Relationship between Collagen and Mineral in Bone Adapted for Different Biological Function

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Thesis submitted for the degree of Ph.D

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I, Pedro Elston, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Bone is a complex material composite of collagen, an organic protein that makes up much of mammalian connective tissue, and mineral, the inorganic calcium phosphate that provides stiffness. In nature bone is subjected to a number of forces that can differ drastically depending on the animal species and location in the body, hence bone material has adapted to suit particular functions through a series of modifications to its composition either in quality or in quantity. The exact nature of the relationship between the mineral and collagen phases are not well understood in terms of the impacts on material properties, nor is the process of mineralisation. Therefore the subject of this thesis is examining the nature of this relationship with a view to increasing our knowledge of bone material to better facilitate clinical treatment of bone disorders, the greatest of which is osteoporosis, estimated to affect 3 million individuals in the UK, the cause of 500,000 fractures yearly. To this end the following studies utilise two emerging in-vivo technologies, Spatially Offset Raman Spectroscopy (SORS) and Reference Point Indentation (RPI).

The results of the experiments show that bone properties, and indeed mineralisation is highly related with aspects of collagen cross-linking and fibre organisation. They also revealed that mineralisation levels do not correlate well with local bone properties, suggesting that the current approach to fragility evaluation which relies on analysing mineral content and distribution, is not adequate. It provides evidence that suggests Raman spectroscopy can detect specific collagen modifications which correlate with bone properties.

The work presented here aids our understanding of the relationships between mineral, collagen and material properties and furthers the use of in-vivo technologies which is of great significance in their respective developments as potentially widespread clinical tools. The ability to access the organic phase of bone and the bone properties both directly and locally could enable a more accurate, immediate and without ionising radiation alternative to current detection of bone defects and fragility.

Impact Statement

Bone diseases are a huge blight on medical services around the world, with approximately 30% of the global population currently afflicted by some form of bone impairment, and the treatment of osteoporosis alone costs the EU €37 billion annually. These defects can arise genetically, as in the case of osteogenesis imperfecta, or as a factor of age and the effects of the hormonal changes in menopause, as in osteoporosis, or through deficiencies, such as a lack of vitamin D which results in rickets. The lifespan and quality of life of the individuals affected, as well as the costs of detection, prevention and treatment are the basis for much research into bone, a massive and incredibly varied field of study with over 2500 publications monthly in recent years. The work undertaken in this thesis is directly applicable to further experimentation in academia, and has implications for developing technologies in business both from a diagnostic perspective and the creation of drugs for treatment (any by extension governmental policy), as well as impacting clinicians and patients on the ground level.

This research focusses on the factors integral to the detection of poor material quality, on the premise that the inorganic mineral phase currently used to make such deductions through X-ray analysis are inadequate and instead observes modifications to the collagenous organic portion of bone tissue with regards to their contribution to the strength of bone. While many studies have been conducted on the various effects of modifications to collagen with regards to diseases, relatively few have compared healthy tissues of varying nature to better understand how these modifications function. This thesis uniquely observes bone tissue from a single species, and probes the mineral and collagen aspects to provide an insight into the relationship between these two components and the resulting material properties.

This thesis utilises novel techniques which have the potential to revolutionise the detection and potentially the treatment of these diseases. The data gathered here will further the research efforts of the technologies used, as well as expand our knowledge of bone composition. Spatially Offset Raman Spectroscopy has the potential to be a widespread clinical tool that can, without ionising radiation, detect both the organic and inorganic phases of bone and observe subtle changes in chemical composition. It has been used in a number of trials and is still in its early phase of development. The use of it in these studies supports its use by comparing the material properties of bone to spectra, determining that it is indeed capable of visualising specific modifications to collagen which correlate strongly with material properties. Further to this is the use of micro-indentation, a novel technique that with minimal invasiveness can directly ascertain material properties in-vivo. Again this technology is under trial in many studies, but has not yet sufficiently been proven able to differentiate between cohorts in the literature, which the data in this thesis aids.

Conventions, Abbreviations, and Formulae:

Conventions and Definitions

Material properties – The attributes of a material taken in isolation, i.e. a small cube of bone.

Mechanical Properties – The attributes of a material taken as a whole structure, including the geometric architecture.

Strength – The ability of a material to endure elastic deformation before experiencing failure.

Toughness – The amount of energy a material can withstand before experiencing failure.

Elasticity – The ability of a material to recover from deformation.

Hardness – The ability of a material to resist changes in shape due to force.

<u>Abbreviations</u>

ACP – Amorphous Calcium Phosphate

AGEs – Advanced Glycation End-products

AN – Antler

ATP – Adenosine triphosphate

BMP – Bone Morphogenic Protein

DB – Bulla

DEXA (DXA) – Dual-Energy X-ray Absorptiometry

DNA – Deoxyribonucleic Acid

ECM – Extracellular Matrix

EDTA - Ethylenediaminetetraacetic Acid

EDX – Energy-dispersive X-ray Spectroscopy

FEM – Finite Element Model

GAGs – Glycosaminoglycans

HCL – Hydrochloric Acid

HP – Pyridinoline, Hydroxylysyl-pyridinoline (also known as PYD in some citations)

HPLC – High Performance Liquid Chromatography

IR Spectroscopy – Infrared Spectroscopy

LP – Deoxypyridinoline, Lysyl-pyridinoline (also known as DPD in some citations)

MC – Metacarpal

MRI – Magnetic Resonance Imaging

NCP – Non-collagenous Protein

OA – Osteoarthritis

OI – Osteogenesis Imperfecta

OP – Osteoporosis

PCA – Principle Component Analysis

PEN – Pentosidine

Pi – Inorganic Phosphate

PPi – Pyrophosphate

pQCT – peripheral Quantitative Computer Tomography

PTM – Post Translational Modification

PYD – Pyridinoline

PYR – Pyrrole

RER – Rough Endoplasmic Reticulum

SEM – Scanning Electron Microscopy

SORS – Spatially Offset Raman Spectroscopy

TEM – Transmission Electron Microscopy

Formulae

Stress = Force / Area

Strain = (Elongated length – Original length) / Original length

Scientific Engagement: Talks and Conference Attendance

- Elston, P., Churchwell, J., Goodship A., Kerns, J.G., Birch H., 2016. The Relationship between Mineral to Collagen Ratio, Ultrastructure and Mechanical Properties: Differences within a Single Species. *Orthopaedic Proceedings,* Vol. 98-B. Abstract of Oral Presentation at BORS Glasgow 2016.
- January 2016 – Invited Speaker "Higher Education" Wilson's School Scientific Research Day
- October 2017 – Invited Speaker "10 Minute Research" Mayday Hospital Patient Outreach Event

Conference Attendance

- Infrared and Raman Discussion Group 212 (2015, UCL)
- Government Policy & Research (2016, UCL)
- British Orthopaedic Research Society (2016, Glasgow University)
- Infrared and Raman Discussion Group 216 (2016, UCL)
- TCES Net Conference: Robotics and Tissue Engineering 2017, Stanmore UCL)
- British Orthopaedic Research Society (2017, Imperial College London)
- Infrared and Raman Discussion Group 219 (2017, UCL)

Contents

Introduction and Summary

Bone is a hybrid material formed of an organic collagenous framework and inorganic mineral deposits. The sturdy matrix formed from these two components provides the skeletal framework of over 60,000 unique living species of vertebrates. However, the bone material in different species and from different sites within the same species often have very different mechanical requirements. For example, material properties required in the legs of an elephant are very different from those in the wings of a bat; hence the composition of bone, both architecturally and chemically, differs according to biological function. The relative quantities of the mineral and collagen components are not fixed and a significant amount of material strength (60-70% (Ammann & Rizzoli, 2003)) can be derived from the amount of mineral present. The current gold standard in fragility assessment for humans is a DEXA (Dual-Energy X-ray Absorptiometry) scan which can access the mineral phase in terms of density. For the purposes of fracture prediction in a clinical setting, this level of accuracy is not adequate, and highlights the need for a better understanding of where bone strength originates from and the processes that control mineral levels.

The thesis opens with a description of bone composition in the first chapter, and underlines important biological processes involved in the mineralisation process. It goes on to introduce key concepts in mechanical testing and how the material properties of bone can be measured. The next chapter gives an overview of the relevant literature, examining changes to the chemical composition of bone and subsequent effects on material properties. The third chapter examines the main techniques used in the study, giving an overview of each method and the theory behind them.

Chapters four through eight provide the experimental data from the research conducted on bone material and collagen. They seek to answer the main research question behind this thesis:

Are chemical changes to type-1 collagen associated with the mechanical properties of bone and levels of mineralisation?

The first experimental chapter (chapter three) brings together a wide range of techniques to access the collagen and mineral phases of three distinct bone material sourced from a single species; red deer (*Cervus elephus*). It reports that there are many differences between the bones beyond a change in the mineral to collagen ratio and compares two approaches to measuring material properties and bone properties. The forth chapter explores type-1 collagen cross-linking, in the same bones, and correlates it to the bone properties accessed in chapter 4, finding an association between levels of pyridinoline and pentosidine cross-linking, and the composition of bone.

Chapter five probes the chemical differences of type-1 collagen within a single bone. The goal of this chapter was to ascertain the level of variation in collagen chemically and understand the impact it has on a smaller scale. The sixth chapter seeks to replicate the mineralisation process in demineralised bone, showing that the uptake of mineral may be dependent on a multitude of factors. The final practical chapter (seventh) observes the bone properties along the length of human tibiae. Previous studies (Buckley, Kerns, Parker, Goodship, & Matousek, 2014) have shown a variation in the chemical aspects of the cortices of long bones.

These experimental chapters attempt to shed light on some of the larger questions on bone formation and composition: What is the precise nature of the mineralisation process? How is the full 100% of material strength determined and how can we measure it? It is for this reason that this thesis employs the use of both Spatially Offset Raman Spectroscopy (SORS), and micro-indentation, techniques which have in-vivo applications and could potentially lead to more accurate clinical diagnostic tools for fracture prediction and potentially prevention.

The thesis continues with a discussion of the experimental chapters and potential future work with questions raised, followed by the conclusions drawn and a re-examination of the original hypothesis.

Chapter 1 – Bone Material

1.1 Bone structure overview

The mammalian skeleton has a wide range of functions: It provides an internal protection for vital organs, acts as a store for metabolic components and participates in homeostasis (mineral, yellow marrow, heavy metals, salts for pH balance, and calcium), holds red marrow which creates red blood cells, and it is an anchor for muscles to create the mechanical action of the body. Its composition reflects these functions, therefore bone is not simply optimised for the transmission of force. This thesis looks at bone primarily as a material, however it is important to recognise that these factors have an impact on the design of the structure and behaviour of bone beyond their individual scope (for example the need for red marrow precludes bone from being solely constructed to resist fracture). Bone is therefore a compromise of these needs, which must be accounted for when making comparisons, taking into account the context in which the bones operate.

Bone is a very broad descriptor for the material that makes up the skeletal system across a multitude of species with different mechanical requirements. It can vary in composition dramatically, providing a range of function from an extremely hard surface for cracking nuts in avian rostrum (beaks) (Currey, Zioupos, Peter, & Casinos, 2001), to strong material capable of bearing heavy repeated loads (e.g. the human femur), to stiffly-walled echo chambers in the ears of whales (Nummela, Reuter, Hemilä, Holmberg, & Paukku, 1999), to tough material that can sustain multiple fighting blows through its flexibility in antlers (Coman et al., 2007). This variety in composition of material is referred to as heterogeneity. This also exists within species, for example the bones in the vertebral column of a horse are drastically different from its legs in terms of the forces they withstand as well as the functions they serve. Individual bones are also heterogeneous, as bone material is denser in the central regions (diaphyses) of long bones, than at the ends (epiphyses) (Cowin & Telega, 2003). This has also been shown to occur on the millimetre scale within a single bone along the length of the diaphysis (Buckley et al., 2014), with bone material being adapted on the millimetre scale by subtle changes in its composition to serve different functions.

