calcium importance between findings in the osteopenic cases from literature and the data here (from animals with adequate calcium levels).

Low maternal vitamin D status has been demonstrated to have a more immediate impact on foetal bone development within humans by Mahon *et al* [137]. Deficient and insufficient maternal 25(OH)D serum levels at 34 weeks of pregnancy was found to be associated with increased femoral metaphyseal cross-section and femur splaying index (a measure of femur shape derived from metaphyseal cross-section divided by femur length) of infants at 19 and 34 weeks of age *in utero* when compared to 25(OH)D replete mothers. Findings from Viljakainen *et al* [216] showed bone mineral content and cross sectional area of tibiae were higher and larger in 10-11 day old newborns from higher vitamin D intake mothers (found to positively correlate with maternal serum 25(OH)D levels measured post-natally and cord blood 25(OH)D levels). This modulation of bone shape has been evidenced in the vitamin D depleted offspring within this study, whereby the femur midshaft (diaphysis) area is reduced. The Mahon and Viljakainen outcomes suggest that this reduction may originate from differences in *in utero* development as a consequence of vitamin D challenge, the effect of which is still present in male rats at 21 days of age.

In contrast to this however, Lawlor *et al* of the Avon Longitudinal Study of Parents and Children (ALSPAC) has found no associations between maternal 25(OH)D concentrations within any trimester and childhood bone mineral content at nine to ten years of age from a sample of 3960 mother-infant pairs [197]. This is similar to our findings where no changes in bone mineral density were found and appears to suggest the influence of vitamin D is limited to bone shape rather than mineral content. The investigators of ALSPAC have suggested in light of their findings and the available literature that it is more plausible that maternal 25(OH)D levels will affect BMC at birth and early life

rather than later on in childhood of the 9-10 year olds studied. This would also appear to be the case in our work, where the age of rats studied were around the weaning stage.

It is unclear from the body of our data how or why maternal vitamin D outside of calcium provision is affecting offspring bone development. A molecular mechanism modulating the behaviour of bone cell function is not apparent from analysis of *Runx2*, *Col1*, *Opn* and *Ocn* gene expression as no differences were present between control and vitamin D deficient animals. Progeny from vitamin D challenged mothers have previously been demonstrated to have reduced lung function due to a change in cell numbers and cell apoptosis behaviour [217] and this may be true too within bone health. The broad experimental approach adopted here is unlikely to detect subtle changes and it is assumed RNA collected from the proximal femur is representative of the cell biology state of the bone overall. Differences may be present in RNA from the midshaft region alone but this was not possible due to the three-point bending and fracture toughness testing.

A limitation of the experimental approach we adopted is that further in-depth analysis was restricted due to accommodation of a range of techniques to provide an overview of bone quality within these samples. In particular, we have restricted our biological analysis to four well researched genes known to influence maturation of osteoblasts and it may be the case that vitamin D has a more refined impact upon bone cellular interaction and regulatory networks. As mentioned, this experimental model has been demonstrated to deplete serum vitamin D levels in offspring whilst calcium levels remain comparable to healthy cases, though no blood analysis has been conducted to confirm this, which would be useful to conclude the timeframe where vitamin D sufficiency was achieved again.

In summary, our study aim was to determine the effect of vitamin D deficiency during *in utero* life on offspring bone development after 21 days of age. Investigation has shown that maternal vitamin D deficiency caused a reduction in bone shape at the femoral mid-shaft of male offspring leading to reduced mechanical capability found experimentally and confirmed computationally. Of interest is that, although the cross-section of the femoral midshaft was affected by maternal vitamin D status, actual bone quantity was not, and highlights a change in bone shape that others have reported [137, 216] although

this did not affect the buckling ratio. Data from bone gene analysis, micro-architecture analysis and material property analysis found no further differences. There were no differences found in any of these areas in the female animals regardless of maternal dietary background.

Vitamin D deficiency has been increasingly highlighted as a modern public health concern globally [212] and the impact on this on foetal development is an active area of research. As seen in this study, vitamin D status has been implicated in the healthy development of a number of organs despite limited impression on size or growth trajectory [196]. Although important for maintaining bone health in childhood and adulthood, the role of *in utero* vitamin D as a factor for future bone health is still widely debated. The findings here indicate that vitamin D has an immediate effect on bone development during foetal life that is still observable into the early life of rats. No changes in gene expression were found so it is still unknown whether this is a persistent adaptation with an epigenetic component. Further investigation of this is required to inform upon the importance of maintaining healthy vitamin D levels and any required supplementation during pregnancy.

Following this study, we have also analysed the effect on bone development of *in utero* vitamin D deficiency in rats at a later stage in life (140 days of age). Evaluating a later timepoint provides further information as to whether the reduction in bone area found in the male femur midshafts (and hence a reduction in mechanical competency) is a continued effect of vitamin D deficiency throughout the course of life (chapter 7). If this indeed is true, then it is possible that this will highlight an observable effect of *in utero* vitamin D deficiency resulting in epigenetic changes.



## Chapter 7

# Effect of *in utero* Vitamin D Deficiency on Rodent Offspring Skeletal Health at 140 Days Old

Work accepted for poster presentation at Singapore, DOHaD World Congress 2013, oral presentation at Southampton, UK, internal Faculty Research Conference 2014 and for oral presentation at Barcelona, Spain, WCCM 2014.

Fracture toughness testing and reference point indentation testing assisted by Dr Tom Jenkins, Bioengineering Research Group, University of Southampton.  $\mu$ CT scanning assisted by Dr Stuart Lanham, Bone and Joint Research Group, University of Southampton. RNA extraction and cDNA synthesis assisted by Kate White, Bone and Joint Research Group, University of Southampton.



## 7.1 Introduction

A primary consequence of current global population ageing is the increasing prevalence of non-communicable diseases, such as osteoporosis, accompanied by economical and societal considerations [102]. Osteoporosis is a common disease amongst elderly people and increases the risk of bone fracture due to a number of possible alterations to cellular and physical bone health. In the UK, it is predicted that one in two women and one in five men over 50 years old will suffer a fracture of any kind [218], with subsequent treatment currently costing the NHS £2.3 billion per annum and set to rise [219].

Typically, osteoporosis sufferers experience decreases in bone mass, differences in structural arrangement of the trabecular bone [220] and increased intracortical porosity at old age (over 65 years old in humans) [221]. Recent work has highlighted the probability that the underlying bone chemistry is altered [172], which is linked to reduced fracture toughness [222, 223]. The pathogenesis of osteoporosis is yet to be fully understood and a wide array of risk factors have been identified. Frequently mentioned osteoporosis risk factors include gender, age, usage of glucocorticoid medication, dietary calcium and vitamin D intake, ethnicity and behavioural aspects such as smoking [170]. Generally, these factors affect two important predictors of osteoporosis fracture risk; peak bone mass and post-peak bone mass loss [224]. With the aforementioned economic burden incurred by osteoporosis, there is great interest in studying how risk can be reduced by understanding the roles of separate factors towards achieving optimal peak bone mass or by lowering the rate of post-peak bone mass loss and hence inform preventative and further therapeutic measures.

One particularly fascinating aspect of modern disease prevention is the developmental origins of health and disease (DOHaD) paradigm, whereby early life events modify development and cause persistent changes that modulate disease likelihood in adult life [117, 225]. Indeed, strong links between circulatory disease mortality, and adult bone mass, to birth weight have been demonstrated [226]. Regarding skeletal health, evidence is accruing whereby osteoporosis is one such chronic disease that can be influenced by experiences within the foetal and early post-natal periods [227–229] and that fracture risk can be determined by the various skeletal maturation phases throughout the entire

lifecycle [230]. Supporting this, measurements of weight at birth and one year of age have been shown to correlate with bone mineral content at both the lumbar spine and hip in adulthood [231, 232] and cohort studies have also highlighted increased height and weight gain at multiple stages of life, are related to increased bone cross-sectional area at early old age (60 - 64 years old) [233].

Despite recognised links between low vitamin D levels and compromised bone health in children (rickets) and adults (osteomalacia) [234, 235], the impact of *in utero* vitamin D deficiency upon offspring future skeletal health remains poorly understood. With an estimated one billion people worldwide suffering from deficient levels of vitamin D (according to World Health Organisation definitions) [236], a comprehensive understanding of *in utero* vitamin D levels and the relationship to subsequent bone health in adulthood, and risk to osteoporosis, is required.

Currently, the evidence regarding the importance of vitamin D levels *in utero* towards later life bone health is inconclusive, and further evidence is required to inform policy [208]. Acute effects of low maternal vitamin D levels have been related to foetal femur splaying, and hence a modification in bone development [137]. Studies investigating bone health further into the life course have shown that reduced vitamin D concentration at late pregnancy leads to a reduction in both lumbar spine and whole body bone mineral content of children at nine years of age [133], with suggestions that the low mineral accrual associated with low vitamin D levels also affect post-natal growth [237]. In contrast, longitudinal studies investigating maternal vitamin D and offspring bone mineral content, at nine and ten years of age, have yielded no correlations [197].

Increased fracture risk associated with osteoporosis can be attributed to a number of biological and physiological parameters [114, 115, 238]. Clinically, evaluation of bone health is assessed through measurement of bone mass using DEXA scans [239]. Building upon this, a number of techniques have been developed to augment data from DEXA data with the goal to ultimately provide a more comprehensive analysis of bone health clinically. As such, nano-indentation [240], micro-indentation [166], fracture toughness [45] and micro-architecture [154] studies have all been conducted *ex vivo*. The breadth

of analysis methods now available to measure bone health reflects the complex nature of different biological and mechanical factors that must be considered to track bone development. The current chapter has examined the bone health of 140 days old rats that have experienced complete vitamin D depletion during *in utero* life.

### Hypothesis

“Animals from a low vitamin D background will demonstrate persistent changes in bone health, resulting in compromised bone in adulthood”.

### Objectives

Therefore, our objectives were to provide information on the effect of *in utero* vitamin D deficiency on;

- i) the bone biological environment through osteoblast gene expression analysis by reverse transcription PCR (RT-PCR),
- ii) the structural cortical and trabecular bone form by CT scanning,
- iii) the cortical bone mechanical competency by reference point indentation (RPI) and three point bend strength testing and
- iv) bone material properties by mineral density measurements and fracture toughness testing. Consideration of each of these components of bone quality will determine if any individual features of bone have a predominant role in setting bone fracture resistance.

## 7.2 Methods

### Sample Preparation

140 day old male and female Sprague-Dawley rats were used for this experiment, with  $n = 8$  for each gender and maternal dietary condition (either control or vitamin D deficient, see section 3.1.1 for more detail). Animals were weighed and after sacrifice stored at  $-80^{\circ}\text{C}$  until required. Hind limbs were rapidly thawed in a water bath and both femora were retrieved and prepared for subsequent testing. An overview of experimental procedures for each sample can be viewed in figure 7.1.

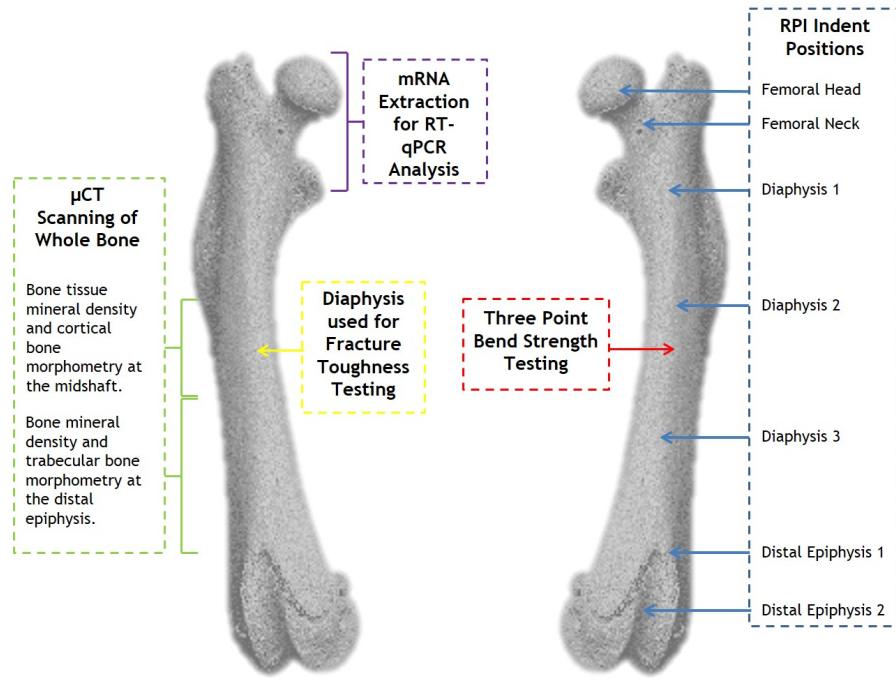


Figure 7.1: Summary of Experimental Methods. The diagram displays the methods used to measure different bone health outcomes from the maternal dietary vitamin D deficient study. Both left and right femora were excised from each sample and subsequently utilised for gene expression, micro-mechanical performance, micro-architecture, material properties and organ level mechanical competence analysis.

### Osteoblastic Gene Expression Analysis

Gene expression was measured using RNA obtained from the right proximal femora and was performed using RT-qPCR. Further details of the RT-qPCR procedure can be found in section 3.2.1. Expression of target genes were presented in relation to a housekeeping gene and as  $2^{-\Delta Ct}$  values. *r18s* was used as a housekeeping gene, *Runx2*, *Col1*, *Opn* and *Ocn* were used as target genes.