Individual bones interact with soft-tissues such as tendons, which connect bone to muscle, and ligaments, which join one bone to another, at locations known as entheses (Benjamin et al., 2006). These allow bones to provide structural support and movement via the contraction and relaxation of muscles. On the outer surface of bone is the periosteum, a fibrous membrane that coats the outer layer of bone (except joint surfaces), providing it with a strong collagenous matrix for attachment to other tissues as well as a nourishing inner 'cambium' layer which allows for the passage of blood and has a progenitor-cell rich matrix,

containing the yet non-specialised cells responsible for the formation and maintenance of bone (Dwek, 2010).

On the macroscopic level, bone material can be split into cortical bone (also known as compact bone), and cancellous (also known as trabecular or spongy) bone (figure 1.1). Cortical material is dense and compact, providing much of the skeletal mass (80%) (Li & Jee, 2005) but only 40% of volume (Cowin & Telega, 2003), and is found primarily in the diaphysis of long bones, but also in the outer layer of much of the skeleton. Its primary functions are load bearing and support, acting as levers to facilitate movement through muscles action, as a store for calcium, and providing protection to internal organs. The inner layer of cortical material is usually coated in another fibrous membrane called the endosteum, with similar function to the periosteum, and provides a bridge to the medullary cavity, the location of red or yellow marrow, and cancellous bone.
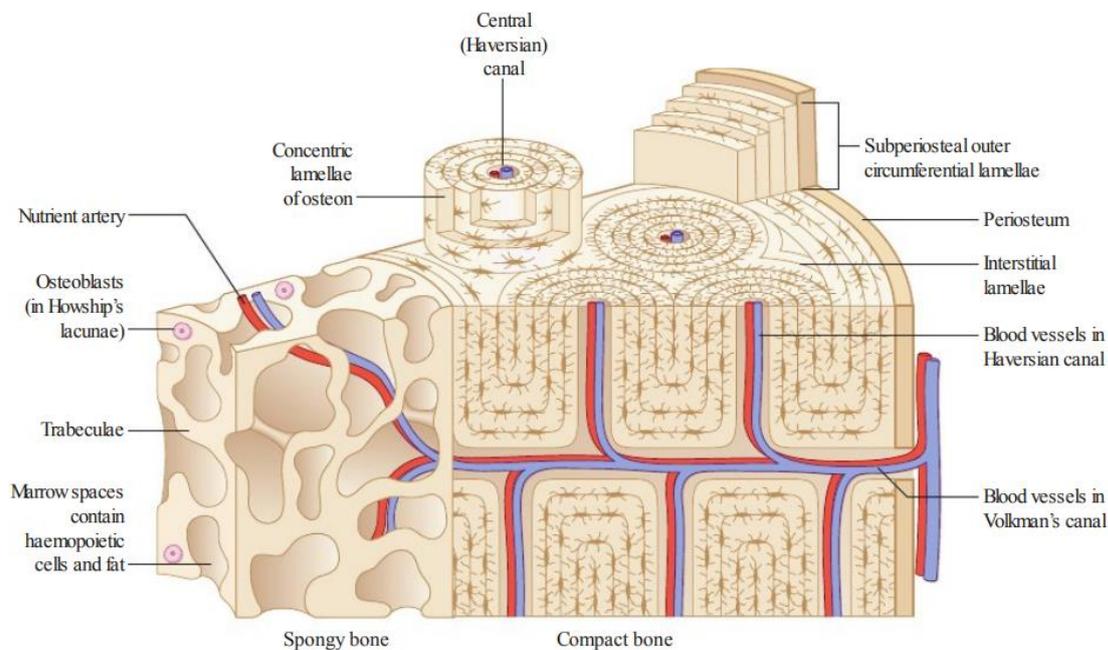
Figure 1.1 – A cross-section of the functional units of bone tissue (Naish & Syndercombe Court, 2015)

The cortical layer itself is made up of microscopic columns called osteons, the functional unit of bone material, which contain a central vascularised 'Haversian' canal, joined by perpendicular Volkmann's canals. The osteons contain the cells (osteocytes, differentiated osteoblasts that no longer produce bone material) responsible for producing bone material, as well as providing a matrix for chemical exchange, assisted by canaliculi, small channels in the hard material which allow for cellular communication (Aarden, Nijweide, & Burger, 1994) and passage of aqueous materials. The bone itself is synthesised by osteoblasts, and deposited around the cell. These osteoblasts once surrounded with bone material become inactive and convert to osteocytes, which are responsible for maintaining bone material, and

are possibly involved in the active remodelling of bone in response to stress to better adapt it to function (Aarden et al., 1994). The bone is organised in concentric circles of lamellae, which surround the central canal, housing osteocytes in small gaps called lacunae (Scott, 1988). A third cell type, the osteoclast, formed by multiple mononuclear cells merging, including osteoblasts, has the role of bone resorption (Soysa, Alles, Aoki, & Ohya, 2012). This cell is found on the outer perimeter of an osteon and removes bone material, allowing for continuous remodelling, essential for maintaining strength and repairing micro-fractures, as well as adapting to stresses on the material (Teitelbaum, 2007).

Cancellous material (figure 1.2), found at the epiphyses of long bones, and internally in vertebrae, ribs and the hips among other locations, is a web of porous bone tissue filled with marrow, providing a shock absorbing component to the mechanical abilities of bone (John D. Currey, 2002). It is markedly less stiff than cortical bone (10.4 GPa for trabecular, 18.6 GPa for cortical on average), and is considered fundamentally a different material as there is not a linear relationship between the density of these types of bone, and the tensile strength (Rho, Ashman, & Turner, 1993). Cancellous material is made up of trabeculae, interconnected struts and plates of bone material with a sponge-like appearance. These are formed in the same manner, with bundles of osteons forming the web of bone tissue. During the early stages of life in vertebrates, all bones of the skeleton are made of trabecular bone and the to-be cortical regions are gradually converted by the laying down of parallel osteons to form a tight structure (Levine, 2012). The arrangement of hard tissue allows for markedly more compression in multiple directions, and the additional flexibility of the soft tissue which fills the interconnected spaces make it an ideal transmitter of force (Rudman, Aspden, & Meakin, 2006).
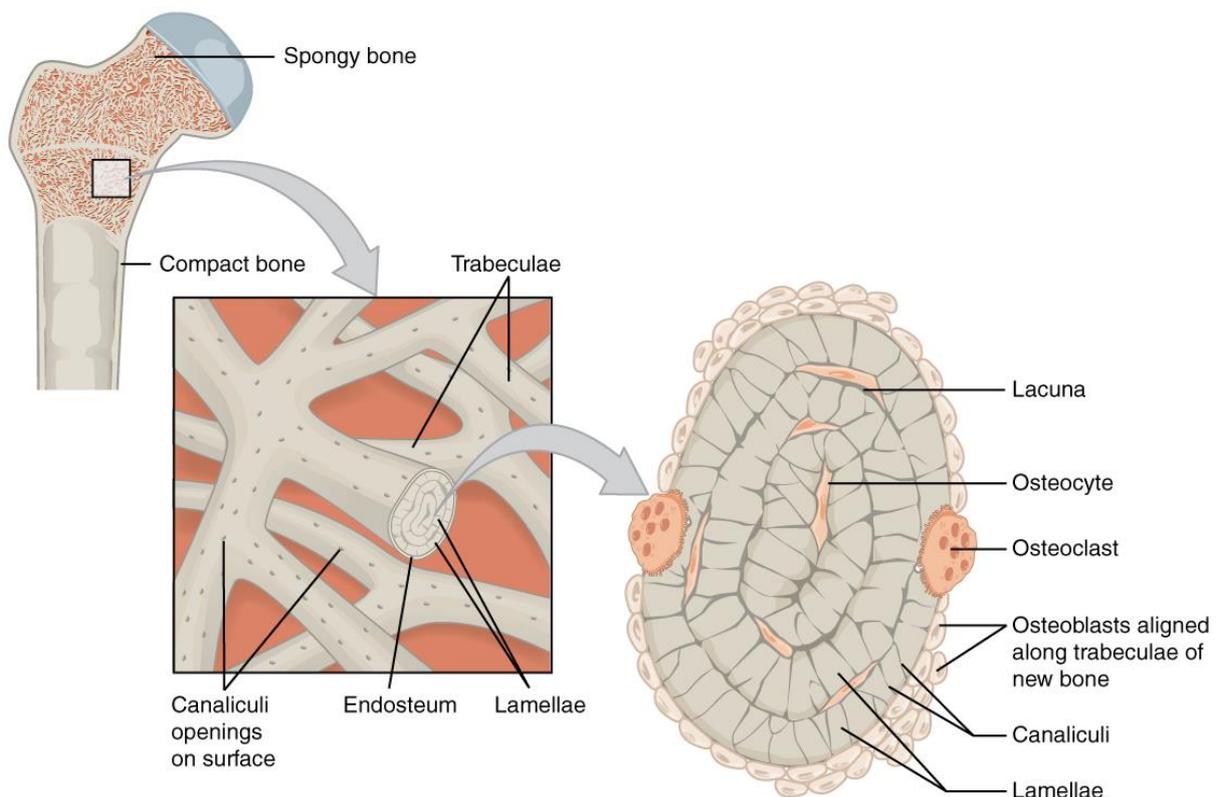
Figure 1.2 – The structure of cancellous bone (Le et al., 2017)

1.12 Modelling and remodelling

Bone exists in a variety of forms, fulfilling different mechanical functions and withstanding different kinds of forces (e.g. compression on vertebra, torsion on limbs, bending on ribs), but is made of the same basic structure outlined above. To achieve these functions bone is modelled as it grows into the appropriate shape, according to its distinct genetics, and has the ability to adapt to a change in loading requirements (Goodship, Lanyon, & McFie, 1979). This is possible through a balanced combination of bone resorption and deposition in a process known as remodelling.

Remodelling is a constant process that continues after bone growth is completed, and happens over three main stages (Hadjidakis & Androulakis, 2006):

- Resorption – Osteoclasts, likely activated by the apoptosis of osteocyte cells within the lacuna in response to either damage or age (Li & Jee, 2005), form a tight seal against lamellae on the osteon with a ruffled surface. They then break down bone material by secretion of H^+ ions through hydrochloric acid, and proteases in vesicles. The resultant breakdown of bone material is endocytosed (taken into the cell), and exocytosed (released from the cell) back into the extracellular matrix, allowing the

osteoclast to efficiently break down and remove new material (Väänänen, Zhao, Mulari, & Halleen, 2000).

- Reversal – Mononucleated progenitor cells, named osteogenic cells or osteoprogenitor cells are activated, producing osteoblasts (Delaisse, 2014). Teams of osteoblasts fill the gap region left by the osteoclastic activity.

- Formation – Osteoblasts begin secreting osteoid, the organic component of bone material, and subsequently mineralise it. Osteoblasts can become surround and trapped within the secreted material, and subsequently differentiate into osteocytes. Meanwhile the outer-osteoblasts will continue to create bone material until the original space is refilled, at which point they will form an outer lining on the newly remodelled osteon and enter a semi-dormant state (Rucci, 2008).

This process of cell action is known as the Basic Multicellular Unit (BMU), although it is not known precisely how the cells communicate, there are long since identified coupling mechanism that ensures they act in unison (Frost, 1964). Osteoclast formation is mediated by the RANK receptor on progenitor cells, which is activated by RANKL secreted by osteoblasts and stromal stem cells (Boyce & Xing, 2007). In addition to this osteoprotegerin (OPG), which is also secreted by osteoblasts, antagonistically binds to RANK to prevent interaction with RANKL to prevent differentiation (Udagawa et al., 2000). Cortical bone is remodelled at a rate of 20-40µm/day, forming the cylindrical osteons with lengths of approximately 2000µm and diameter of 150-200µm, orientated by the direction of dominant loading (Petrtýl, Heřt, & Fiala, 1996). Up to 100 osteoclasts begin this process, with thousands of osteoblasts following in their wake, with the process affecting between 2% and 5% of bone mass in humans yearly (Parfitt, 1994). This process is much faster in trabecular bone, as it is less dense, and results in the formation of osteons known as hemi-osteons (Kassem, Rungby, Mosekilde, & Eriksen, 1992). However without mechanical loading it has been shown that these cells can become uncoupled in function, and bone material can be removed without being replaced (Mosekilde, 1990; Nomura & Takano-Yamamoto, 2000).

Bone is a highly responsive material, able to adapt to the needs placed upon it over time. This theory was first put forth by Wolff in 1892, on the basis that trabecular bone appeared to conform to mathematical models of the directions and forces of stress placed on joints (Wolff, 1986). It has been proven in practise that disused limbs do not form correctly, with only 30-50% of the natural bone mass being present (Robling & Turner, 2009). The opposite has also proven true, whereby exercise and induced stresses on bone has resulted in increased growth in dimensions and mass (Charalambous, 2014). It has also been shown that the increase in mechanical strength in response to loading is not just mass based, but due to an efficient tuning of the material, confirmed in a study on rats where functional

loading increased strength in a particular direction by up to 64%, with only an 7% total increase in bone mass (Robling & Turner, 2009). Exercise also has an impact on bone turnover as a function of age, with less turnover occurring as a response to exercise in the adult skeleton, whereas young individuals create more mass in response (Nomura & Takano-Yamamoto, 2000).

The process of bone remodelling under stress is mediated by cellular activity. In order to respond to stresses, cells must be able to detect stress and respond to it, converting kinetic information into chemical responses which signal osteoclasts and osteoblasts to intervene in specific areas, in specific quantities. Osteocytes are thought to be the main mechano-sensors in bone, due to the fact that they are spread in a vast web throughout bone tissue, and outnumber osteoblasts and osteoclasts combined at a factor of 20:1, as well as existing in extremely confined spaces within bone material making them more sensitive to stress on bone material (Takano-Yamamoto, 2014). Osteocytes communicate through calcium-based signals and paracrine signalling molecules such as adenosine triphosphate (ATP) (Jørgensen, Geist, Civitelli, & Steinberg, 1997), and express certain growth factors in response to loading such as IGF-I (Lean, Mackay, Chow, & Chambers, 1996).

The way in which osteocytes sense stress has competing theories, it is known that dynamic loading (repeated short loads) induces more bone formation than static loading (a single compressive load over a long period) (A. G. Robling, Duijvelaar, Geevers, Ohashi, & Turner, 2001), which suggests that osteocytes are stimulated by the movement of the extracellular fluid surrounding them. Therefore it has been put forth that these fluid forces bow the external proteins which are responsible for attaching the osteocyte to bone, causing a strain in the cell membrane which elicits an internal response (Han, Cowin, Schaffler, & Weinbaum, 2004), or that the cell membrane itself is directly stretched causing ion channels to open resulting in an increase in calcium-based signalling (Wang, McNamara, Schaffler, & Weinbaum, 2007). By a similar pathway, many progenitor cells respond to loading or increased pressure from blood flow by differentiating into appropriate cells (Yamamoto et al., 2003). Remodelling is preceded by the programmed cell death (apoptosis) of osteocytes, in response to the glucocorticoids in the extracellular matrix, responsible for detaching the proteins which hold osteocytes to bone material (Plotkin, Manolagas, & Bellido, 2007). The apoptosis process is likely stimulated by the aforementioned stresses, however also occurs during bone disuse which is accompanied by insufficient osteoblastic response leading to bone loss (Noble et al., 2003).

<u>1.2 Bone Material</u>

Bone material refers to the amalgamation of compounds that form the functional unit of bone. The main components of bone material (table 1.3) can be split into organic and inorganic. By mass, inorganic mineral (mineral) accounts for approximately 70% of dry weight, with organic material in the form of collagen (25%), and other proteins (5%). The mineral is a modified crystalline hydroxyapatite with base formula $Ca_{10}(PO_4)_6(OH)_2$ (Bilezikian, Raisz, & Martin, 2008) which is housed in and around a type-1 collagen triple helix structure (Grynpas, 1993). The combination of the stiff mineral aspect, and the flexible fibrous collagen, results in a material more sturdy than the sum of its parts, specifically to resist fracture in its given role (Rabie, Bakr, & Urist, 1997).

Average Long Bone Material Component	Approximate Wet Weight Percentage (%)
Mineral	55-65
Collagen	15-25
Non-collagenous proteins	3-5
Water	10-20

Table 1.3 – The approximate contents of bone material in human long bones (X. J. Li & Jee, 2005).

<u>1.21 Collagen</u>

There are 28 distinct types of collagen, each classified as such as they contain a triple-helix as part of their structure and are found outside of cells. They are named using roman numerals, the most common of which appear in table 1.4. The amount of the molecule that contains the triple-helix can vary from 10% in collagen XII to 96% in collagen I (also known as type-1 collagen) (Ricard-Blum, 2011). Each collagen has three separate polypeptide chains, which can vary from 662 to 3152 amino acids (Gordon & Hahn, 2010), known as alpha chains (using the symbol α). The composition of which can involve three identical chains, as in collagen VII, or a heterotrimer of three distinct chains, as in collagen XI, or a mid-way with two chains of a single type, and one different, as in collagen I. However there is further diversity as hybrid molecules can exist, for example collagen XI can form using a single alpha chain from collagen V (Mayne, Brewton, Mayne, & Baker, 1993; J. J. Wu, Weis, Kim, Carter, & Eyre, 2009). Adding to this are a number of post-translational modifications that alter key sites of the collagen molecule, altering its functions.

Types	Locations
Type I	Bone, skin, dentin,cornea,blood vessels, fibrocartilage and tendon
Type II	Cartilaginous tissues
Type III	Skin, ligaments, blood vessels and internal organs
Type IV	Basement membrane in various tissues
Type V	Blood vessel wall, synovium, corneal stoma, tendon, lung, bone, cartilage and skeletal muscle

Table 1.4 – The most common types of collagen and their locations (Goonoo, Bhaw-Luximon, & Jhurry, 2014)

Collagen is the most common protein in mammals and in part it makes up skin, internal organs, cartilage, blood vessels, the lens of the eye, and bone to name but a few. The most abundant version which is of interest in this study is type-1, accounting for 95% of the collagen present in the human body (Fratzl, 2008). Type-1 collagen is a set of 3 polypeptide chains that form a triple helix structure with two alpha1 chains and one alpha2 chain (figure 1.5). In these amino acid chains there are repetitive patterns of the amino acids 'Glycine – X – Y' where 'X' and 'Y' are usually proline and hydroxyproline respectively (Peter Fratzl, 2008), although the most common formations are 'Glycine – Proline – Z' and 'Glycine – Z – Hydroxyproline' where Z is an amino acid other than those already described (Szpak, 2011). Initially the pre-collagen chains are translated from genes COL1A1 and COL1A2 on ribosomes along the rough endoplasmic reticulum (RER). The chains, known as pre-procollagen, have a signal sequence which directs them to the endoplasmic reticulum proper, where they are cleaved and released. The chains are immediately subject to post translational modification, a series of alterations which impact how collagen forms (Kivirikko & Myllyla, 1985).
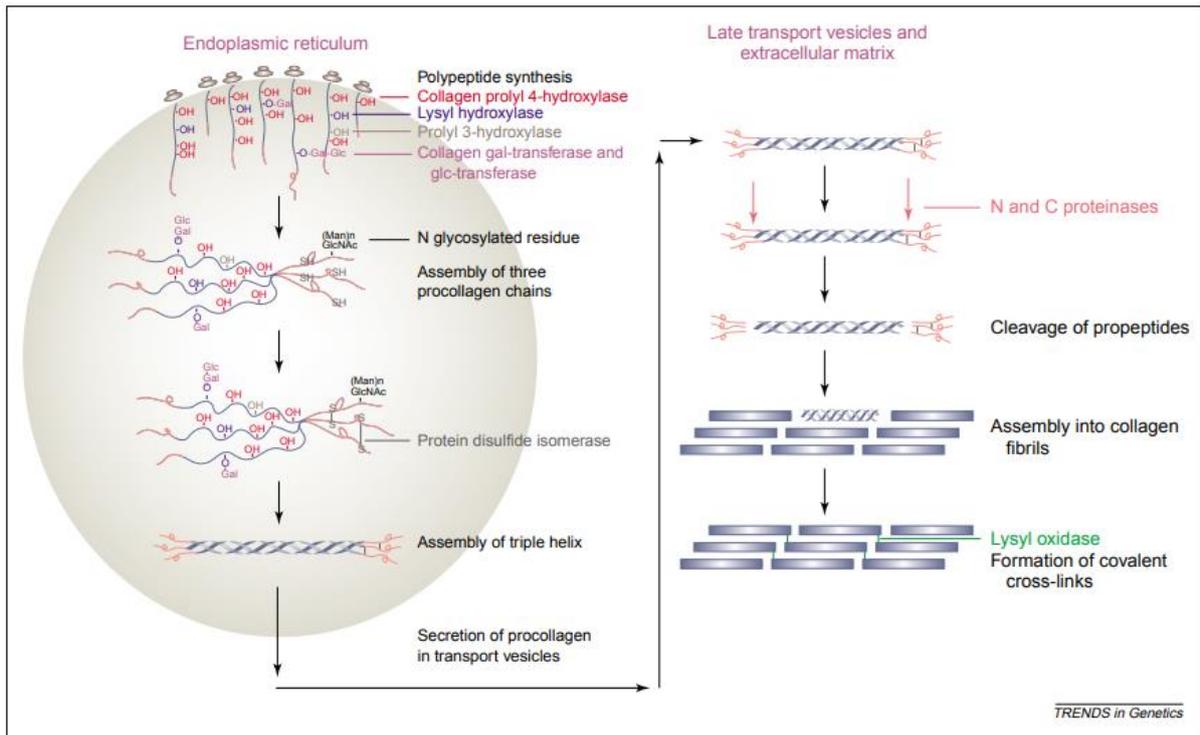
Figure 1.5 – depicting the process of collagen formation from synthesis to assembly (Johanna Myllyharju & Kivirikko, 2004)

Post translational modification involves the X and Y portions of the alpha helix being modified, determining their ability to form cross-links which are critical for viscoelasticity and tensile strength (Paschalis et al., 2004). These modifications include the hydroxylation of proline to form hydroxyproline, which accounts for 14% of type-1 collagen (Reddy & Enwemeka, 1996) and is required for helix formation (Kivirikko & Myllyla, 1985). They also include conversion of lysine to hydroxylysine, required for cross-link formation and further glycosylation (Kivirikko & Myllyla, 1985). The absence of these adjustments is well documented and results in a variety of bone defects such as osteogenesis imperfecta (OI) among others, stemming from mutations in the genes of the required enzymes (Bank et al., 1999; Byers & Pyott, 2012; J. Myllyharju & Kivirikko, 2001; Johanna Myllyharju & Kivirikko, 2004). However relatively few studies exist on the impact of subtle variation to these modifications.