### $\mu$ CT Bone Architecture Analysis

Left side femora were imaged by  $\mu$ CT using a SkyScan 1176 in vivo scanner (Bruker micro-CT, Kontich, Belgium) at 18  $\mu$ m resolution (source voltage of 65 kV, current of 385  $\mu$ A, 0.5 mm Al filter and exposure time of 390 ms). Reconstruction was performed in NRecon software (version 1.6.4.6, Bruker) before microarchitectural analysis was conducted using CTAn software (version 1.11.9.3, Bruker) at the cortical bone regions from the midshaft and trabecular bone regions from the distal epiphysis. Further details of this procedure and on parameters measured can be found in section 3.3.1.

### Mineral Density: $\mu$ CT

Bone mineral density (BMD) measurements were obtained from trabecular bone regions and tissue mineral density (TMD) values were obtained from the cortical bone regions from the  $\mu$ CT data calibrated against hydroxyapatite phantoms of known density. Further details of this procedure can be found in section 3.3.1.

### Bone Micromechanics Analysis

Indentation was performed with a Bident reference point indenter (Active Life Scientific, Santa Barbara, USA) at seven sites along the anterior surface; the femoral head, femoral neck, three points along the midshaft, and twice at the distal epiphysis (one at the top of the condyles and one between the condyles). Data was recorded using

the built-in Biodent software and measured variables were total indentation distance (TID), indentation depth increase (IDI), creep indentation distance (CID) and energy dissipation. Further details of this procedure can be found in section 3.4.1

#### Three-Point Bending Analysis

The left femora were mechanically tested until failure within a three point bend arrangement using an electromechanical testing machine (Instron, High Wycombe, UK) to measure strength - different machines were used for fracture toughness and strength due to the higher loads necessary for fracture in the strength testing). Samples were placed on two supports of 10 mm span within a water bath filled with HBSS so samples stayed hydrated and loaded at  $0.01 \text{ mm s}^{-1}$  until fractured. Maximum loads were recorded from experimental data. Further details of this procedure can be found in section 3.4.3.

#### Fracture Toughness Analysis

The right femora were measured for fracture toughness through notched (three-point) bend testing. Samples were placed on a 6 mm span with the anterior wall of each femur notched and tested in tension. Loading was performed at  $0.001 \text{ mm s}^{-1}$  on an Electro-force 3200 mechanical tester (Bose, Minnesota, USA), with load and displacement data recorded. Fracture surfaces were then imaged through scanning electron microscopy (JSM-6500F, JEOL, Peabody, USA) and utilised for determining fracture toughness. Further details of this procedure can be found in section 3.4.2.

#### Statistical Analysis

It has been demonstrated that skeletal development and the role of vitamin D differs according to gender. As such, the experiment was split according to gender before analysis of control and vitamin D deplete samples was performed. Statistical analysis was performed using unpaired t-tests, with Welchs correction to account for unequal

standard deviation on data sets (Graphpad Prism, La Jolla, USA). All data sets were tested for Gaussian distribution and significance determined at  $p < 0.05$ .

### 7.3 Results

This study utilised Sprague-Dawley rats following *in utero* vitamin D depletion as a model for skeletal development challenge. Analysis of mean weights of each dietary group demonstrated mean male vitamin D deplete weight to be lower than the male controls (541 g and 580 g respectively), although this did not reach statistical significance (data in table E.1, Appendix E). Negligible weight difference was observed between the two female conditions.

#### Osteoblastic Gene Expression Analysis

Osteoblastic mRNA retrieved from the femoral bone was analysed using qRT-PCR to assess the gene expression profile. The averaged normalised  $2^{-\Delta Ct}$  values for the male and females groups are presented in figures 7.2 and 7.3. Comparison of the expression levels of established osteogenic genes throughout bone formation (*Runx2*, *Col1*, *Opn* and *Ocn*) between the two dietary conditions yielded limited statistical differences (*p* values are presented in table E.2, Appendix E).

For osteopontin, the increased gene expression found in the male deplete group was statistically significant (*p* = 0.02). Typically, gene expression levels tended to be higher in the male deplete group. This was in contrast to observations in the female cohort, where, aside from *Col1* levels, the control group showed higher average osteogenic gene expression.

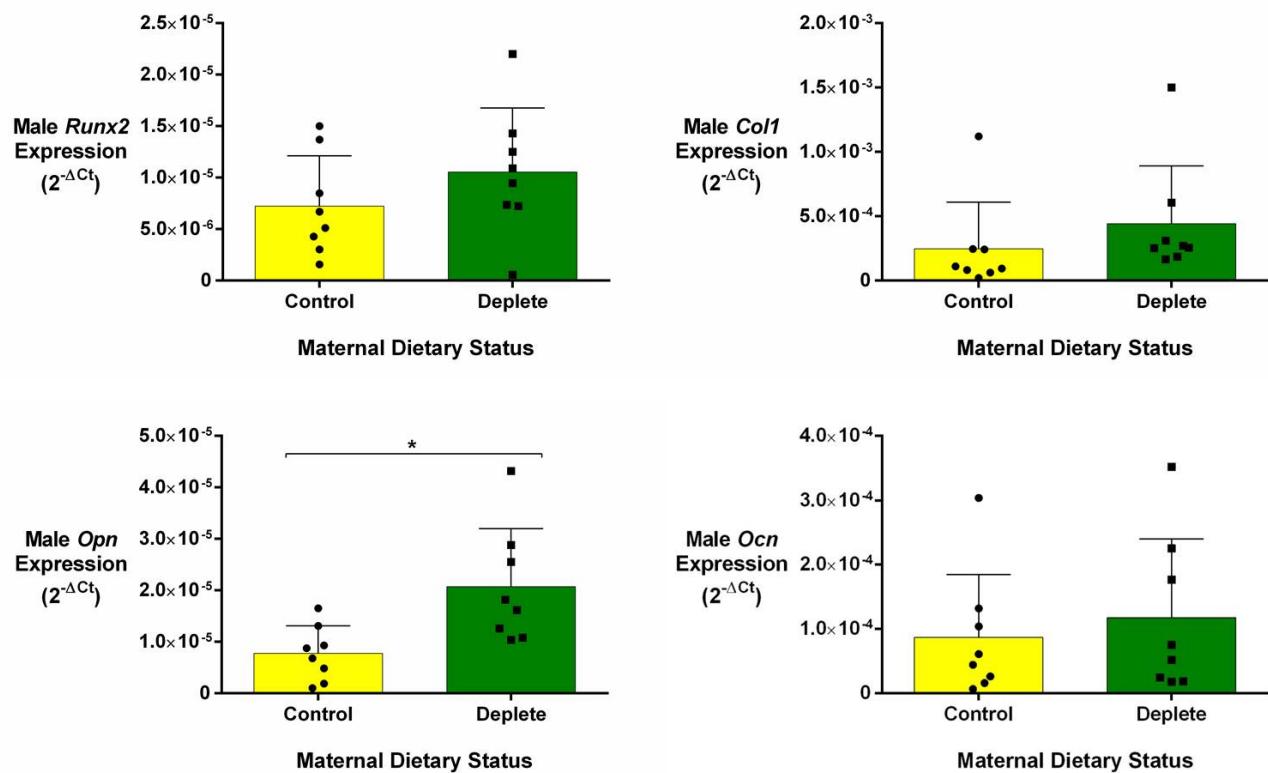


Figure 7.2: Vitamin D Day 140 Male Osteogenic Gene Expression. RT-qPCR data for gene expression of osteoblastic RNA from male samples, data points represent  $2^{-\Delta Ct}$  values for each sample normalised to internal housekeeping gene (*r18s*). Means for all four genes were found to be higher in the deplete group, with *Opn* found to be significantly different ( $p = 0.02$ ).  $n = 8$  for each condition. Mean and standard deviation presented.

#### $\mu$ CT Analysis: Bone Architecture and Mineral Density

$\mu$ CT analysis was undertaken on all femora to determine changes in bone architectural and density data. The bone tissue mineral density (TMD) measurements of the cortical bone within the midshaft region of interest produced negligible differences ( $p = 0.67$  for males and  $p = 0.10$  for females) between the diet groups in both genders and very little variation between samples. Analysis of the bone mineral density (BMD) of trabecular bone at the distal femur also produced limited evidence of changes due to the different diet backgrounds (figure 7.4).

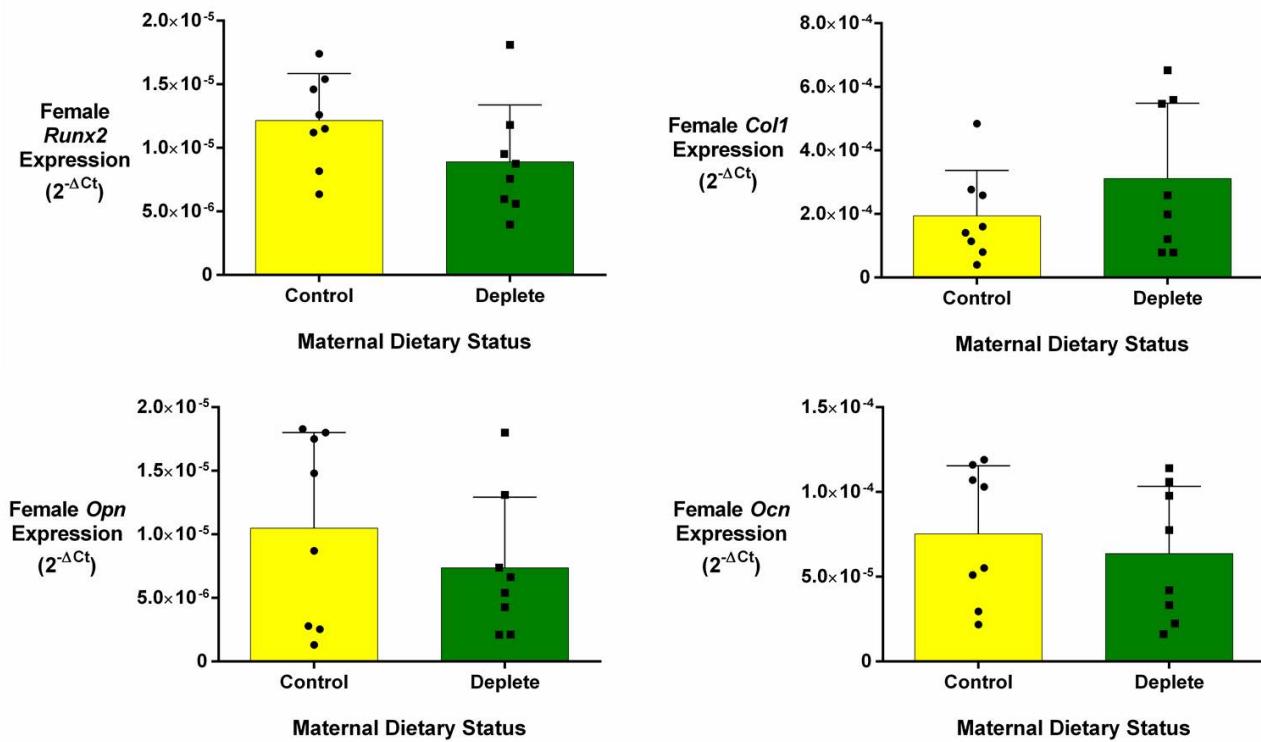


Figure 7.3: Vitamin D Day 140 Female Osteogenic Gene Expression. RT-qPCR data for gene expression of osteoblastic RNA from female samples, data points represent  $2^{-\Delta Ct}$  values for each sample normalised to internal housekeeping gene (r18s). No statistical significance was found between the control and deplete groups. Aside from *Col1*, mean gene expression was found to be higher in the control diet group.  $n = 8$  for each condition. Mean and standard deviation presented.

$\mu$ CT assessment of cortical bone architecture (mean cross-sectional area, mean cortical bone area, mean bone area fraction and mean cortical bone thickness) at the femur midshaft and trabecular bone architecture (bone volume to tissue volume percentage, trabecular number, trabecular thickness and trabecular separation) at the distal femur produced no changes based on dietary background that reached statistical significance for either gender (figure 7.5).