After modification, the polypeptide chains spontaneously fold into the collagen triple helical shape inside the endoplasmic reticulum (Zhu & Kaufman, 2014). The 3 chains form the quaternary structure called a procollagen approximately 300nm in length and 1.5nm in diameter (Fratzl, 2008). Procollagen is then packaged at the Golgi apparatus, and secreted from the cell by exocytosis in vesicles. At this point, outside the cell, the N and C terminuses are cleaved and the unit is known as a tropocollagen. The tropocollagen units come together to form micro fibrils and then these join to form fibrils. To do this, the base unit of

tropocollagen joins staggered by 67nm to the next tropocollagen (Petruska & Hodge, 1964). This leaves gaps in the structure as 67nm can't be divided into 300nm length (Fratzl, 2008) where it is likely that mineral can be formed and stored. After this aggregation, the enzyme lysyl oxidase acts on lysine and hydroxylysine residues to form aldehyde groups critical for cross-linking (Smith-Mungo & Kagan, 1998). Cross-linking holds the tropocollagen fibres in place with covalent bonding, increasing their stability (Grynpas, 1993). The fibres are then known as osteoid, the organic un-mineralised component of bone.

1.22 Cross-linking

Type-1 collagen forms a variety of cross-links, and begins this process immediately after formation with the modification of the collagen molecule by the enzyme lysyl oxidase. Aldehyde groups formed by the enzyme lysyl oxidase spontaneously react with lysines and hydroxylysines on adjacent tropocollagen to form immature cross-links (Rodriguez-Pascual & Slatter, 2016), which mature over a number of months or years to form permanent cross-links (Eyre, Paz, & Gallop, 1984; Terajima et al., 2014; Yamauchi & Shiiba, 2008; Yamauchi & Sricholpech, 2012). These permanent cross-links are essential for good bone formation. For example a defect in PLOD2, the gene coding for the enzyme lysyl hydroxylase, which converts lysine residues in the telopeptides of collagen, results in Bruck syndrome, a form of OI (Van der Slot et al., 2003).

Collagen cross-linking can be split into 3 principle categories (figure 1.6) (Saito & Marumo, 2010):

- Lysyl oxidase mediated cross-links, characterised by their immaturity and divalent nature
- Mature trivalent pyridinoline (PYD) and pyrrole (PYR) cross-links (formed from the Lysyl oxidase mediated immature cross-links)
- Glycation or non-enzymatic cross-links such as pentosidine (PEN)
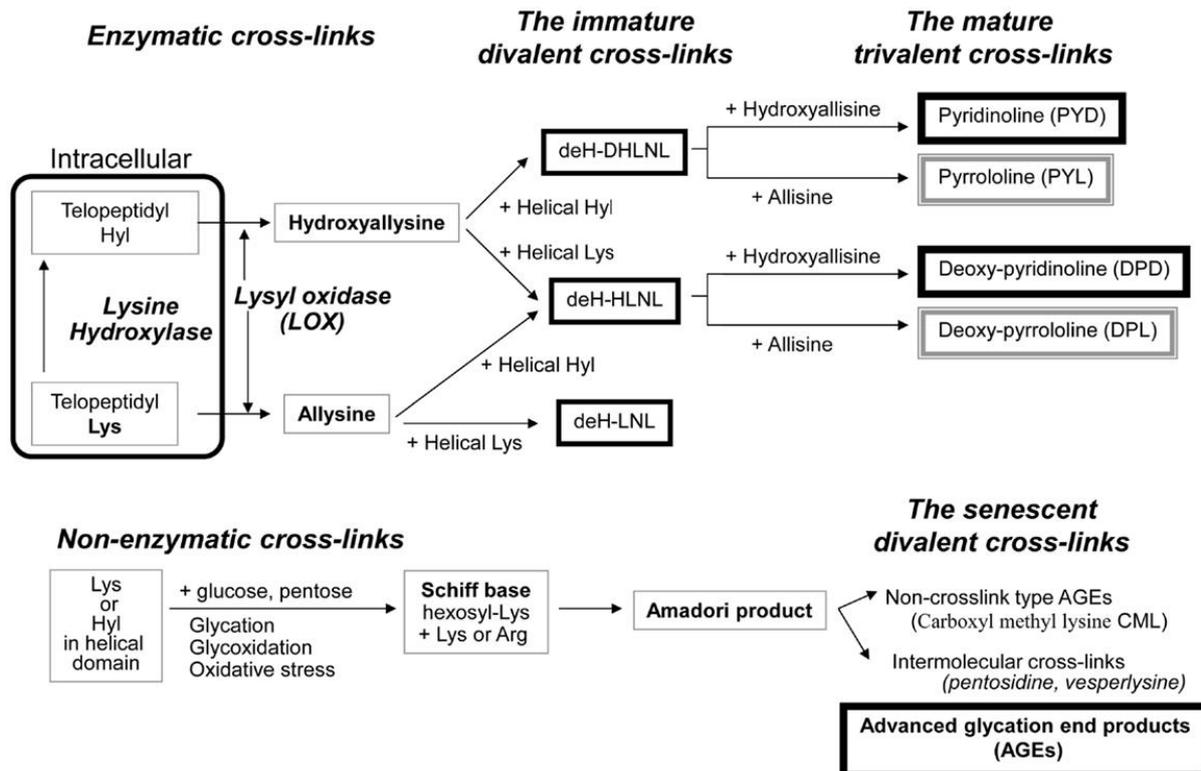
Figure 1.6 – The cross-links of type-1 collagen (Saito & Marumo, 2010)

Enzymatic immature cross-links are essential to bone strength as they mature into more stable bonds (Knott & Bailey, 1998). Of the mature cross-links, hydroxylysyl-pyridinoline (HP or PYD) and lysyl-pyridinoline (LP or DPD), can be taken as a ratio to act as a biomarker for certain disease, as the HP:LP ratio is relatively constant in healthy individuals (Tanimoto et al., 2004)(Lindert et al., 2015). Pyrrololine (PYL) has a similar reaction pathway to pyridinoline cross-links (Brady & Robins, 2001), and is associated with the structural organisation of trabecular bone (Banse et al., 2002).

Advanced glycation end products (AGEs) such as pentosidine are thought to have negative effects on the mechanical function of bone (Vashishth, 2007). Pentosidine is a well-documented AGE that increases in quantity with both individual and tissue age, and has been shown to decrease flexibility of collagen and permeability of tissues (Paul & Bailey, 1996). It has also been shown that the quantity of these cross-links does not impact collagen or mineralisation negatively (Viguet-Carrin et al., 2008), and that they are associated with lower bone mineral density and fragility (Viguet-Carrin et al., 2006).

1.23 Mineralisation

The process of bio-mineralisation, also known as calcification, is an ongoing process throughout a vertebrate's lifetime. The fibrous collagen scaffold is supplemented, and eventually outweighed, by a mineral component. This process is mediated by a number of

internal and external influences, including the age and gender of the individual in question. There is a host of complex biological systems of promoter and inhibitory mechanisms as well as feedback from stress and strain on the bone material itself which influences mineral deposition, although the exact mechanism for the control of mineralisation level is not clear.

Interwoven in the organic collagenous matrix are the mineral crystals, these are made of modified hydroxyapatite with the base formula $Ca_{10}(PO_4)_6(OH)_2$ (Rey, Combes, Drouet, & Glimcher, 2009), with substitutions of all regions being common (Cacciotti, 2016). The hydroxide portion of the molecule is frequently replaced with a carbonated group or a halogen (usually fluorine or chlorine), and in the extreme this substitution can have adverse effects on function (Everett, 2011).

The process begins with osteoblasts secreting a form of nanoscopic mineral into the extracellular matrix (Orimo, 2010), osteoblast membrane vesicles collect Ca^{2+} and inorganic phosphate ($H_2PO_4^-/HPO_4^-$), and transport them into the extracellular matrix (Anderson, 1995). These nano-hydroxyapatites migrate into the collagen fibrils and undergo heterogeneous nucleation (Glimcher, 1959; Glimcher, Hodge, & Schmitt, 1957; Rey, Miquel, Facchini, Legrand, & Glimcher, 1995) in the gap regions of collagen fibrils. They form small platelets that line up parallel to the collagen structure (Jäger & Fratzl, 2000), and then grow by aggregation, eventually exceeding the size of the gap region (Paro, Hossain, Webster, & Su, 2016). They are also found on the surfaces of fibril structures (Fratzl & Weinkamer, 2007; H. S. Gupta & Zioupos, 2008). The hydroxyapatite once cemented in position also offers some level of protection to collagen, making it more resistant to enzymatic activity when present (Grynpas, 1993).

However, the exact mechanism of mineralisation is unknown, as the structure of mineral is not perfectly understood nor how it is transported into the collagen network. Early theories suggested a simple precipitation model from a calcium and phosphate ion rich solution, where calcification occurs if there is a suitable template for mineral to form on (Urry, 1974; Westenfeld et al., 2009). However in recent years it has been shown that mineral forms a transient amorphous calcium phosphate (ACP) before becoming crystallised, suggesting that the process is more complex (Mahamid, Sharir, Addadi, & Weiner, 2008). Some studies posit that even mitochondria are involved in the process, assisting in delivering calcium and phosphate ions directly into the mineralising matrix via membrane bound vesicles (Veis & Dorvee, 2013).

<u>Factors Affecting Mineralisation</u>

<u>Phosphate Levels</u>

Mineralisation depends highly on the homeostasis of inorganic phosphate, one of the most critical minerals involved in cellular processes, being essential to energy transfer in the form of adenosine triphosphate (ATP). Up to 85% of phosphate found in the body is in bone (Khoshniat et al., 2011), but not all of it is directly integrated in bone material. Inorganic phosphate (Pi) can be found joined in a pair to form pyrophosphate (PPi) with an ester bond, this molecule can be found in the extracellular matrix of bone tissue, as well as being present in the blood stream (Russell, Bisaz, Donath, Morgan, & Fleisch, 1971), and is a natural by-product of cellular processes. PPi when in deficiency can lead to hyper-mineralisation, leading to excess hydroxyapatite formation in the skeleton, however in excess it results in a marked decrease in mineral deposition (Narisawa, Fröhlander, & Millán, 1997). Therefore the concentration, and ratio to Pi needs to be controlled for proper mineral deposition. Osteoblast membrane vesicles exclude PPi for transport to the extracellular matrix, instead PPi is expelled via a multi-pass transmembrane protein known as ANKH (Sapir-Koren & Livshits, 2011). A defect in the gene that expresses this protein leads to excess PPi concentrations in the extracellular matrix and causes catastrophic bone mineral loss (Ho, Johnson, & Kingsley, 2000).
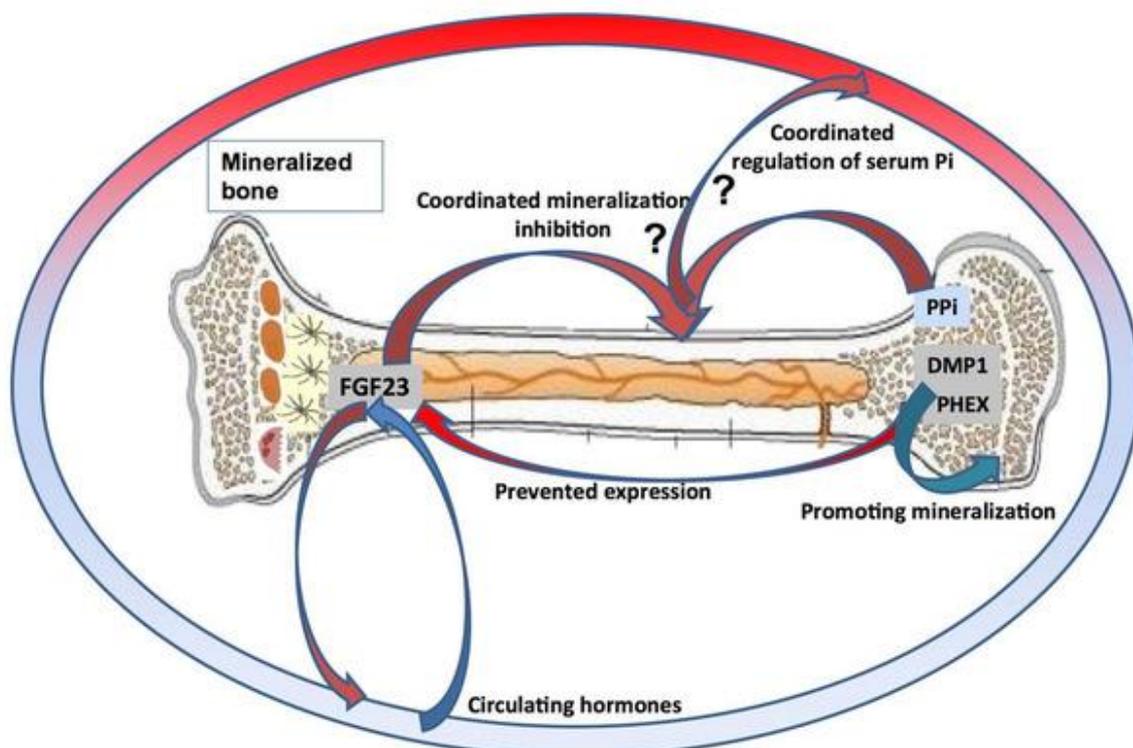
Figure 1.7 – Pi and PPi related promoters and inhibitors (Sapir-Koren & Livshits, 2011)

Levels of PPi are also controlled by an enzyme found on the surface of osteoblasts known as TNAP, which simultaneously removes PPi from the matrix while increasing Pi levels by cleaving the PPi molecule in two (Murshed, Harmey, Millán, McKee, & Karsenty, 2005). Alkaline phosphatase is a preparative enzyme found in the ECM which is involved in either breaking down ester-phosphates to increase the phosphate concentration or to remove pyrophosphate (Mundy, 1999). Further, PHOSPHO1 (phosphatase, orphan 1) is another enzyme which is found 100 fold in mineralising locations, and is essential to the regulation of PPi levels (Houston, Stewart, & Farquharson, 2004), although the mechanism has not yet been elucidated. This is further complicated by a number of hormones such as FGF23, and proteins (PHEX, DMP1), which are involved in Pi and PPi regulation (figure 1.7) (Sapir-Koren & Livshits, 2011). PPi acts as an inhibitor of mineralisation through three potential pathways (Addison, Azari, Sørensen, Kaartinen, & McKee, 2007):

- Directly binding to aggregating hydroxyapatite crystals, preventing further expansion of the structure.
- As a signalling mechanism, directly affecting osteoblastic activity.
- Prevention of the TNAP enzyme production.

Non-Collagenous Proteins (NCPs)

While this study is focussing on the impact of type-1 collagen, which makes up 95% of bone protein, there are a number of other proteins essential to construction of healthy bone which need to be considered when discussing mineralisation, and any changes this thesis associates with modifications to type-1 collagen. The extracellular matrix contains the following important proteins: bone sialoprotein, osteocalcin, osteonectin, osteopontin, fibronectin, bone morphogenic proteins (BMP), and other growth factors (Aszódi & Bateman, 2000).

Osteoblasts can associate with these proteins through transmembrane receptors, known as integrins, which bind to specific sites on proteins, to direct their actions (Damsky, 1999). In knockout mice the absence of certain integrins leads to impaired bone formation (Zimmerman, Jin, Leboy, Hardy, & Damsky, 2000). The presence of integrins are also essential to osteoclastic activity, as without binding the osteoclast to the surface of mineralised collagen, resorption will not take place sufficiently (Saltel, 2004). The integrin β1, is largely responsible for adherence to collagen fibrils, and when inhibited leads to a 40% reduction in bone resorption (Helfrich et al., 1996). They are also present on the membranes of osteocytes, facilitating extracellular communication, potentially part of the signalling mechanism that attracts osteoclastic activity for bone remodelling (McNamara, Majeska, Weinbaum, Friedrich, & Schaffler, 2009).

Osteopontin is a highly phosphorylated protein which actively inhibits hydroxyapatite formation by binding to crystals while also promoting osteoclastic activity (Vincent & Durrant, 2013). It is produced by osteoblasts, osteoclasts and osteocytes, and has a curious composition. The C- and N- terminus fragments are promoters of mineralisation, while the central inhibitory region contains multiple post-translational modifications to add phosphate groups (Boskey, Christensen, Taleb, & Sørensen, 2012). This central region's ability to bind to hydroxyapatite mimics the action of PPi, therefore it can be regulated by an enzyme that breaks down the central structure to provide both additional Pi to the extracellular matrix, as well as removing the inhibitory portion and releasing the terminuses which promote mineralisation, this is performed by an enzyme known as ALP (Alkaline Phosphatase) (Addison et al., 2007).

Bone sialoprotein (BSP), constituting about 8% of the NCPs, is a potential cell activator as well as acting as one of many 'phosphoproteins', a group of proteins modified post-translationally to include a phosphate group which is thought to bind calcium and be involved in mineral nucleation (Chen, Shapiro, & Sodek, 1992; Mundy, 1999; Roach, 1994). BSP is present during embryonic stages of life, being associated with membrane vesicles which deliver the calcium and phosphate to the extracellular matrix for hydroxyapatite formation (Harris et al., 2000). It is of a similar structure and shares many functions with osteopontin, being from the same group of NCPs, called SIBLINGs (small integrin-binding ligand N-linked glycoprotein) (Staines, MacRae, & Farquharson, 2012).

Osteocalcin is the most abundant non-collagen protein, at around 20% (Al-Qtaitat, 2014). It is produced by osteoblasts, although its role is unclear, being reported as a calcium binder (George & Veis, 2008; Termine, 2007), but also as a retarder of mineralisation and acts as a chemical signal for osteoclastic activity (Price, Williamson, Haba, Dell, & Jee, 1982). However its effects on mineralisation are minimal, despite the quantity present in the matrix (Ducy et al., 1996). A deficiency in osteocalcin leads to increased bone formation, however it may have a far greater role in the endocrine system as a hormone to regulate glucose levels in tissues (Wei & Karsenty, 2015).

Osteonectin has been shown to be crucial to bone remodelling and mineral mass in knock-out studies (Vincent & Durrant, 2013). Despite the nomenclature, osteonectin is found in a host of mineralised and non-mineralised tissues (Bradshaw, 2012), and binds itself to both the mineral and collagen phases in bone at opposing terminuses, thought to facilitate mineralisation (Termine et al., 1981). It has been shown in knockout studies to be integral to normal collagen levels in the ECM, with an overexpression leading to excess levels of collagen (Rosset & Bradshaw, 2016; Trombetta-eSilva, 2012). As osteonectin has a direct

effect on the production of procollagen, it is reasonable to think it may have an impact on fibril formation and the final state of collagen in bone material.

Fibronectin is a high-weight, dimer molecule that has binding functions in the ECM, similar to integrins found in the membranes of cells. It is implicated in wound healing, providing a framework for cell adhesion and repair, as well as promoting clotting, and is critical during embryogenesis where its removal results in non-viability (Valenick, Hsia, & Schwarzbauer, 2005). The molecule also serves as a binding site for collagen, prioritising denatured material, as it may be involved in the removal or redistribution of damaged tissues (Pankov, 2002).

Bone Morphogenetic Proteins (BMP) are a group of over 20 unique proteins which are essential to bone growth as signallers and receivers, and are used clinically to increase mineralisation levels (Lavery, Swain, Falb, & Alaoui-Ismaili, 2008). There are many functions associated with BMPs, many of which are key to bone and cartilage formation, as well as enzymatic activity on procollagen (Bragdon et al., 2011). However they are too numerous and beyond the scope of this review section to cover each individually, but are thoroughly reviewed by Bragdon et al. (2011).

Others

Oestrogen plays a large role in mineralisation, with the decrease of this hormone in post-menopausal women being directly linked to bone loss and osteoporosis (Callewaert, Venken, Boonen, & Vanderschueren, 2010). Oestrogen has the role of potentially preventing the premature apoptosis of osteocytes, therefore retaining bone mass (Tomkinson, Reeve, Shaw, & Noble, 1997), as well as inhibiting osteoclast formation through decoy receptors and reduction of osteoclastogenic cytokines (Pacifici, 2009).

Summary

There are many factors that affect the mineralisation levels in bone tissue that work in a delicate balance where any one over or under-expression of a particular molecule can result in poor bone material. This study does not seek to investigate the roles of impacts of these important aspects of bone formation, however it is important to emphasise that in the context of analysing the impact of modifications to the collagen unit that these proteins and hormones play a significant role that must be taken into account.

1.3 Water

Water is the third largest component of typical long bones by weight (up to 25% (Wilson et al., 2006)), providing biological processes a medium for transport and reaction in areas with a diameter as small as 0.1µm in cortical bone (Fernández-Seara, Wehrli, & Wehrli, 2002), as

well as increasing the stability of collagen (Grynpas, 1993). It is a crucial component for bone material properties as dry bone is far more brittle than wet (Sedlin & Hirsch, 1966). However its effect on material properties is more complex than simply providing hydration, water is involved from the macroscopic organ scale, through to the molecular structure of collagen (figure 1.8).
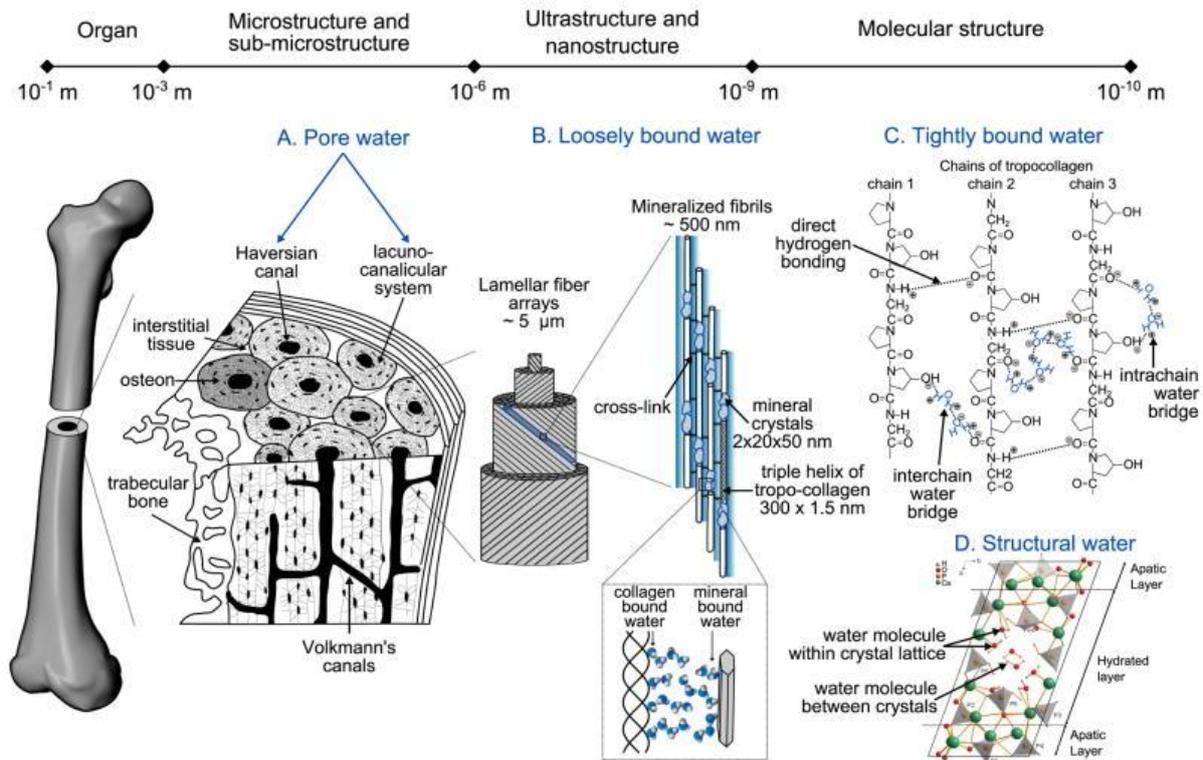
Figure 1.8 – Water at different organisational levels of bone (Granke, Does, & Nyman, 2015)