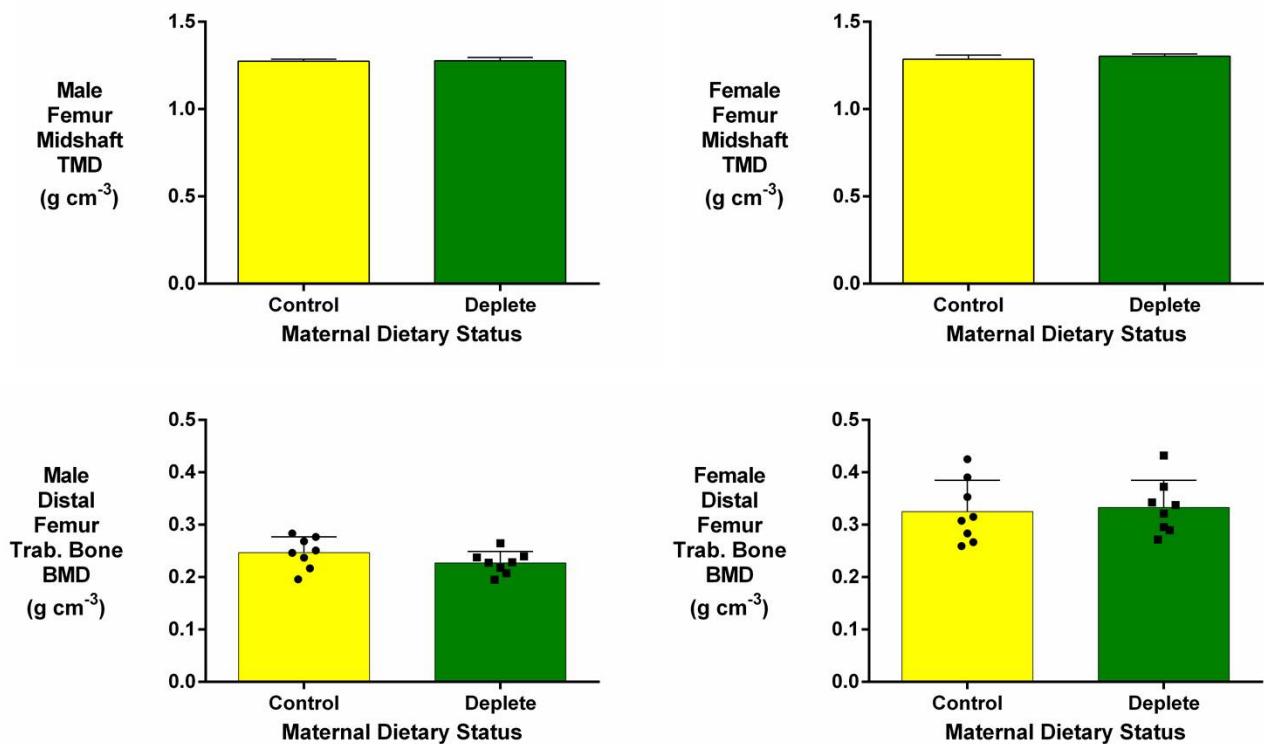


Figure 7.4: Tissue mineral density measurements for cortical bone at the femora midshaft region and bone mineral density values for trabecular bone located at the distal femur regions. Values were calibrated against a hydroxyapatite phantom of known density. For both the TMD and BMD measurements, little difference was found between the two dietary backgrounds.  $n = 8$ , mean and standard deviations shown.

The male control cohort showed evidence of greater bone mass compared to the vitamin D deplete group although this was not statistically significant. Mean cortical bone area, cortical thickness, trabecular bone to volume percentage and trabecular number were all higher in the control samples. Similarly, female control cortical bone measurements were higher for total midshaft area and bone area (figure 7.6). However, this trend was less clear when assessing the trabecular architecture.

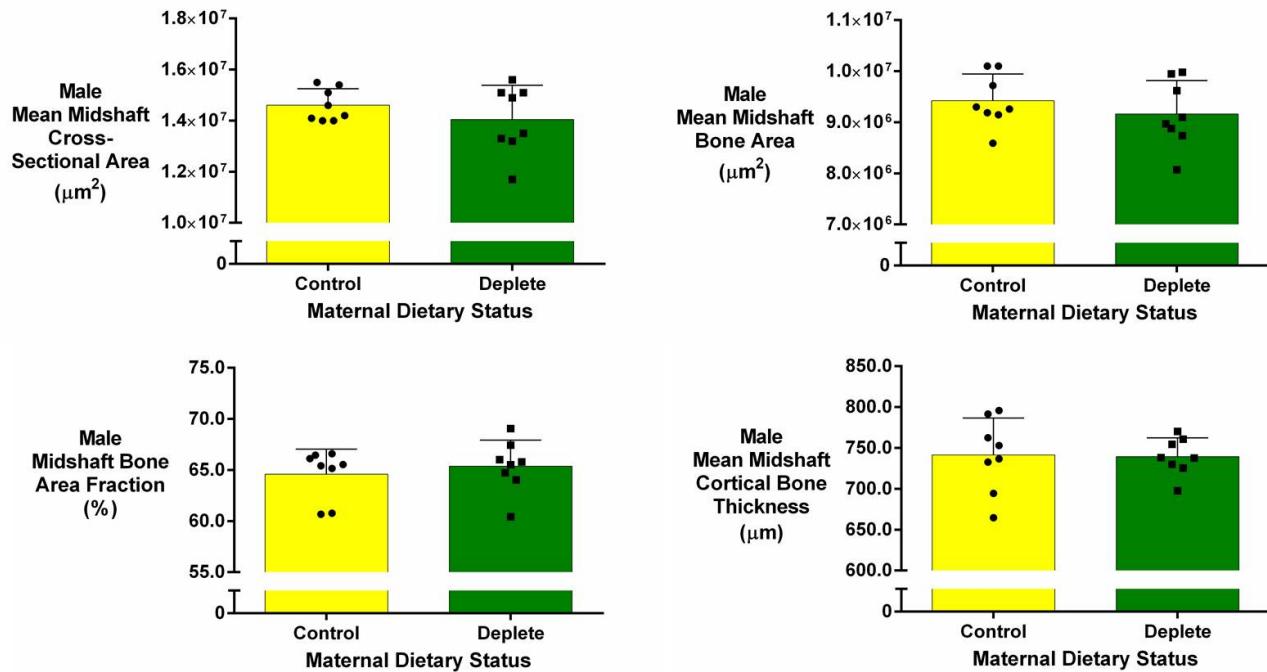
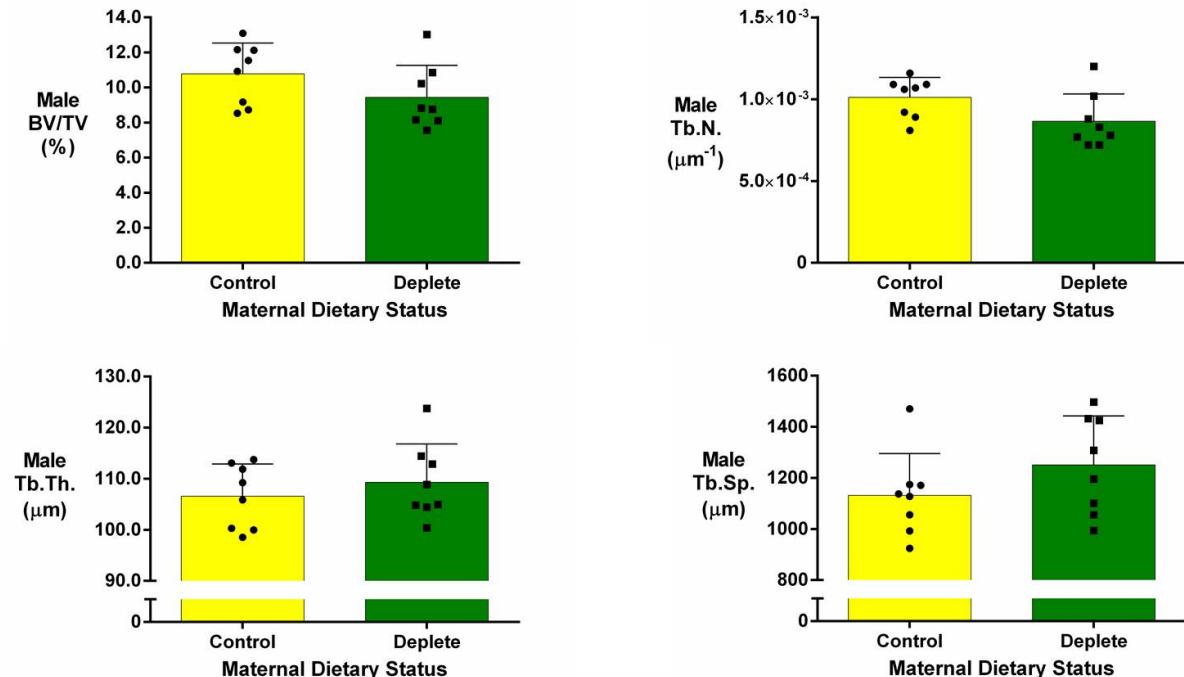
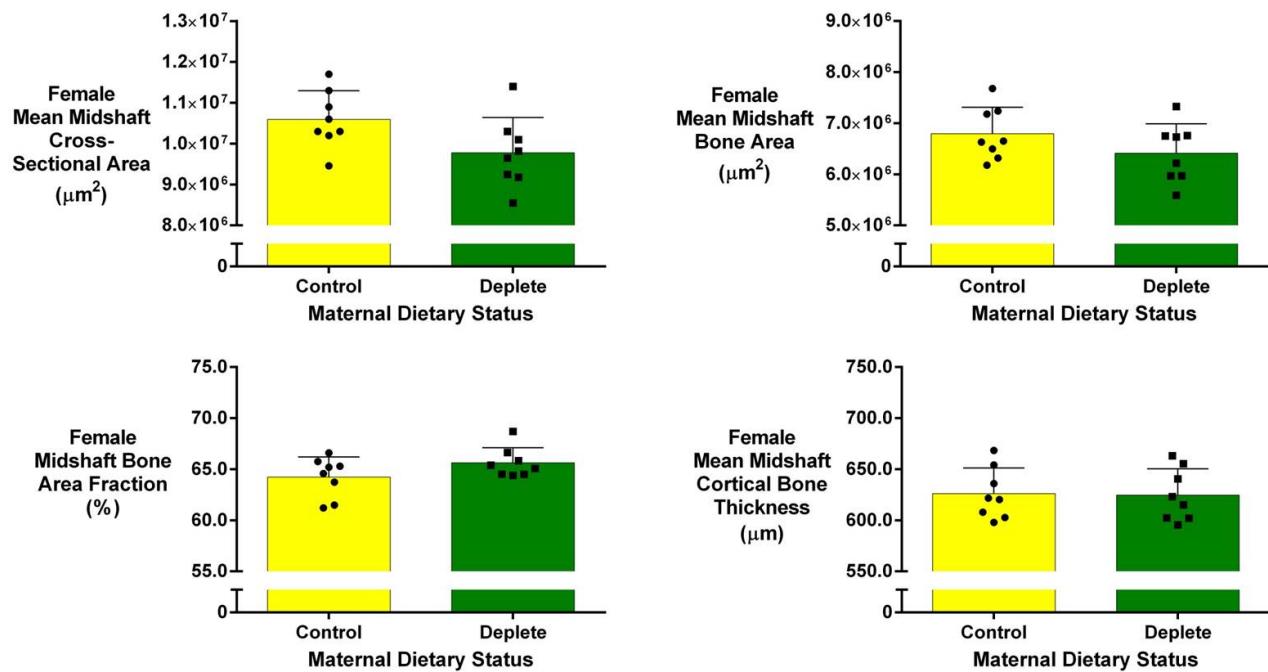
Femur Midshaft: Cortical BoneDistal Femur: Trabecular Bone

Figure 7.5: Architectural analysis of male cortical and trabecular bone. Cortical bone from the midshaft of femora and trabecular bone from the distal femora were analysed for volume and 3D morphology. No significant results were reached for any of the parameters assessed.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Femur Midshaft: Cortical Bone



Distal Femur: Trabecular Bone

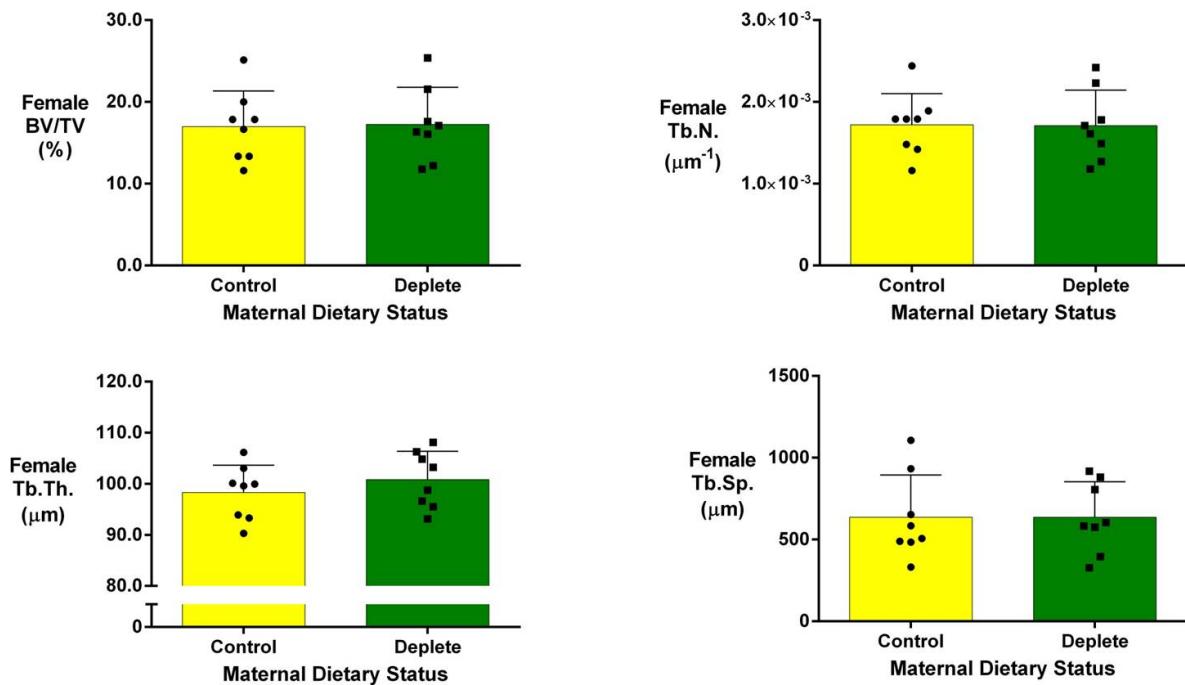


Figure 7.6: Architectural analysis of female cortical and trabecular bone. Cortical bone from the midshaft of femora and trabecular bone from the distal femora were analysed for volume and 3D morphology. Similar to the male cohort, no significant results were reached for any of the parameters assessed.  $n = 8$  for each dietary condition, mean and standard deviation presented.