Water exists in three main locations in bone tissue:

- Free within pores, providing hydrostatic support, which confers stress more evenly to bone tissue. Structural studies have indicated that water acts as a sacrificial layer for stress, dispersing when bone material is put under pressure as the first line of deformation (Wilson et al., 2006). The water in this category is found in Haversian and Volkmann canals, vascular spaces, and lacuna-canalicular systems.

- Loosely bound, found on the surface of collagen fibrils and in the interstitial spaces between mineral and collagen in mineralised tissue. This provides a medium between bound and unbound water, where water is closely associated with collagen and hydroxyapatite structures.

- Bound in the matrix, forming bonds between collagen chains, and increasing hydrogen bonding availability. This also includes water found within the crystal structure of hydroxyapatite. This water being directly responsible for a large proportion of the extra flexibility lost when dried (Granke et al., 2015).

In the first instance during bone formation, osteoid can be described as hyper-hydrated collagen, with a high density of loosely bound water which is slowly displaced during the mineralisation process (Robinson, 1975). There is strong evidence to suggest that water also plays a role in the mineralisation process by binding to the crystal surface of hydroxyapatite to assist in orientation for aggregation into larger crystals (Yan Wang et al., 2013).

The role of water in conferring strength is not easily defined, however it has been shown that the removal of hydration leads to an increase in stiffness and Young's modulus (Evans & Lebow, 1951), and a marked reduction in toughness (Yan, Daga, Kumar, & Mecholsky, 2008). The loss of pore or free water has been shown not to have a major effect on the mechanical properties of bone material, however the effect of this water loss on post-yield loading (bone which has begun to deform or fail in response to stress) results in a significant amount of additional deformation (Nyman et al., 2013), as it does provide some hydraulic support to the strength and stiffness of the material (Liebschner & Keller, 2005). However the bulk of strength is associated with bound water, directly affecting the ability of hydroxyapatite crystals to disperse energy through ductile sliding (Eberhardsteiner, Hellmich, & Scheiner, 2014). It also directly affects the properties of the collagen component, with the loss of bound water resulting in a contraction of the collagen molecule, affecting its flexibility (Fratzl, Fratzl-Zelman, & Klaushofer, 1993).

In studies where water has been substituted for another solvent such as ethanol to observe the impact of water-based bonding, water bridges (water mediated hydrogen bonding) linking tropocollagen molecules are removed and replaced with collagen to collagen hydrogen bonding, strengthening the stiffness of fibrils at the cost of reduction in flexibility (Nalla et al., 2005; Pashley et al., 2001). Bound water has also been directly correlated with the strength of human cortical bone (Horch, Gochberg, Nyman, & Does, 2011), similarly to bone density and the strength of human cortical bone (Keller, Mao, & Spengler, 1990), implicating it as a key player in role of determining material properties. However the presence of bound water, as well as unbound water is not controlled by any modifications to the water itself, or by the concentration of water. Bound water content decreases with age (Currey, Brear, & Zioupos, 1996), likely to other changes in the composition of bone material, including bone mineral density, and the organisation of collagen, the two major locations for bound water content.

<u>1.4 Mechanical Behaviour of Bone</u>

The mechanical properties of bone are very complex, requiring a thorough understanding of how bone material is organised, and a careful selection of methods to measure these properties. As mentioned earlier, bone is an extremely heterogeneous organ, with the chemical composition and arrangement of material differing between locations within a single bone on the millimetre scale. Therefore, assigning a bone a single value for its properties is only relevant to the small area in which it was tested, and a single metric such as stiffness is only valid as one of many metrics unless it has been specifically related to strength in another capacity, such as risk of fracture rates. There are methods which test the integrity of whole bone, i.e. a three-point bending test where the entire organ is put under force until failure, however this still requires points of contact on the bone surface which, due to the heterogeneous nature of bone, results in large amounts of variation. Indeed many papers can easily have opposite conclusions when testing similar material, with perhaps heterogeneity being one of the main contributing factors (e.g. Carriero et al., 2014; Gallant, Brown, Organ, Allen, & Burr, 2013).

The first distinction to be made is between mechanical and material properties, sometimes incorrectly used interchangeably in the literature. Mechanical properties relate to the whole bone, or a large section of it, and take into account the geometric architecture in which it is arranged, making its behaviour much more difficult to predict (Schileo, Taddei, Malandrino, Cristofolini, & Viceconti, 2007). Material properties treats a section of bone as a singular entity, and ignores the larger architecture and its influence on surrounding areas, but retaining information on local hierarchy, focussing in on how an area is composed and reacts to stress (Sharir, Barak, & Shahar, 2008). To fully understand the mechanical properties of a complex material such as bone, you would need to be able to map out the properties of every differing section of material before examining it as a whole, which while attempted by modelling methods, is difficult and cannot be easily translated into patient care. It is usually invasive and destructive to obtain mechanical properties of whole bone material, which is why many methods seek to link particular material properties in easily accessible locations as markers of organ-scale behaviour, to provide an easy and effective clinical approach. Most clinical approaches measure some aspect of the bone composition, usually bone mineral, such as the DEXA (Dual Energy X-ray Absorptiometry) scan for bone density (Stewart, Kumar, & Reid, 2006). However these approaches are only partially successful, such as the DEXA's 60-70% rate of fracture prediction (Geusens, Van Geel, & Van Den Bergh, 2010), in part due to variation between individuals activity and backgrounds, but also due to the technique only accessing partial material information and extrapolating outwards to mechanical properties.

Bone Mineral Density

The density of bone material plays a large role in its strength and stiffness, and is of great concern in ageing humans. From the age of 30 years, as the total bone density of the human skeleton begins to decrease, which can result in age-associated osteoporosis (Swedish Council on Technology Assessment in Health Care, 1997). However mineral density (as measured by DEXA) is not a reliable predictor of fracture risk as it only indicates the presence of mineral, and does not take into account bone quality, for example woven bone (an intermediate stage of bone development with haphazard organisation of collagen), due to its immature structure, would provide mineral content but markedly low stability relative to fully formed bone material (Hernandez, Majeska, & Schaffler, 2004). Nor does DEXA include the ratio of organic to inorganic material, making comparisons across smaller areas more difficult, as the ratio of the two main components of bone are adjusted for function and a reduction in mineral content does not necessarily lead to a decrease in mechanical properties (Curtis et al., 2016). Despite these drawbacks, bone mineral density is the current gold standard in osteoporosis diagnosis and is used in the definition of osteoporosis.

From a material point of view, an increase in bone density should result in a linear increase in resistance to force, assuming all the material was homogenous. However, bone has innate restrictions to its density, in that it must have room for vascularisation, cells, marrow deposits, and space to react to force so that it has some flexibility even in cortical regions. This means that the function of the specific region of bone regulates its density, and indeed studies have shown that there is no correlation with resistance training and bone density measurements (Layne & Nelson, 1999).

Stress and Strain

Stress can be described as a force acting over an area (figure 1.10), although in reality it is more complex due to the nature of forces acting on bone. A bone loaded in nature will often have multiple forces in different directions, as well as parallel forces in any one location from nearby loading known as shear forces (Fung, Tong, & Bechtel, 2003). Stress would be difficult to quantify in a natural environment, however in mechanical testing where loading is controlled this becomes much more viable. The units of stress are typically measured in pascals (Pa), one of which is a newton over a meter squared (N/m^2) and a measurement of force over area, although this represents a very small force for bone, which is usually measured in megapascals (MPa).

$$\sigma = \frac{F}{A}$$
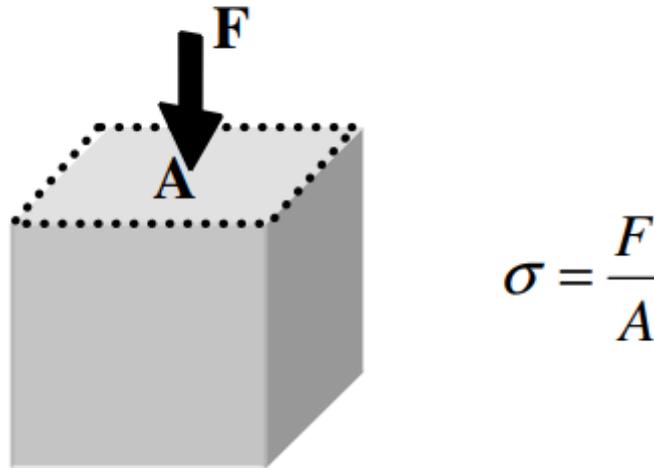
Figure 1.9 – The equation for stress = force over area (Sharir et al., 2008)

Strain is a measurement of the deformation of material, the amount by which the material contracts or stretches in response to stress being placed upon it. Similar to stress it can be described in terms of direct strain, i.e. a singular compressive force as in figure 1.9, or from shear strain, where the material is bent by a perpendicular force. As strain is a measure of the amount of deformation, it is typically measured in unit-less percentage, although can be measured in terms of distance. All loaded bones undergo some amount of strain during activity, with race horses approaching 1% compression in metacarpals, one of the most extreme examples of strain (Muybridge, 1882).

The relationship between stress and strain is a useful descriptor of materials, as according to Hooke's law, there is a linear relationship between stress and strain in elastic materials (such as bone). Describing each potential force on an object however, becomes very complicated in nature with the number of available dimensions, but a single force is relatively straightforward. In an isotropic material (one that does not have a preferred orientation and will react the same if the force was applied in any direction) the only variables are the amount of force applied, and the amount of deformation observed. Cortical bone is considered to be transversely isotropic, being able to be measured parallel to ordered osteons, or orthogonal to them, increasing the number of variables that need to be controlled. This is further complicated when bone arrangement is not so tightly packed or is a specialised bone which is tuned to react differently to stress in different areas.

Young's modulus (also known as the modulus of elasticity) is a measurement of the relationship between stress and strain, with the symbol E and the same unit as stress (Pa),

as strain is unit-less. The higher the value for Young's modulus the greater amount of force is required to achieve the same amount of deformation. When a sample is compressed, it becomes shorter in the direction of compression, but some of this compression will result in an increase in the width of the object. Poisson's ratio is calculated from the ratio of horizontal strain caused by compression, and the negative vertical strain resulting from it, describing this change in width relative to change in height (Shahar et al., 2007).