Bone Micromechanics Analysis

Reference point indentation (RPI) was conducted to assess bone micromechanical behaviour, which has been suggested as a comparative measure of bone fracture toughness [166]. Investigation into femur micromechanics using RPI showed that bone located at the midshaft is more resistant to indentation forces (smaller indentation depths) when compared to bone at the femoral head and femur condyles, and was true for every sample (figure 7.7). Comparison of control and vitamin D deplete femora at individual sites highlighted no differences and this was consistent with all seven sites tested along the femur and testing parameters (total indentation distance, indentation distance increase, indentation distance upon constant load and energy dissipation, tables E.4-E.8, Appendix E). These results were found in both male and female groups (figure 7.7).

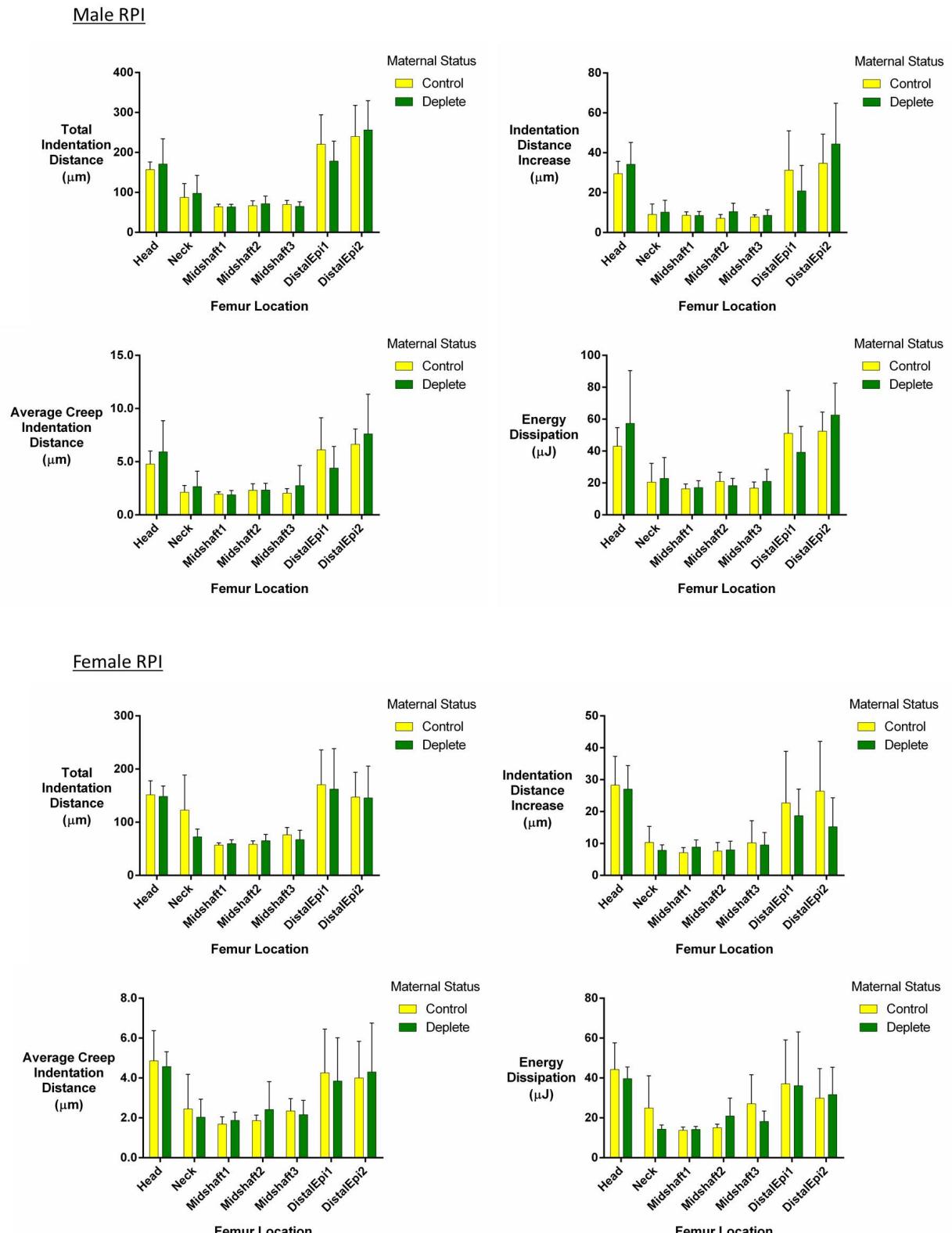


Figure 7.7: Reference point indentation results along femora. Mean indentation values are displayed for a range of parameters measured during the indentation cycle; total indentation distance, indentation distance increase, average creep indentation distance and energy dissipation. No significant differences were found at any location for either gender when comparing control against deplete.  $n = 8$  for each dietary condition, mean and standard deviation presented.

### Fracture Toughness and Strength Analysis

Fracture toughness analysis was performed on  $n = 6$  for male controls and  $n = 6$  for male deplete; and  $n = 7$  for the female control and deplete groups due to uncontrolled loading of samples during experiment set-up. Similarly, three-point bending analysis was performed on  $n = 7$  for controls for both male and females and  $n = 8$  for the deplete groups, again due to sample fracture during test set-up.

Notched bend testing of samples is an established method to measure material response to the presence of a critical flaw when loaded, hence evaluating material properties independent of geometry and mass. Analysis of the material properties of bone at the midshaft through fracture toughness testing (figure 7.8) showed negligible changes ( $p = 0.77$  for males and  $p = 0.83$  for females) regardless of dietary background.

Three-point bending testing in contrast is a measure of the collective response to load from material, structural and mass components. Similar to fracture toughness, the maximum load of the femoral midshaft measured through three point bend testing (figure 7.9) presented no significant differences. Interestingly, fracture toughness values for males and females were similar in magnitude, whereas the maximum load values obtained differed noticeably (mean male control, maximum load = 236.8 N and mean female control, maximum load = 173.1 N, table E.4, Appendix E), indicating material fracture toughness is consistent across genders for bone, with differences in mechanical response derived from structure or mass changes.

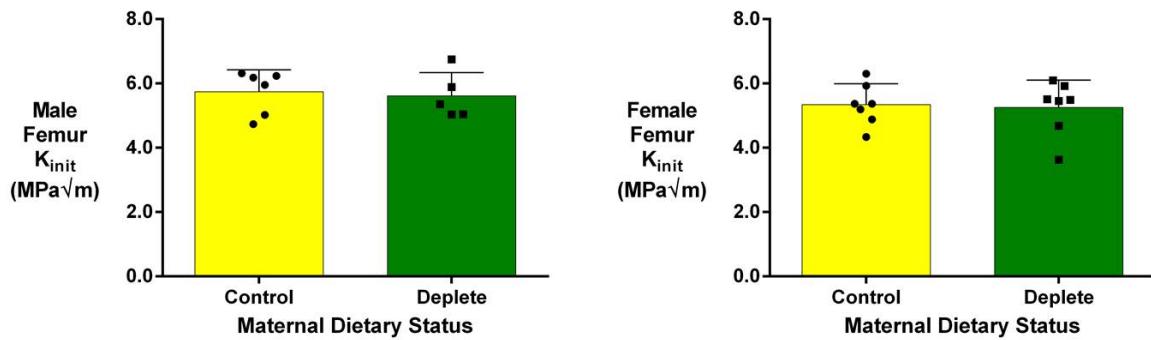


Figure 7.8: Femur fracture toughness. Fracture toughness results from notched bending tests, no differences were found between the control and deplete groups. Male control  $n = 6$ , male deplete  $n = 5$ , female control  $n = 7$  and female deplete  $n = 7$ . Mean and standard deviation presented.

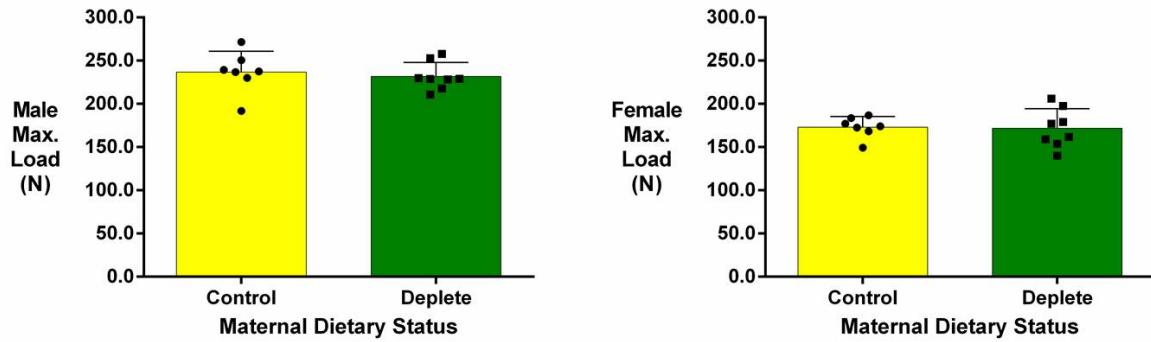


Figure 7.9: Femur maximum load. Maximum load results from three-point bend testing of femora. No differences were found between the two diet conditions for either gender. Male control  $n = 7$ , male deplete  $n = 8$ , female control  $n = 7$  and female deplete  $n = 8$ . Mean and standard deviation presented.

## 7.4 Discussion

The current study examined whether *in utero* vitamin D levels impacted upon adulthood skeletal health with regards to osteoblast phenotype, bone fragility and bone mass. Analysis of offspring at 140 days of age, from mothers deplete of vitamin D during pregnancy, and using weight as a surrogate measure for general health, animals from the control and experimental groups showed no differences despite altered vitamin D supply during foetal life. More specific to bone health, the mRNA expression, micro-architecture, micro-mechanics, bone material and organ-level mechanics were not found to be statistically significant for all comparisons between the control and deplete groupings, aside from osteopontin expression in males.

There has been recent evidence suggesting that development of many common diseases can be modulated in the early stages of life [1, 117, 119, 126, 226], promoting the significance of considering risk factors in earlier timeframes than is traditional. Osteoporosis is a significant problem and is associated with reduced bone strength, causing the characteristic increase in fracture likelihood [99]. Reduced bone strength itself can result from changes in bone structure, material properties and bone mass [241] and as such, modification of these parameters throughout the lifecourse can change an individual's susceptibility to osteoporosis [242, 243].

The model utilised in this study created a condition of complete vitamin D deficiency during foetal life, as evidenced by Eyles *et al* using the same procedure [244], before animals were transferred to normal conditions after birth. Therefore, any modulation of skeletal parameters would be determined solely by the difference in vitamin D provision *in utero*. It was anticipated that the severe vitamin D depletion would induce detrimental changes to skeletal mechanics originating from disruption of 'normal' bone cell activity. It has been shown that nanoscale heterogeneity can improve the ductility of bone, providing a mechanism of how the cellular environment and events occurring at this level can closely affect bone strength [57]. Regarding cell interaction, active vitamin D metabolites appear to promote osteoprotegerin and hence reduce osteoclastogenesis through the RANKL pathway during remodelling [245, 246], whilst vitamin D also appears to promote the expression of several osteogenic related genes such as osteocalcin

and alkaline phosphatase [199]. Utilisation of similar rat models of *in utero* dietary vitamin D deficiency have shown reduced trabecular bone health in offspring at 140 days old and differences in mineralisation [209]. However, the results obtained within this rodent cohort indicate that vitamin D during foetal life, within the age group examined, would appear to have no bearing on bone health in adulthood.

Current interest in the role of vitamin D in pregnancy towards future health is such that longitudinal studies, such as the Southampton Womens Survey and Maternal Vitamin D Osteoporosis Study (MAVIDOS) [128, 247, 248], are employed to investigate the effects of vitamin D. This has occurred, in part, due to the observation that previous studies into the role of *in utero* vitamin D levels on future bone health have been conflicting, with a need for randomised trials to provide stronger evidence [208]. Javaid *et al.* found positive correlations between maternal vitamin D levels during the last trimester and with bone size and mineral content at nine years age [133]. However, no data was collected on child vitamin D status, thus the findings may reflect environmental conditions experienced during childhood as well. Similarly, children from low vitamin D during pregnancy were found to have lower cross-sectional area at the tibia as measured through peripheral computed tomography at 14 months of age [249]. Conversely, the Avon Longitudinal Study of Parents and Children (ALSPAC) [197] found no association between 25(OH)D3 levels of mothers during pregnancy and bone mineral content of children at nine to ten years old in a larger cohort study (3960 mother and offspring pairs compared to 77 assessed by Javaid *et al.*) suggesting again that the duration of any skeletal effect diminishes with offspring age.

Whilst a more distinct physiological response was expected as a consequence of complete deficiency of vitamin D, these results do imply that a number of compensatory mechanisms could be active and at play. Reviews of studies using human and animal patients highlight physiological changes that occur to mothers during pregnancy, ensuring delivery of the necessary calcium to the foetus through increased intestinal calcium absorption independent of vitamin D [210]. Similarly, vitamin D receptor knockout mice were able to maintain normal skeletal development [250], showing that vitamin D may not be critical towards skeletal health. Findings from Kovacs *et al.* state that vitamin D status in neonatal, childhood and pubescent life is more influential on skeletal health

than events experienced *in utero* [210], implied by the current results. With only one time point analysed, it was not possible to investigate this effect further.