Material properties and Bone properties

Material properties when referred to in this thesis describes the behaviour of bone as defined by materials science tests, such as Young's modulus. Bone properties refer to the description of bone behaviour as defined by other techniques, such as those of the Biodent (Active Life Scientific, USA) instrument.

The stiffness of a material is defined as its ability to resist deformation, and is dependent upon the elastic modulus as well as the geometry of the structure (Boughton et al., 2018). In a material such as bone the ability to resist deformation is linear in the elastic range of bone, and in this range no permanent deformation occurs. However, at higher levels of stress this linear relationship fails (yield point, at which after this point deformation becomes plastic), and then reaches its maximum stress tolerance at its 'ultimate strength' at which point the bone fractures (figure 1.10).
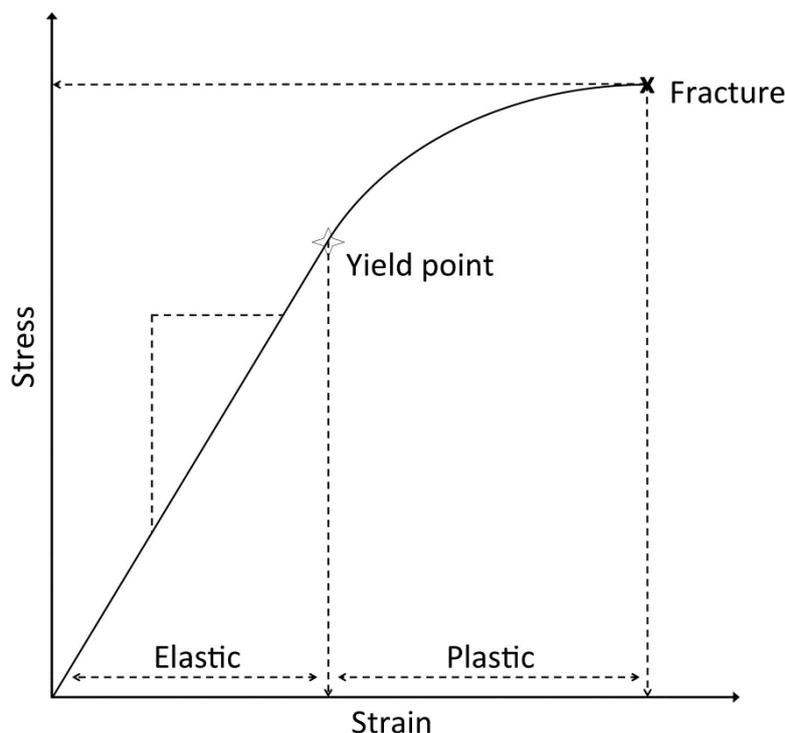
Figure 1.10 – A graph of stress vs strain in bone material (adapted fromForestier-Zhang & Bishop, 2016)

The hardness of a material is described as the ability to resist plastic deformation, measured by the size or depth of an impression using a known force and indenter. This is frequently

measured using micro- or nano-indentation (Cowin & Telega, 2003). Creep is the tendency of a material to deform plastically when presented with stress and can occur beneath the yield point. In bone this can be represented during indentation, where there is a mixture of elastic and plastic deformation. The toughness of a material can be defined as the amount of energy it is able to absorb before the point of failure, in bone this would be the area under the stress/strain curve in figure 1.10 up until the point of fracture.

1.5 Summary

This chapter has described the nature of bone material, its composition, and its material and mechanical properties. It has highlighted the important roles of the organic components with regards to regulating bone as a material, and touched on some of the methods used to analyse bone material. It has been said already that the current DEXA scan method is only 60-70% effective in predicting fragility due to its inability to access the organic, mainly collagenous phase of bone (Ammann & Rizzoli, 2003). Thus radiographic methods of obtaining information on bone are missing a large proportion of the bone material itself, where it is likely that the additional strength unaccounted for by these methods is located.

Chapter 2 – Methods Used to Probe Bone

This chapter describes the main relevant analytical techniques used to probe bone material in the literature, examining their contributions and discussing their effectiveness. It has a particular focus on in-vivo techniques and their applications, as one of the goals of this thesis is to contribute to knowledge that will work towards better clinical techniques for the analysis of human bone.

Bone has been thoroughly examined by many methods, from the whole bone level mechanical testing, to micro-scale radiographic techniques, nano-scale microscopy, and quantitative chemical measurements of constituents that appear in the region of parts per billion. It is impossible to cover each of the hundreds of techniques that have been used over the years within this chapter, so focus will be placed upon the main contributors to our current understanding of bone material, in-vivo clinical techniques, and those used in this study.

2.1 Bone Imaging In-vivo

Radiography is one of the first ways in which bone material was examined, with the first X-ray image being taken in 1895 depicting a human hand using a simple emitter and receiver system, with the X- in X-ray being coined as it was a 'mystery ray' (Howell & Arbor, 2016). An X-ray is a form of electromagnetic radiation, a photon, on the shorter wavelength end of the spectrum at 0.1-10nm. It is also a type of ionising radiation, carrying enough energy within the beam to remove electrons from atoms, giving them charge and potentially leading to the breaking of chemical bonds and the production of free-radicals, highly reactive particles that can cause unwanted chemical reactions and permanent damage to living organisms where DNA is modified. X-rays have the ability to easily pass through soft tissue, but are blocked by the denser mineralised material in bone, making them ideal for in-vivo imaging of bone. The original, and simplest setup involves an X-ray source projector, a detector sensitive to X-rays, and the object to be scanned in between the two. The rays that are not absorbed by the denser phases of the object create a negative image, similar to a shadow, on the detector. This has the major disadvantage of turning a 3D object into a 2D image, meaning any detail along the depth of the object is compacted, leaving a large amount of information open to interpretation as you're only presented with an average density along a plane.

This limiting factor has since been overcome, with the invention of the CT (computed tomography), a technique that involves a much larger dose of radiation, but provides a 3D image for analysis. This is achieved through an axial and helical (also known as spiral scanning, figure 2.1) method by which the object is scanned in the traditional projection method, but from multiple angles creating an array of slices which can be joined together. Cube units named voxels act as the measure of resolution, where a voxel is similar to a pixel in that it describes one uniform piece of information for a region. The data is then mathematically processed to provide a full 3D model in which 2D slices can be selected and examined as if they had been scanned as separate entities, allowing more information to be probed (Tepper, 2008).
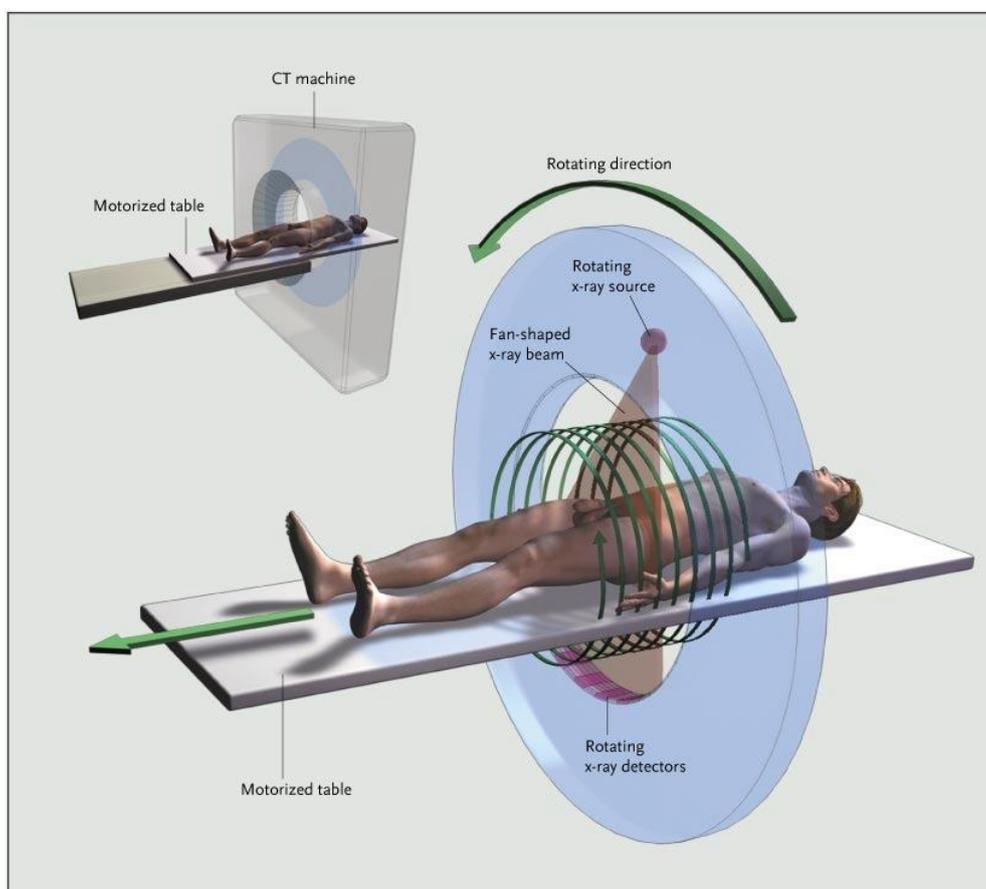
Figure 2.1 – Operation of a CT scanner, with the central green spiral indicating the travel of the X-ray source
(Tepper, 2008)

pQCT

Peripheral quantitative computer tomography is a method designed for measuring bone mineral density in smaller compartments of limbs such as ankles or wrists. A motorised stage passes an object through a ring, stopping to allow x-rays to be taken from multiple angles to create a series of image slices akin to the computed tomography method

described above. This supplementary technique allows thin slices of bone to be quantified in terms of its mineral content, with images being coloured artificially to represent the changing density of the material (Muller, Ruegsegger and Ruegsegger, 1989). It is also capable of automatically analysing regions of trabecular and cortical material through computational algorithms, and assigning the locations for easy diagnosis for clinicians who preferentially use certain areas of the body as markers for bone defects, such as the metatarsal of the foot (Chaplais et al., 2014). Particularly high resolution pQCT instruments are in use that can access information down to 100µm^2 (MacNeil & Boyd, 2007), providing information on trabecular architectural organisation to which changes to organisation have been associated with a reduction in bone strength (Goldstein, 1987). pQCT has also been used to access the muscle-bone attachment sites in detail (Rauch et al., 2001), as well as examining muscle and fat sites based on cross-sectional area in which there is an absence of bone material (Stagi, Cavalli, Cavalli, De Martino, & Brandi, 2016). CT scanners have approximate resolution of 0.5mm, which allows for an overview of the microstructure (Lin & Alessio, 2009).

DEXA

The Dual energy X-ray Absorptiometry method, currently used to evaluate bone mineral density in elderly patients for early signs of osteoporosis, involves two low-powered X-ray sources angled at a single detector to create a single averaged reliable image. Compared to CT, this technique offers a markedly lowers resolution of approximately 4mm (Mazess & Barden, 1988). The bone mineral density is reported as a t-score, an expression of the difference in number of standard deviations from the general population. Depending on the organisation and country, this can also be referred to as a z-score, a more accurate version which uses a database of age matched controls (Kling, Clarke, & Sandhu, 2014). It has the disadvantage of simple X-rays in which information is compacted and only an average is produced, meaning information on the bone cannot be separated into cortical and trabecular for further analysis as with pQCT.