To summarise, minimal differences in gene expression, bone architecture, bone mechanics and bone material measures at multiple sites and scales were observed when compared to controls. These findings were replicated in both male and female animals, indicating that vitamin D depletion during *in utero* life has limited impact on skeletal health of rats at 140 days old. Higher *Opn* levels in the deplete group suggests that there may be an altered osteogenic phenotype due to the dietary differences that has persisted through to adult life. Osteopontin has been shown to regulate bone mineralisation by providing calcium ion binding sites and can influence the fracture mechanism of bone matrix [60, 251]; as such, any changes in transcript levels highlight a difference in bone maintenance. However, it is also possible that this result may be explained as a consequence of the repeated t-tests performed on the RT-qPCR datasets, with the likelihood of finding significant results at the  $p < 0.05$  level increased to 18.5% for the four genes reviewed. Considering this and the weight of evidence gathered from the other testing procedures, the conclusion is that vitamin D depletion, within the rodent sample, resulted in minimal changes to bone health in the sample cohort studied.

This is the first study, to our knowledge, whereby vitamin D depletion has been applied *in utero* to determine effects on biological, structural and mechanical features of bone health in adulthood. In conclusion, no effect was found in animals experiencing different vitamin D levels during foetal life. Similarities in skeleton pathophysiological response between the humans and rats have been confirmed. Thus, these results indicate a minimal role of vitamin D within the developing and modelling phase of skeletal health up to early adulthood and the attainment of peak bone mass [142]. Previous studies looking into the effect of vitamin D on rat offspring skeletal health have shown an impairment of proximal femur trabecular bone structure that is associated with deficient backgrounds [209]. This region of bone was not analysed during this investigation due to a focus on bone mechanics outcomes at other areas of the femur but this does highlight the possibility of site specific differences that may occur as a result of changes persisting from *in utero* experiences. It is possible that maternal calcium provision independent of the vitamin D pathway has occurred during pregnancy or that any differences in skeletal

health from birth has been remediated during childhood following the resumption of a normal diet.

In conclusion, the evidence from this rodent study suggests that no persistent changes regarding bone health has remained at adulthood (140 days old) as a consequence of *in utero* vitamin D depletion. Further study is required to confirm the presence of earlier effects in younger animals and to determine whether a mechanism of compensation is active to preserve bone health with implications therein for skeletal development and modulation.



## Chapter 8

## Discussion



## 8.1 Summary of Thesis Outcomes

This thesis has examined the process of bone development and how mechanical competence is established throughout the different levels of bone tissue hierarchy. Importantly, the contributory factors of bone mechanical development and establishment were assessed within healthy and challenged skeletal models. Primarily, investigation into whether *in utero* vitamin D levels impacted upon future skeletal health (at 21 and 140 days of age in rats) with regards to osteoblast gene expression and bone quality parameters was conducted. A reduction in male femur midshaft bone area and maximum load capacity was observed in rats from *in utero* vitamin D depleted cohorts at 21 days old, indicating *in utero* vitamin D deficiency has an adverse effect on femur development in male rats in early life. No differences were observed in female rats despite the different vitamin D conditions examined. Furthermore, no differences were observed in either gender at 140 days old. These observations highlight a gender specific response in offspring to maternal vitamin D depletion and that changes in femur structure in males are recovered following resumption of a control diet after birth.

Additionally, pilot studies into i) the effect of *in utero* low protein on female second generation offspring and ii) the relationships between biological, structural, compositional and mechanical bone indices at multiple length scales were completed. No differences in bone gene expression or bone mechanical analyses were observed between female offspring from control and protein restricted diets (during grand-maternal pregnancy). These findings indicate no persistent effect of low maternal protein dietary status on bone health in second generation offspring. Within the second pilot study, relationships were found between gene expression levels and indentation distances, cortical bone quantity, fracture toughness and whole bone mechanics. Further research is recommended to provide further details on the causation of these relationships and the gender-specificity observed in a number of relationships.

## 8.2 Discussion of Results

Evidence of the Barker hypothesis or developmental origins theory of disease has shown that the aetiology of many diseases include a component of early life adaptations due to environmental influences that consequently modify disease susceptibility in later life [117, 126, 226]. The relevance of this in terms of skeletal development is vast. There is already established evidence that factors such as peak bone mass are a cumulative response to mineral accrual throughout the first thirty years of life in humans and an important factor in subsequent risk of osteoporosis [252]. However, despite studies indicating links between early-life health to bone density and geometry, the mechanisms involved with driving this effect remain unknown [228, 230]. From a clinical perspective, uncovering and validating the action of any mechanisms involved in the modulation of skeletal development will be considerably advantageous to facilitating efforts in preventative healthcare and minimisation of bone strength reduction and fracture likelihood that are characteristic features of osteoporosis [99].

Investigating skeletal development is a uniquely complex task and characterising bone formation, maturation and maintenance a challenging prospect. For a complete understanding of skeletal development, it is necessary to bridge between the biological mechanisms of cellular action and initial bone formation through to the highly evolved mechanical behaviour of bone derived across the hierarchy/length scales and bone quality factors (figure 8.1). The implications of achieving this understanding between the interplay of multiple disciplines within bone include the possibility of targeting influential factors affecting bone strength during treatment and aiding regenerative strategies in bone tissue engineering.

This interaction between the biological and mechanical environments was explored in chapter 4 and more thoroughly in chapter 5. Much work has been conducted to investigate the regulatory pathways involved in osteogenic cell behaviour and the influential components that determine whole bone strength and fracture [172, 194, 223]. Recently this has been aided by analytical techniques with improved measurement sensitivity (such as nano and microindentation [42, 43, 166]). In chapter 4, measurements of gene

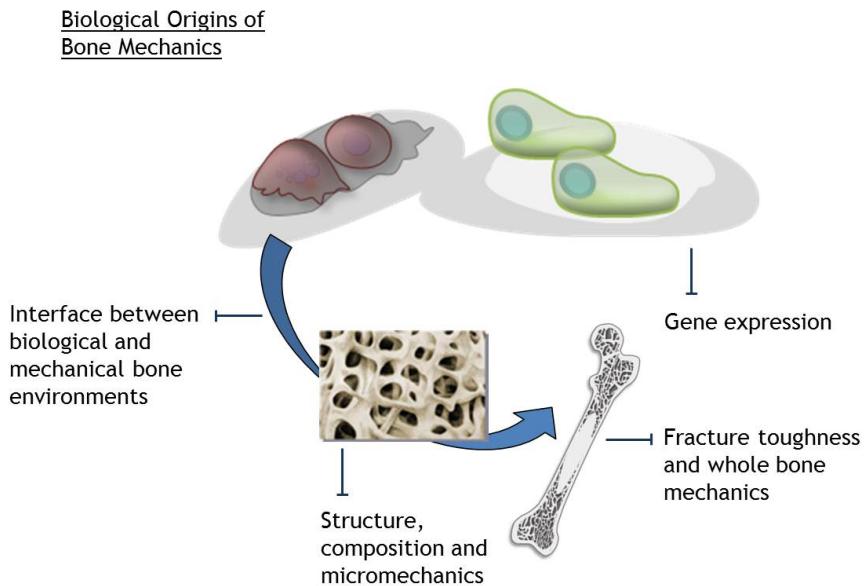


Figure 8.1: Link Between Bone Biology and Mechanics. Cellular events can influence modifications of bone structure, composition and mechanics. A greater understanding of the links between cellular activity and bone quality metrics will provide more detail as to which parameters are key in establishing bone strength at the organ level.

expression, reference point indentation (RPI), fracture toughness and whole bone mechanics of 70 day old female rats showed relationships between increased *Runx2* and *Alp* expression and greater indentation (CID) values. Additionally, increased last cycle CID was found to be related to greater fracture toughness. The increased gene expression levels are believed to indicate an early stage of bone formation and osteoblast maturation [179] and so related to bone tissue still to be fully mineralised. This results in bone that is less resistant to indentation (particularly at the periosteum where appositional growth is occurring and where indentations were performed) yielding the greater distances measured by RPI. The relationship between increased indentation distance and greater fracture toughness was contrary to expected results, with RPI previously shown to induce similar damage to bone at the micro-scale similar to the origins of fracture damage [166]. Therefore, higher indentation distances are to be expected to correlate with lower fracture toughness. This result is however in support of findings by Carriero *et al* [181] who observed that RPI results are unable to predict fracture toughness obtained through notched bend testing (the method used here). It is clear that further research is warranted to further explain these findings.

Following these results, further analysis of the relationships between gene expression, micro-architecture, bone composition, fracture toughness and micro and whole bone mechanics in 140 day old rats were conducted (chapter 5). Once again, with a different strain, age and cohort of rats, gene expression (*Runx2* and *Col1*) was found to be related to increased indentation distances. These findings are similar to results found in chapter 4 and provide further evidence, in particular with *Runx2* levels, that expression of early osteogenic genes are related to less mature bone in rats. In contrast, it was found that expression of a late osteogenic gene (*Ocn*) was related to increased indentation distance measured by RPI. No satisfactory explanation for this was found within existing literature and re-emphasises the need for further experimentation to validate whether this increase in gene expression is related to a greater *Ocn* protein presence within the bone matrix (through proteomic approaches [253]). Additionally, with *Ocn* known to control bone crystal growth [194], further detail of the mineral phase of bone (such as with Raman spectroscopy [172], a technique capable of providing compositional data) may explain the relationships found in our study between *Ocn* expression and RPI data.

Progression within this area will establish a link between the biology and mechanics of bone that will be key to recognising the mechanistic differences between healthy and diseased bone and the causes therein. Understanding the genetic determination of bone strength is a complex task, not least due to the multifactorial nature of contributors towards bone strength. Inter-species differences in laboratory mice (B6 and C3H) in terms of bone mineral density; site-specific bone mechanics; and cortical and trabecular bone morphology, have been confirmed [254] and emphasises the importance in genetic establishment of bone strength factors. Indeed investigation into the same two strains of mice have shown differences in response to tibial mechanical loading, with phenotypic changes associated to corresponding changes to bone formation and resorption gene expression levels [255].

More recently, isolation of novel genes influential towards bone strength in knockout mice (either by affecting signalling pathways, structural proteins, enzymes involved with matrix formation and methylation mechanisms) are beginning to be uncovered by methods such as high-throughput phenotype screening [256]. A review of genetic regulation of bone strength conducted by Adams and Ackert-Bicknell [257] highlights 75 quantitative

trait loci in the rat genome and around 50 in the mouse genome that have been implicated to possibly direct bone geometry, size, matrix and quality. Each one of these mapped genomic regions often contains hundreds of genes and the challenge is to isolate candidate genes within loci that have the greatest impact on bone strength phenotype, again representing the ongoing research and highly complex nature of identifying genes linked to bone strength.

In relation to the findings within this thesis, of significant interest will be to expand the range of genes examined to provide enhanced detail of bone physical adaptation at different stages of osteoblast maturation and, furthermore, to investigate protein and bone matrix mineral composition to link gene expression, bone mechanics and structure together. Recent research between mechanics and structure has begun to elucidate the relationships between fracture and toughening events at the protein and lamellar level [61, 258] to overall bone strength. It is also apparent that bone matrix proteins also have roles beyond mechanical functions. Thus, consideration of protein roles on cell activity, signalling and hormonal pathways on bone development [194, 259, 260] would provide and inform a more comprehensive model.

Within this body of work, we have attempted to track the process of bone mechanical development using a multidisciplinary approach to measure a number of different components of bone health outcomes. Although these results highlight further experimental work is required, the current studies have demonstrated the possibility of measuring a number of different bone quality parameters from one biological sample through the application of a range of experimental techniques that will be useful in assessing bone disease models.

Experimental work in this project has examined the programming of the skeleton due to early life cues and if this was subsequently translated into persistent alterations throughout life. Epigenetic changes have been proposed as the foremost mechanism of these persistent changes [118, 174], serving as precursors for increased disease risk in later adulthood. In chapter 4, second generation female rats from grand-maternal protein

restricted diets during pregnancy exhibited no differences in gene expression, bone micromechanics, fracture toughness and whole bone mechanics. Findings from Lanham *et al* [131, 175] that also investigated maternal protein restriction during pregnancy found initial weight differences in first generation offspring from low protein mothers (higher at birth but lower at four weeks of age when compared to controls). Analysis of mechanics within the same study found low protein background rats had weaker femoral heads and tibia midshafts, whilst femoral necks and vertebrae were stronger at 75 weeks of age. These changes highlight how maternal diet can affect skeletal development of the offspring, with observable consequences in later life. The results obtained here show that the effects of low protein during pregnancy do not, in this cohort, manifest in second generation rats in terms of skeletal health. In contrast, existing research has demonstrated the role of grand-maternal obesity and high fat in modifying hepatic gene expression and increasing tumour incidence within second generation offspring [173, 176]. Overall, these results imply there are nutrient and tissue specific effects in terms of nutrition and healthy offspring development. Here we have observed grand-maternal protein status to have negligible influence on offspring skeletal development.

There is currently conflicting evidence from human studies over the importance of vitamin D during pregnancy on offspring future bone health [133, 137, 197]. Despite a recognised and established role of vitamin D in childhood and adulthood bone health [234, 235], it is unclear whether there is a similar importance within *in utero* life. Vitamin D is a strong candidate for epigenetic modulation due to known interactions between the vitamin D receptor and responsive elements in target genes [261], such as VDR protein interaction with HATs and HDACs [204]. There is thus a possible role in the developmental origins of bone diseases. Investigation of this effect was conducted within an animal model of maternal vitamin D depletion during pregnancy and subsequent impact on offspring skeletal health.

Resultant offspring health at 21 days of age after vitamin D depletion *in utero* was assessed in chapter 6. Osteoblast gene expression, mineral density, cortical bone architecture, fracture toughness and whole bone mechanics of femora were measured and showed males from vitamin D deplete backgrounds experienced a reduction in femur mid-shaft area despite no reduction in bone area. Furthermore, there was also a reduction

in femur maximum load during three-point bend testing. Interestingly, no differences were observed in female rats. Subsequent further analysis of male midshaft area using finite element analysis (FEA) also found a reduction in predicted strength within vitamin D deplete animals. Lanham *et al* utilising the same rat model of maternal vitamin D depletion showed impaired trabecular bone development at 0 and 140 days of age in female proximal femora from the depleted background [209]. These results when evaluated together indicate that vitamin D levels are important in determining the course of femoral bone development. Interestingly, there was also a gender specific response to low vitamin D levels that resulted in manifestation of bone development adaptation at different stages of life, highlighting the importance of considering hormonal influences and interactions therein with bone formation.

In chapter 7, investigation into the effects of vitamin D depletion during *in utero* life on post-birth skeletal development demonstrated no significant differences in gene expression, micromechanics, mineral density, cortical and trabecular bone architecture, fracture toughness and whole bone mechanics when animals at 140 days of age were studied. We reasoned that this may signify a compensatory framework in place post-birth to minimise the effects of adverse conditions encountered during *in utero* life. Certainly in terms of the male cohort this is evident whereby differences seen at 21 days of age have been ameliorated upon resumption of a control diet post-birth. At 140 days of age, within rats, endochondral growth is concluding, with bone mineral density levels plateauing [262], such that no information was gathered about bone loss after peak bone mass. Studies on this effect within human cohorts have only looked at pre-pubescent ages at the time of writing, with linked effects to foetal bone shape [137] and conflicting conclusions drawn with childhood cohorts [133, 197]. Therefore, an ambiguity surrounds early childhood and how skeletal development is modulated and this may be a reason as to why opposing findings have been reported. It has been suggested that low maternal vitamin D will result in low stores within newborns and that this will be maintained due to continued low vitamin D exposure (particularly in cases where vitamin D intake is derived from breastfeeding) [237]. It may be that this early period of sustained low vitamin D is more influential in early skeletal development and programming. The critical implication from the observations within this study is that it appears maternal dietary vitamin D challenge and repercussions on offspring skeletal health can be recovered with

a sufficient diet after birth, suggesting vitamin D sufficiency during early childhood is essential.

One of the weaknesses of our approach was the use of repeated t-tests for significance at the  $p < 0.05$  level. As mentioned in section 7.4, testing of RNA for expression of four genes increased the probability of finding false positive results to 18.5 %. A similar case can be argued for the differences found in mean midshaft cross-sectional area of the femur in males at 21 days of age (section 6.3). However, confidence intervals are provided in Appendices D and E to examine the effect sizes of differences between control and vitamin D deplete groups and as investigations were exploratory in nature, large sample sizes for higher statistical power was impractical. Further study of these effects is required in independent studies to confirm or reject the findings within this thesis.

In summary, the objectives of this project were to examine how bone mechanical competence is established and how this is modified during early life as a consequence of nutrition. Research into the bone biology environment and cellular action and relationships to bone structure and mechanics have shown relationships between gene expression and micro-mechanics. However, a more detailed assessment of protein within the bone matrix is recommended to provide a bridge between gene expression and mechanical performance (not conducted within this thesis due to experimental design to investigate the role of vitamin D, which is more influential in gene regulation [261]). Maternal low protein diet was observed to have a negligible effect on bone health within second generation offspring although maternal vitamin D depletion was found to be detrimental to bone health at 21 days of age in rats. The detrimental effects were recovered by 140 days of age following resumption of a normal control diet after birth. Further research into this effect is clinically relevant in patients with low vitamin D levels during *in utero* life in order to promote healthy skeletal development in childhood.

### 8.3 Future Work

The findings from this thesis indicate that *in utero* vitamin D influences offspring bone development in the early life period of rats, an effect that can be remedied by adulthood upon resumption of a control diet after birth. Osteoblast gene expression from femur bone was related to bone mechanics and structure. Suggestions for future investigations to develop the findings here include:

1. *In utero* vitamin D deficiency and offspring bone health at old age. An experimental design limitation of the vitamin D deficiency studies was that the latest time point assessed was at 140 days of age. It would be of interest to measure bone health outcomes at later time points following *in utero* vitamin D deficiency, particularly as osteoporosis is a chronic disease usually presenting during later life. For example, Lanham *et al* [175] have demonstrated that maternal low protein can cause adaptations to femur trabecular structure in female offspring at 75 weeks of age (525 days). It is understood that remodelling activity within rats becomes predominant over modelling at around 3 to 12 months depending on which bone within the skeleton [142]. Therefore, a time point after this period would incorporate analysis into the remodelling process and features such as the coupling mechanism between osteoblasts and osteoclasts can be interrogated for sensitivity to *in utero* vitamin D status. Of particular interest would be the induction of osteoporosis in female rats by ovariectomy [263] to observe any differences in response between vitamin D depleted and control cohorts and whether osteoporosis symptoms are more pronounced if *in utero* vitamin D deficiency has been experienced.
2. As highlighted in section 7.4, foetal calcium provision and accrual has been observed to occur independently of vitamin D status [210] and indeed vitamin D receptor knockout models have maintained normal skeletal development [250]. In contrast however longitudinal human studies have suggested the possibility of calcium and vitamin D interaction to maximise foetal bone growth [264]. To provide further clarity on calcium status within low vitamin D mothers, maternal serum calcium levels can be measured before conception, during pregnancy and immediately after birth. If indeed calcium is provided independently of vitamin D (such

as through maternal bone resorption) then it is to be expected that serum calcium levels will be observed to rise during pregnancy when compared to before conception and after birth.

3. Cellular studies into the effect of vitamin deficiency during *in utero* life. We were unable to conduct any cellular studies to support the gene expression data gathered due to sample storage (and subsequent transportation) after culling. Investigation into maternal low protein has shown offspring osteoblasts to be modified in terms of proliferation and alkaline specific activity [9] and similar data from a vitamin D deficient model will provide further data as to the origins of the femur differences seen in the day 21 animals.
4. Component analysis of bone matrix. Characterisation of the bone matrix in terms of protein and mineral composition will provide more detail between cellular gene expression and micromechanical competence. Inclusion of techniques such as Raman spectroscopy can determine differences in crystallinity, mineral composition and mineralisation, which have been related to bone mechanical properties [172, 265]; whilst measurement of protein content can be achieved using proteomics [253], ELISA assays and immunohistochemical staining [61], as non-collagenous proteins have been shown to influence bone fracture toughness behaviour [60, 61]. With this additional information, it will be further possible to elucidate the cause and effect pathway between the bone biological environment and modulation of whole bone mechanical behaviour.
5. Markers of typical genes expressed during osteocyte maturation could also be included into future studies to provide more detail into the bone remodelling machinery and the status and inter-relations of osteoblast, osteocyte and osteoclast health. Typically, genes such as *E11* [266], *Dmp1* (dentin matrix protein 1) and *Sost* (sclerostin) [267] have been utilised to characterise osteocyte-like cell lines and primary isolated osteocyte cell cultures. Similarly, the effect of dietary changes on osteoclast activity can also be investigated. Genes such as *Trap* (thrombin receptor-activating peptide), *Mmp-9* (matrix metalloproteinase-9) and *Rankl* (receptor activator of NF- $\kappa$ B ligand) are routinely used [255, 268] to measure osteoclast activity. Therefore, expanding the range of genes tested for expression levels

within bone samples will provide a more comprehensive overview of bone health in disease models.

## 8.4 Conclusion

In summary, these studies demonstrate a multi-disciplinary approach to bone health investigation that can provide a number of metrics for a comprehensive analysis of skeletal development. The importance of additional measures to BMD to predict bone fracture risk is well documented [27, 253, 269] although progress has been restricted due to the inherent difficulty in understanding the interdisciplinary link between the osteogenic environment and microscale bone quality. An understanding of how this contributes to whole bone mechanics is equally difficult but our initial gene expression results, have highlighted candidate biological roots and intermediary steps towards the global mechanical properties. We have applied this approach to investigate the possibility of programming with respect to skeletal development, resulting in the notion of possible natural amelioration of processes to minimise adverse environment conditions. Further work will aim to develop on these concepts to provide a greater appreciation of bone establishment and function and the aetiology of bone diseases for improved therapeutic strategies.



## Chapter 9

## Appendices



## Appendix A

# RT-qPCR Analysis of Human RNA

Presentation of RT-qPCR data on the effect of different culture conditions of human bone marrow cell samples can be seen in figure A.1. Human bone marrow stem cells (HBM-SCs) were isolated from bone marrow samples obtained from Southampton General Hospital through fractionation using Lymphoprep (Axis-Shield, Oslo, Norway). Cells were cultured in basal medium ( $\alpha$ MEM media with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (P/S)). Osteogenic conditions were induced through the addition of 100  $\mu$ l of ascorbate acid and 50  $\mu$ l of dexamethasone per 50 ml of basal media. Comparisons were made between basal and osteogenic conditions and time for culture (7, 14 and 21 days).

At each time point, cells were harvested and homogenised through the use of TRIzol reagent (Life Technologies, Paisley, UK) and subsequent RNA isolation was performed. RNA was then used to generate cDNA through the Superscript Vilo cDNA Synthesis kit (Life Technologies, Paisley, UK) for RT-qPCR. Samples were run in duplicates, with alkaline phosphatase (*ALP*), collagen I (*COL1*) and  $\beta$ -actin measured. Target gene expression was referenced to  $\beta$ -actin as the housekeeping gene, presented data is with normalisation to the basal day 7 condition.

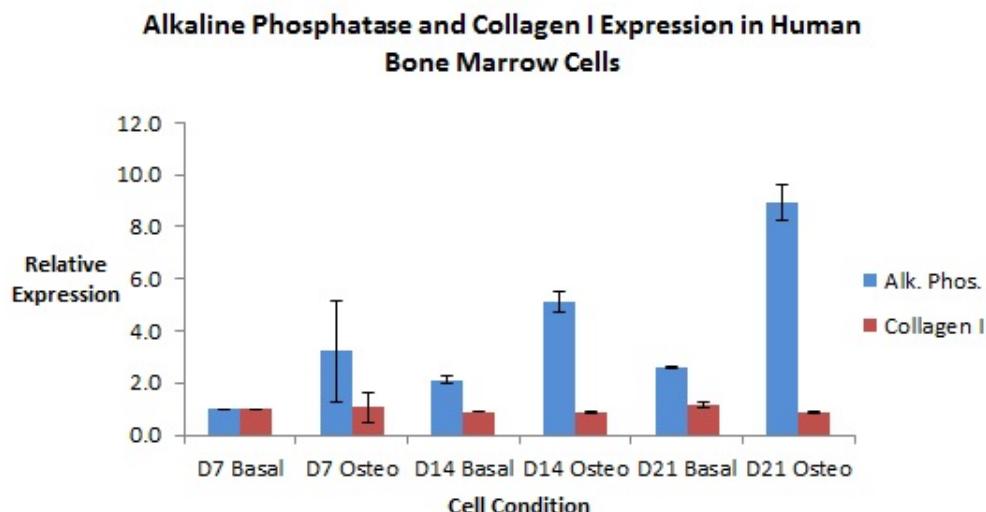


Figure A.1: RT-qPCR Results. Gene expression of cultured cells in basal and osteogenic conditions at timepoints of 7, 14 and 21 days. Relative expression normalised housekeeping gene  $\beta$ -actin expression. Each gene referenced to day 7 basal for subsequent timepoints. Bars represent mean of duplicate runs and standard deviation is shown. One patient sample run in duplicates, averages presented with confidence intervals.

From figure A.1, a clear trend of increasing *ALP* expression over time can be viewed in both basal and osteogenic conditions. At each timepoint, higher levels of *ALP* within the osteogenic samples were also evident. In contrast, *COL1* expression showed little change over time and between different culture conditions.

*ALP* is an early marker of osteoblast differentiation and likely to be involved with the initial stages of mineralisation [68]. These results are indicative of differentiating bone marrow stromal cells down on osteogenic pathway due to continued expansion in culture. Dexamethasone has been shown to increase the rate of differentiation [270] and this is represented within this analysis, where increased *ALP* expression is associated with osteogenic media conditions when compared to basal. It is unclear why no changes were viewed in collagen I expression in either culture conditions or with time. Similar to *ALP*, collagen I is also an early marker of osteogenic differentiation and contrasts against the conclusions drawn from the *ALP* results.

Acknowledging the findings from existing studies on the effects of dexamethasone and

the *ALP* data, it is surmised that the gene expression analysis of *COL1* was erroneous. A possible source of error could originate from the efficiency of the *COL1* primers used. The presence of primer-dimers, evidenced from the collagen I melt curve (figure A.2), may have interfered with the testing procedure. Primer-dimers occur when primer pairs anneal to each other rather than sample DNA and as such increase the fluorescent signal beyond the real effect and introducing inaccuracy. Anecdotal evidence from members within the research group also support this conclusion.

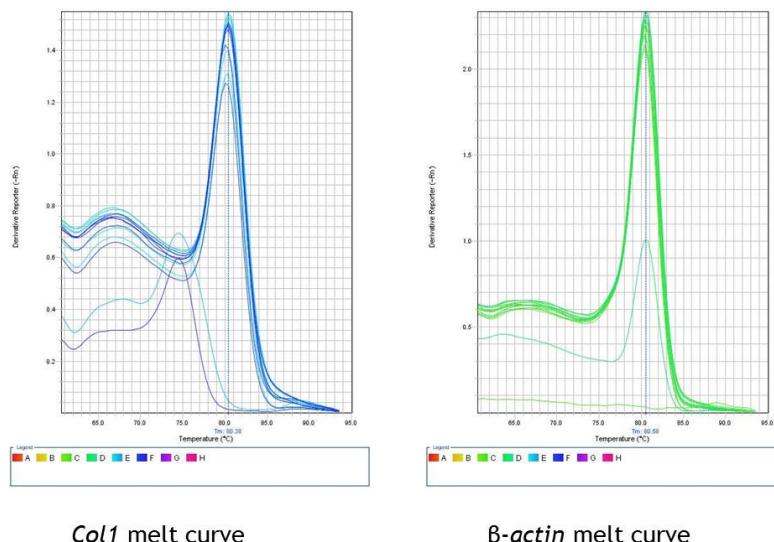


Figure A.2: RT-qPCR Melt Curves. Melt curves for *COL1* and  $\beta$ -*actin*, note how a double peak is present in the collagen I curve at around 66 °C and 80 °C, indicating the presence of primer-dimers. This is not the case with the  $\beta$ -*actin* curve, which has only one prominent peak.

To validate these RT-qPCR findings, immunostaining of specific samples was performed for alkaline phosphatase expression, an established biochemical technique to elicit whether cells have begun to differentiate down an osteogenic lineage [271]. Briefly, the appropriate flasks were removed of media and cells and then fixed with 95% ethanol. Upon removal of the ethanol, cells were washed with phosphate buffer solution (PBS) (Lonza, Verviers, Belgium) and then stained with a Fast Violet Salt and Naphtol AS-MX Alkaline solution (Sigma-Aldrich, Gillingham, UK). Cells were then imaged using a microscope, camera (Carl Zeiss, Cambridge, UK) and Axiovision microscopy 4.6 software.

Images of alkaline phosphatase staining of human bone marrow stem cell samples in both conditions at 14 days culture are shown in figure A.3. Staining was enhanced in osteogenic conditions compared to the basal condition. This shows that at 14 days, cells cultured in the osteogenic media produce greater alkaline phosphatase, indicating that more cells are differentiating down the osteogenic lineage when compared to basal condition cells. These results corroborate with the RT-qPCR data.

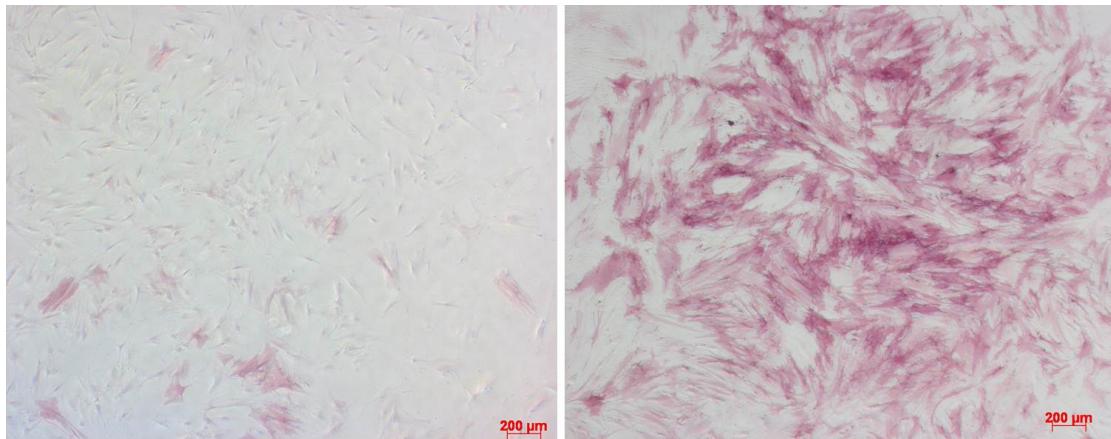


Figure A.3: Alkaline Phosphatase Stain of Human Bone Marrow Stem Cells. Alkaline phosphatase staining of cells cultured in basal (left image) and osteogenic conditions (right image). Both samples were cultured for 14 days in respective conditions then fixed with 95% ethanol. Enhanced expression of alkaline phosphatase was found in osteogenic conditions. Scale bar represents 200  $\mu$ m.

Results from both the *ALP* RT-qPCR and alkaline phosphatase experiments support the expected role of dexamethasone in bone marrow cell differentiation. This validation study confirms the potential of using RT-qPCR for accurate characterisation of molecular events occurring within bone development, which can then be related to any physical changes within the skeleton.

## Appendix B

# $\mu$ CT CTAn Segmentation Tasklist

The tasklist for cortical bone segmentation from the femur midshaft and subsequent analysis of *TMD* and structure was based on Bruker technical notes and is as follows:

1. Thresholding - Automatic Otsu method in 3D space.
2. Bitwise operation - Region of interest = COPY of Image.
3. Reload - Reload image.
4. Histogram - Bone mineral density in 3D space, inside VOI.
5. Reload - Reload region of interest.
6. Thresholding - Automatic Otsu method in 3D space.
7. ROI shrink-wrap - Shrink-wrap in 2D space, stretch over holes with diameter of 20 pixels.
8. 2D analysis - 2D analysis of all results.
9. 3D analysis - 3D analysis with basic values and additional values of structural model index, trabecular thickness, trabecular number, trabecular separation, degree of anisotropy, fractal dimension and number of objects.

Similarly, analysis of trabecular bone was conducted according to Bruker technical notes. After isolation of the distal femur region (figure 3.4, section 3.3.1), bone from the trabecular region was segmented from cortical bone in CTAn by manually tracing inside the endosteal surface (figure B.1) at periodic intervals (around every 40-50 CT images). The ROI was then constructed by interpolating through the manually drawn regions.

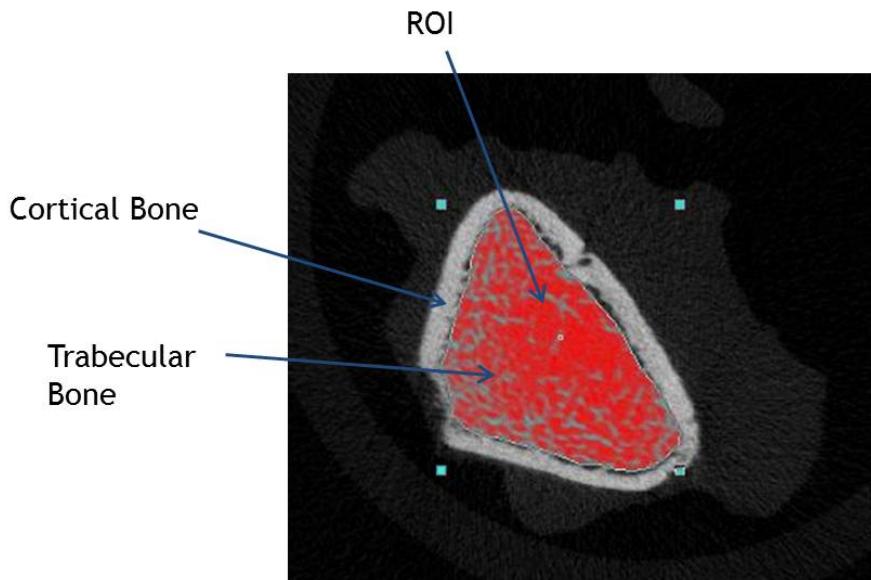


Table B.1:  $\mu$ CT Trabecular Bone Segmentation. Segmentation of trabecular bone regions from cortical bone at the distal femur was performed by manually tracing inside the endosteal surface.

Analysis was then conducted within the ROI with the following tasklist:

1. Thresholding - Adaptive (mean of min and max values) in 3D space, default levels and with pre-thresholding, radius of 6 and constant 0.
2. Save bitmaps - Apply to image inside ROI, file format BMP, convert to monochrome (1 bit), copy shadow projection and copy dataset log file.
3. Despeckle - remove white speckles in 3D space, volume less than 20 voxels, apply to image.
4. 2D analysis - 2D analysis of all results.
5. 3D analysis - 3D analysis with basic values and additional values of structural model index, trabecular thickness, trabecular number, trabecular separation, degree of anisotropy, fractal dimension and number of objects.

## Appendix C

# Molecular Origins of Bone Quality: Correlation Grids

$r$  values were calculated between osteoblast gene expression and micromechanical measures at the femoral head can be viewed in table C.1. No strong relationships were observed in the male samples. Female samples yielded  $r > 0.7$  for TID and *Runx2*, *Ocn* and *Col1* expression levels. In addition, a relationship between *Runx2* expression and IDI was also demonstrated.

Similarly,  $r$  values were calculated between osteoblast gene expression and RPI, TMD, micromechanical, fracture toughness and whole bone mechanics measurements taken at the femur midshaft (table C.2).

Correlation Grid: Male Proximal

	Head				Neck			
	TID	IDI	CID	Energy	TID	IDI	CID	Energy
RUNX2	-0.118	-0.273	-0.200	-0.182	-0.024	0.010	-0.092	-0.079
OC	0.276	-0.036	-0.360	-0.322	-0.019	-0.082	0.258	0.276
OP	0.242	0.166	-0.272	-0.304	-0.254	-0.281	-0.039	0.028
COL1	0.403	0.144	-0.186	-0.199	-0.151	-0.203	0.193	0.199

Correlation Grid: Female Proximal

	Head				Neck			
	TID	IDI	CID	Energy	TID	IDI	CID	Energy
RUNX2	0.933	0.774	0.542	0.583	0.326	0.348	0.281	0.585
OC	0.754	0.684	0.655	0.657	0.536	0.568	0.451	0.553
OP	0.554	0.503	0.446	0.370	0.453	0.387	0.325	0.558
COL1	0.807	0.493	0.331	0.466	0.174	0.224	0.230	0.713

Table C.1: Molecular Origins of Bone Quality Correlation Grid: Proximal Femur. Pearson correlation coefficient values ( $r$ ) between gene expression and RPI measurements at the femoral head and femoral neck. Relationships with  $r > 0.7$  were further assessed for statistical significance.

Correlation Grid: Male Midshaft

	TMD	Ar	Car	Car/Ar	CTh	TID	IDI	CID	Kinit	ForceMx
RUNX2	0.064	-0.468	-0.852	-0.615	-0.858	0.063	0.428	0.253	-0.912	-0.828
OC	0.297	-0.116	0.124	0.320	0.345	0.603	-0.166	0.691	0.227	-0.379
OP	0.523	-0.587	-0.190	0.487	0.144	0.200	0.227	0.481	0.266	-0.370
COL1	0.339	-0.052	0.287	0.469	0.537	0.541	-0.301	0.642	0.277	-0.171

Correlation Grid: Female Midshaft

	TMD	Ar	Car	Car/Ar	CTh	TID	IDI	CID	Kinit	ForceMx
RUNX2	-0.304	0.305	0.609	0.821	0.774	0.616	0.121	0.869	-0.117	0.137
OC	-0.143	-0.152	0.199	0.734	0.489	0.427	0.331	0.572	0.279	0.068
OP	0.111	0.216	0.436	0.599	0.650	0.659	0.376	0.458	0.086	0.508
COL1	-0.124	0.320	0.555	0.657	0.700	0.602	-0.037	0.735	-0.361	0.212

Table C.2: Molecular Origins of Bone Quality Correlation Grid: Femur Midshaft. Pearson correlation coefficient values ( $r$ ) between gene expression and RPI, TMD, micromechanical, fracture toughness and whole bone mechanics measurements at the femur midshaft. Relationships with  $r > 0.7$  were further assessed for statistical significance.

## Appendix D

### Vitamin D Day 21 Data Tables

		Control	Deplete	Diff. Mean	95% C.I.	<i>p</i> value
Male	Weight (g)	52 (9)	49 (6)	-3	-11 to 6	0.47
Female	Weight (g)	49 (15)	57 (12)	+8	-7 to 22	0.28

Table D.1: Vitamin D 21 Days Old Cohort Weights. Study groups for 21 day old animals and mean weights.  $n = 8$  for each group.

		Control	Deplete	Diff. Mean	95% C.I.	p value
Male	<i>Runx2</i> , 2 <sup>-ΔCt</sup>	9.10E-06 (8.65E-06)	9.64E-06 (1.06E-05)	5.43E-07	-9.9E-06 to 10.9E-06	0.91
	<i>Col1</i> , 2 <sup>-ΔCt</sup>	1.74E-03 (1.62E-03)	1.60E-03 (1.68E-03)	0.14E-03	-0.1E-03 to 0.8E-03	0.87
	<i>Opn</i> , 2 <sup>-ΔCt</sup>	5.44E-06 (4.78E-06)	7.73E-06 (9.62E-06)	2.29E-06	-6.1E-06 to 10.7E-06	0.56
	<i>Ocn</i> , 2 <sup>-ΔCt</sup>	3.46E-05 (2.08E-05)	5.75E-05 (7.13E-05)	-2.29E-05	-3.8E-05 to 8.3E-05	0.41
Female	<i>Runx2</i> , 2 <sup>-ΔCt</sup>	5.23E-06 (5.23E-06)	4.96E-06 (2.20E-06)	-2.68E-07	-4.8E-06 to 4.4E-06	0.90
	<i>Col1</i> , 2 <sup>-ΔCt</sup>	9.30E-04 (9.60E-04)	15.11E-04 (11.08E-04)	5.81E-04	-5.3E-04 to 16.9E-04	0.28
	<i>Opn</i> , 2 <sup>-ΔCt</sup>	3.97E-06 (3.50E-06)	5.96E-06 (3.96E-06)	1.99E-06	-2.0E-06 to 6.0E-06	0.31
	<i>Ocn</i> , 2 <sup>-ΔCt</sup>	2.78E-05 (2.31E-05)	4.73E-05 (2.27E-05)	1.95E-05	-0.5E-05 to 4.4E-05	0.11

Table D.2: Vitamin D 21 Days Old Gene Expression

		Control	Deplete	Diff Mean	95% C.I.	<i>p</i> value
Male	TMD	1.28 (0.08)	1.22 (0.05)	-0.06	-0.18 to 0.02	0.12
	Cort Tissue	4.70 (0.70)	4.00 (0.49)	-0.70	-1.35 to -0.05	0.04
	Cort Bone A	1.86 (0.36)	1.53 (0.23)	-0.32	-0.65 to 0.01	0.06
	Cort Area Frac	39.48 (3.28)	38.39 (2.76)	-1.09	-4.34 to 2.17	0.49
	Cort Thick	238.6 (30.4)	214.2 (22.0)	-24.4	-53.1 to 4.3	0.09
Female	TMD	1.24 (0.09)	1.20 (0.09)	-0.04	-0.19 to 0.10	0.45
	Cort Tissue	4.52 (0.57)	4.13 (0.41)	-0.39	-0.97 to 0.19	0.16
	Cort Bone A	1.76 (0.31)	1.54 (0.20)	-0.22	-0.52 to 0.08	0.14
	Cort Area Frac	38.62 (2.31)	37.08 (1.74)	-1.54	-3.89 to 0.81	0.18
	Cort Thick	225.1 (26.6)	210.6 (19.5)	-14.5	-41.3 to 12.3	0.26

Table D.3: Vitamin D 21 Days Old CT

		Control	Deplete	Diff. Mean	95% C.I.	<i>p</i> value
Male	Buckling ratio	5.15 (0.39)	5.29 (0.45)	0.14	-0.30 to 0.21	0.50
Female	Buckling ratio	5.36 (0.37)	5.47 (0.36)	0.11	-0.30 to 0.52	0.56

Table D.4: Vitamin D 21 Days Femur Buckling Ratio.

		Control	Deplete	Diff Mean	95% C.I.	p value
Male	Frac Tough	1.49 (0.54)	1.51 (0.38)	0.02	-0.77 to 0.82	0.93
	Strength	11.43 (3.26)	8.32 (1.94)	-3.11	-6.05 to -0.17	0.04
Female	Frac Tough	1.09 (0.62)	0.86 (0.27)	-0.23	-1.17 to 0.72	0.56
	Strength	8.87 (3.41)	7.31 (2.18)	-1.56	-5.27 to 2.14	0.36

Table D.5: Vitamin D 21 Days Old Midshaft Mechanics

		Control	Deplete	Diff. Mean	95% C.I.	p value
Male	Failure Load	7.02 (1.17)	5.24 (1.09)	-1.78	-3.43 to -0.13	0.04

Table D.6: Vitamin D 21 Days Male Bone Compressive Strength Simulation

## Appendix E

# Vitamin D Day 140 Data Tables

		Control	Deplete	Diff. Mean	95% C.I.	<i>p</i> value
Male	Weight (g)	580 (52)	541 (53)	-39	-95 to 17	0.16
Female	Weight (g)	325 (50)	329 (22)	+4	-39 to 47	0.83

Table E.1: Vitamin D 140 Days Old Cohort Weights. Study groups for 140 day old animals and mean weights.  $n = 8$  for each group.

		Control	Deplete	Diff. Mean	95% C.I.	p value
Male	<i>Runx2</i> , $2^{-\Delta Ct}$	7.24E-06 (4.88E-06)	10.54E-06 (6.22E-06)	3.31E-06	-2.7E-06 to 9.3E-06	0.26
	<i>Col1</i> , $2^{-\Delta Ct}$	2.50E-04 (3.62E-04)	4.43E-04 (4.48E-04)	1.93E-04	-2.4E-04 to 6.3E-04	0.35
	<i>Opn</i> , $2^{-\Delta Ct}$	7.79E-06 (5.31E-06)	20.70E-06 (11.28E-06)	12.91E-06	3.1E-06 to 22.8E-06	0.02
	<i>Ocn</i> , $2^{-\Delta Ct}$	8.69E-05 (9.78E-05)	11.78E-05 (12.21E-05)	3.09E-05	-8.8E-05 to 15.0E-05	0.59
Female	<i>Runx2</i> , $2^{-\Delta Ct}$	12.15E-06 (3.69E-06)	8.91E-06 (4.45E-06)	-3.24E-06	-7.6E-06 to 1.2E-06	0.14
	<i>Col1</i> , $2^{-\Delta Ct}$	19.44E-05 (14.26E-05)	31.19E-05 (23.68E-05)	11.75E-05	-9.7E-05 to 33.0E-05	0.25
	<i>Opn</i> , $2^{-\Delta Ct}$	10.50E-06 (7.51E-06)	7.38E-06 (5.54E-06)	-3.12E-06	-10.0E-06 to 40.0E-06	0.36
	<i>Ocn</i> , $2^{-\Delta Ct}$	7.53E-05 (4.02E-05)	6.37E-05 (3.97E-05)	-1.16E-05	-5.5E-05 to 3.1E-05	0.57

Table E.2: Vitamin D 140 Days Old Gene Expression

		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TMD	1.274 (0.012)	1.277 (0.018)	0.003	-0.013 to 0.020	0.67
	BMD	0.247 (0.030)	0.228 (0.021)	-0.019	-0.048 to 0.009	0.16
	Cort Tissue	14.61E+06 (0.64E+06)	14.05E+06 (1.33E+06)	-0.56E+06	-1.73E+06 to 0.60E+06	0.31
	Cort Bone A	9.43E+06 (0.52E+06)	9.16E+06 (0.65E+06)	-0.26E+06	-0.89E+06 to 0.37E+06	0.39
	Cort Area Frac	64.6 (2.45)	65.39 (2.54)	0.79	-1.88 to 3.47	0.54
	Cort Thick	741.5 (45.1)	739.4 (22.9)	-2.1	-41.7 to 37.6	0.91
	Trab BV TV	10.79 (1.75)	9.44 (1.82)	-1.35	-3.26 to 0.57	0.15
	Trab Tb N	1.01E-03 (0.12E-03)	0.81E-03 (0.17E-03)	-0.20E-03	-0.30E-03 to 0.01E-03	0.07
	Trab Thick	106.6 (6.3)	109.3 (7.5)	2.7	-4.7 to 10.1	0.44
	Trab Sep	1132 (163)	1251 (192)	119	-72 to 311	0.20
Female	TMD	1.286 (0.023)	1.303 (0.013)	0.017	-0.004 to 0.037	0.10
	BMD	0.325 (0.060)	0.333 (0.052)	0.008	-0.052 to 0.068	0.79
	Cort Tissue	10.60E+06 (0.70E+06)	9.78E+06 (0.86E+06)	-0.82E+06	-1.66E+06 to 0.03E+06	0.06
	Cort Bone A	6.80E+06 (0.52E+06)	6.42E+06 (0.57E+06)	-0.38E+06	-0.97E+06 to 0.2E+06	0.18
	Cort Area Frac	64.24 (2.00)	65.64 (1.46)	1.40	-0.47 to 3.27	0.13
	Cort Thick	626.2 (25.1)	624.7 (25.7)	-1.5	-28.7 to 25.7	0.91
	Trab BV TV	16.98 (4.35)	17.26 (4.51)	0.28	-4.47 to 5.03	0.90
	Trab Tb N	1.72E-03 (0.38E-03)	1.71E-03 (0.43E-03)	-0.01E-03	-0.45E-03 to 0.43E-03	0.97
	Trab Thick	98.3 (5.3)	100.8 (5.5)	2.5	-3.3 to 8.4	0.37
	Trab Sep	636 (258)	636 (217)	0	-257 to 257	<0.99

Table E.3: Vitamin D 140 Days Old CT

		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TID	66.83 (6.23)	66.87 (9.04)	0.04	-8.38 to 8.46	0.99
	IDI	7.86 (0.64)	9.24 (2.12)	1.38	-0.41 to 3.17	0.11
	CID	2.11 (0.21)	2.34 (0.72)	0.23	-0.38 to 0.84	0.42
	Energy	18.04 (3.42)	18.85 (3.19)	0.81	-2.74 to 4.36	0.63
	Frac Tough	5.74 (0.68)	5.62 (0.72)	-0.12	-1.10 to 0.85	0.77
	Strength	236.8 (24.0)	231.9 (15.9)	-4.9	-28.6 to 18.9	0.66
Female	TID	63.92 (5.71)	64.09 (8.75)	0.17	-7.88 to 8.21	0.96
	IDI	8.34 (2.51)	8.82 (1.93)	0.48	-1.93 to 2.89	0.67
	CID	1.97 (0.24)	2.15 (0.61)	0.18	-0.34 to 0.71	0.44
	Energy	18.67 (4.83)	17.81 (4.07)	-0.86	-5.67 to 3.93	0.70
	Frac Tough	5.34 (0.65)	5.26 (0.84)	-0.08	-0.97 to 0.80	0.83
	Strength	173.1 (12.3)	171.9 (22.4)	-1.2	-21.4 to 18.9	0.90

Table E.4: Vitamin D 140 Days Old Midshaft Mechanics

RPI (Head)		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TID	157.00 (19.09)	171.30 (62.89)	14.30	-39.02 to 67.52	0.56
	IDI	29.56 (6.11)	34.24 (10.95)	4.68	-5.08 to 14.43	0.31
	CID	4.78 (1.23)	5.93 (2.93)	1.15	-1.37 to 3.67	0.33
	Energy	43.08 (11.67)	57.39 (33.06)	14.31	-13.87 to 42.49	0.28
Control		Deplete	Diff Mean	95% C.I.	p value	
Female	TID	151.60 (26.37)	148.60 (19.38)	-3.00	-28.02 to 22.02	0.80
	IDI	28.30 (9.01)	27.06 (7.36)	-1.24	-10.10 to 7.62	0.77
	CID	4.86 (1.51)	4.58 (0.74)	-0.28	-1.61 to 1.03	0.64
	Energy	44.33 (13.28)	39.73 (5.78)	-4.60	-16.08 to 6.88	0.39

Table E.5: Vitamin D 140 Days Old RPI (Head)

RPI (Neck)		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TID	87.38 (34.89)	97.75 (44.67)	10.37	-32.84 to 53.59	0.61
	IDI	9.10 (5.21)	10.21 (5.97)	1.11	-4.91 to 7.13	0.70
	CID	2.13 (0.65)	2.68 (1.44)	0.55	-0.70 to 1.80	0.35
	Energy	20.56 (11.75)	22.86 (13.06)	2.30	-11.04 to 15.64	0.72
		Control	Deplete	Diff Mean	95% C.I.	p value
Female	TID	122.90 (65.81)	72.63 (14.55)	-50.27	-105.60 to 5.10	0.07
	IDI	10.36 (5.00)	7.89 (1.67)	-2.47	-6.72 to 1.77	0.22
	CID	2.45 (1.73)	2.04 (0.90)	-0.41	-1.94 to 1.11	0.56
	Energy	24.98 (16.11)	14.38 (14.38)	-10.60	-24.09 to 2.89	0.11

Table E.6: Vitamin D 140 Days Old RPI (Neck)

RPI (Pos. 6)		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TID	220.50 (73.69)	178.50 (49.65)	-42.00	-109.40 to 25.38	0.21
	IDI	31.29 (19.71)	20.89 (12.77)	-10.40	-28.49 to 7.69	0.23
	CID	6.11 (3.02)	4.40 (2.03)	-1.71	-4.51 to 1.08	0.21
	Energy	51.13 (26.81)	39.28 (16.20)	-11.85	-36.09 to 12.39	0.31
		Control	Deplete	Diff Mean	95% C.I.	p value
Female	TID	170.50 (65.30)	162.40 (76.12)	-8.10	-84.34 to 68.09	0.82
	IDI	22.73 (16.16)	18.71 (8.33)	-4.02	-18.25 to 10.23	0.55
	CID	4.26 (2.19)	3.85 (2.17)	-0.41	-2.75 to 1.92	0.71
	Energy	37.09 (22.03)	36.20 (26.87)	-0.89	-27.33 to 25.55	0.94

Table E.7: Vitamin D 140 Days Old RPI (Pos. 6)

RPI (Distal Femur)		Control	Deplete	Diff Mean	95% C.I.	p value
Male	TID	240.30 (77.58)	256.40 (73.11)	16.10	-64.74 to 96.99	0.68
	IDI	34.79 (14.50)	44.41 (20.39)	9.62	-10.82 to 30.07	0.32
	CID	6.64 (1.44)	7.61 (3.73)	0.97	-2.22 to 4.17	0.51
	Energy	52.43 (12.01)	62.60 (19.95)	10.17	-7.85 to 28.20	0.24
		Control	Deplete	Diff Mean	95% C.I.	p value
Female	TID	147.40 (46.56)	145.90 (59.83)	-1.50	-59.31 to 56.31	0.96
	IDI	26.44 (15.57)	15.29 (9.03)	-11.15	-25.12 to 2.823	0.11
	CID	4.00 (1.84)	4.30 (2.46)	0.30	-2.04 to 2.64	0.79
	Energy	29.93 (14.77)	31.68 (13.70)	1.75	-13.54 to 17.04	0.81

Table E.8: Vitamin D 140 Days Old RPI (Distal Femur)

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