The disadvantages of X-ray based scanning are very clear, despite their effectiveness and widespread use. The ionising radiation element can cause cell death and dangerous mutations resulting in cancers, making repeated scans a considerable risk, especially in CT scans where multiple scans are taken over a longer period. They also are practically incapable of observing the organic phase of bone in-vivo, making a full picture of the state of the material impossible, resulting in suboptimal analysis of the condition of patients, and subsequent diagnosis and treatment.

MRI

Magnetic Resonance Imaging is a non-ionising technique which uses a strong magnetic field to align the spin of (usually) hydrogen atoms (protons) in water within the body. The magnetic field is oscillated, causing the protons to emit a radio signal which is measured by detection grids, which attenuate to the amount of time it takes the proton to return to its original spin. Altering the magnetic field, by rapidly switching the electromagnetic coils on and off, allows the measurement of these radio signals from different locations, enabling the scanner to identify tissues by location. This is frequently combined with contrast agents in-vivo, used to mark certain aspects of tissue for analysis. MRI has shown to be more effective at determining bone metastates than CT scans (Gandage, Kachewar, Aironi, & Nagapurkar, 2012), however an MRI costs roughly 10x the amount per scan compared to DEXA and requires more specialist knowledge and analysis, making its widespread use prohibitive in the context of bone scans. An additional development is the µMR, a scaled-down version of the traditional MRI (with a resolution of 2-3mm) with the ability to resolve much smaller structures, including trabecular spacing and can be used in-vitro (Genant, Engelke, & Prevrhal, 2008).

Finite Element Model

FEM is a computer-based analysis approach that incorporates bone imaging into its input. The main goal of this analysis method is to create a model of bone which can describe the stresses and strains on it induced by mechanical loading to predict fracture risks. Typically the input data comes from pQCT or CT scans and has been used successfully to predict fractures in models, however the transition to clinical use is hampered by the complexity of the models and the amount of data required to provide accurate analysis (Lang et al., 1997; Schileo et al., 2007). Figure 2.2 depicts a comparison between human femurs, one healthy (left) and one osteoporotic (right), showing the amount of predicted strain, where negative strain is describing physical instability under compression. It raises interesting questions as often the patterns of stress on bone material are not considered when, for example, evaluating bone mineral density in relation to fracture. Studies typically draw a correlation between fractures and measurements at a specific site (not necessarily the point of potential fracture), rather than observing the operation of the actual site of fracture. The patterns of loading stress can be used to identify key sites for observation, leading to more effective diagnoses in the prediction of bone strength (Zysset, Dall'Ara, Varga, & Pahr, 2013).
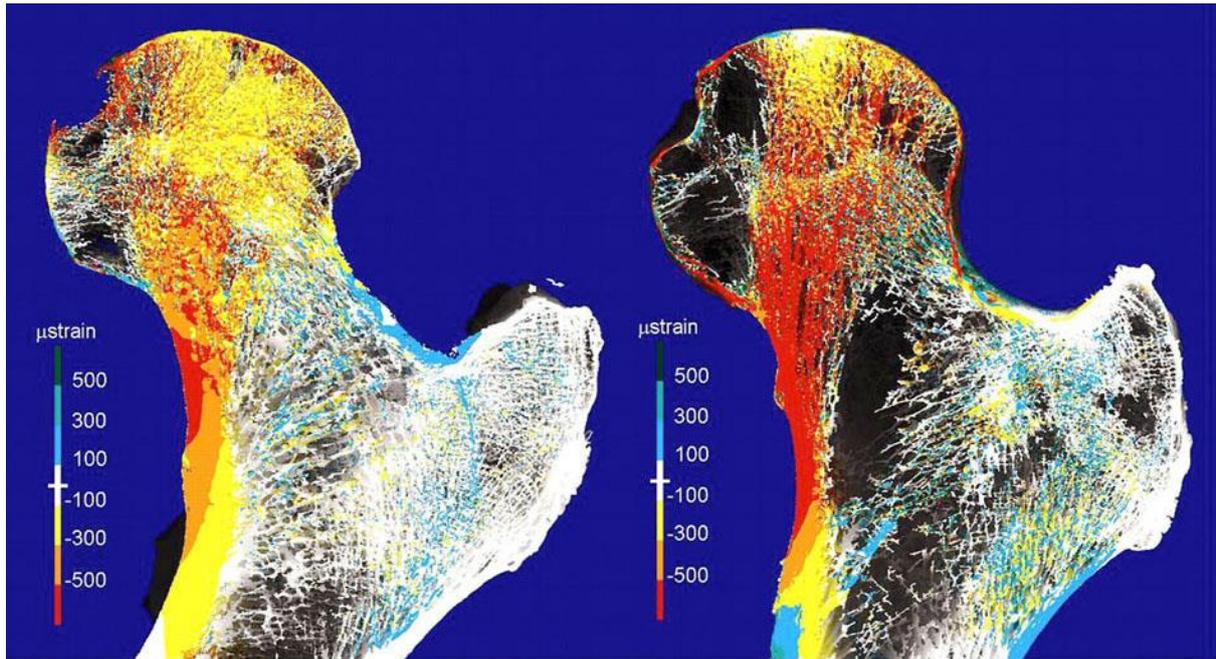
Figure 2.2 – FEM model of trabecular strain dispersal in a normal (left) and osteoporotic (right) femur (Morgan & Bouxsein, 2005)

2.2 Bone Imaging Ex-vivo

Electromagnetic Microscopy

Scanning electron microscopy (SEM) allows for a visual representation of the structure of bone and collagen down to the nanometre scale. By focussing a stream of electrons onto a sample the SEM is able to resolve an image by retrieving signals from the resulting interaction (Reimer, 1998). In backscattered electron mode, information is collected when electrons are fired into the sample and hit the nucleus of an atom, a single high energy electron is returned which allows a certain amount of depth perception between atoms. Secondary electrons result from collecting signals from electrons which have been knocked out of their shells by those fired from the SEM. Each electron fired is capable of knocking thousands of others out of their shells giving back a much larger signal. A positively charged detector attracts the loose or bounced electrons, discerning them by amount of energy, and creates an image based on energy received. Samples are typically coated in gold or palladium as it increases the signal without obstructing the sample, and in the case of bone are frequently dried before viewing as the SEM operates in a vacuum (Boyde, 2012). SEM is also capable of elemental analysis through use of an EDX (Energy-dispersive X-ray Spectroscopy), using the energy emitted from electrons dislodged from the inner shells of atoms in samples by detecting the resulting X-rays. As each element has its own unique structure and atomic shell arrangement, the EDX is able to distinguish between them and can provide ratios of elements, which has been used to show the general uniformity of

mineral composition across bone with differing mineral content (Bloebaum, Skedros, Vajda, Bachus, & Constantz, 1997).

A variation of EM microscopy is the method TEM (transition electron microscopy), offering a significantly higher resolution compared to SEM, with the drawback of a more complicated sample preparation procedure. TEM works on the same principles as SEM with regards to the use of electron emission onto a sample, however it differs by having the detector behind the sample in question. By passing electrons through an object (usually less than 100nm thick), a negative image can be produced by the electrons that do not interact with the atoms of the sample (Boivin, Anthoine-Terrier, & Obrant, 1990). To create a slice of bone thin enough it must first be demineralised with a substance such as formic acid, as mineral makes it extremely difficult to cut sections. The bone is then mounted within a resin block, and is cut using a specialised microtome, with the thin slices being then placed onto grids for viewing. This method has been used to study the lamellar structure of bone tissue in control and osteoporotic samples, showing an alteration in the alignment of osteons, and has also been used to observe cell ultrastructure, observing the morphologies of cells as they differentiate from bone progenitor cells (Qu, Lu, & Leng, 2007). One of the most important contributions of TEM studies has been the analysis of collagen (e.g. figure 2.3) in relation to the location and organisation of the mineral aspect of bone, with recent studies identifying patterns in collagen arrangement resulting in preferential areas of mineralisation (Quan & Sone, 2015).
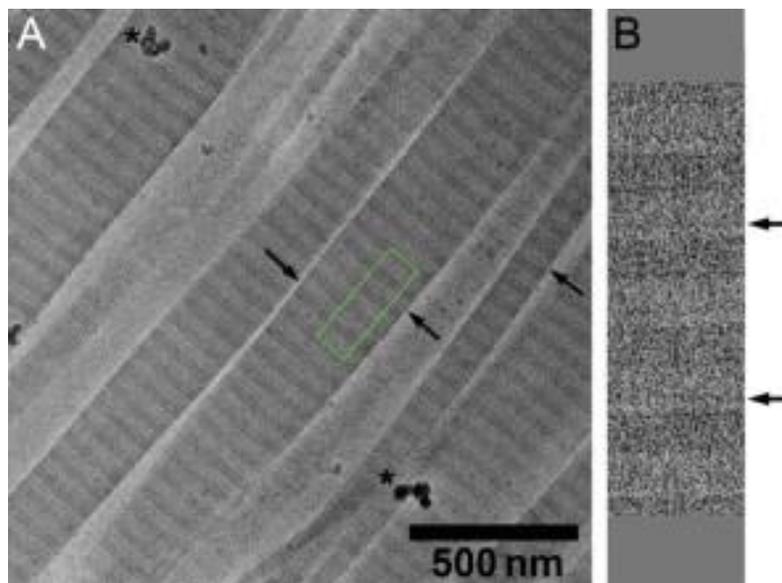

Figure 2.3 – The banding pattern of type-1 collagen in bone tissue (Quan & Sone, 2013)

2.3 Optical Spectroscopy

Infrared spectroscopy (IR) is a spectrum of techniques which involve the interaction of infrared radiation with particles for the study of chemical composition, providing information on the amount of infrared absorbed or transmitted by an object. However, the infrared spectrum is comprised of many wavenumbers, approximately $14000cm^{-1}$ to $25cm^{-1}$ (although most biological studies are conducted in mid-IR, within the $4000 – 400cm^{-1}$ range), and so the first spectrometers would use prisms or gratings to present the sample with each wavelength and record the absorption on a detector, a slow process. The most common modern instrument used is an FTIR (Fourier transform Infrared spectroscopy). It has the advantage over the older IR spectroscopy designs of having a greater signal to noise ratio, faster processing times, with a smaller error on wavelength assignment, and a larger range and resolution (Paschalis et al., 2004). FTIR allows all infrared frequencies to be measured simultaneously using a Michelson interferometer, a configuration of mirrors, one of which moves using a motor. A beam splitter diverts approximately 50% of the beam to the moving mirror, and the rest to a stationary one, the beams are then recombined. A broadband light source (one with all the wavelengths to be measured) is utilised as the mirror moves, each wavelength of light is blocked or transmitted based on wave interference brought about by the change in path distance of the moving mirror. In this way multiple wavelengths of IR light can be directed onto a sample within seconds and processed by a detector. The result is known as an interferogram, which when analysed using a computer algorithm known as a Fourier Transform, creates a graph of light absorption at each wavelength which tells us how the absorption of IR varies across a sample. Each peak is a result of the resulting vibration of molecular bonds when excited by IR radiation, allowing the inference of what is present in a given sample.

FTIR has contributed greatly to the study of bone material, providing information on collagen cross-linking and its relation to disease (Paschalis et al., 2003), as well as allowing the organic and inorganic phases of bone to be compared directly, chemically, within a single technique (Mendelsohn, Hassankhani, DiCarlo, & Boskey, 1989). FTIR has been applied as an in-vivo technique in pilot studies, with the use of fibre-optics to deliver the light source directly into patients, however its effectiveness and need for interpretation of results have prevented it from becoming a widespread diagnostic tool (Li, 2005; Van Nortwick et al., 2009). In addition to this, FTIR is sensitive to water, which introduces further difficulties when observing biological samples, especially in-vivo.

Raman Spectroscopy
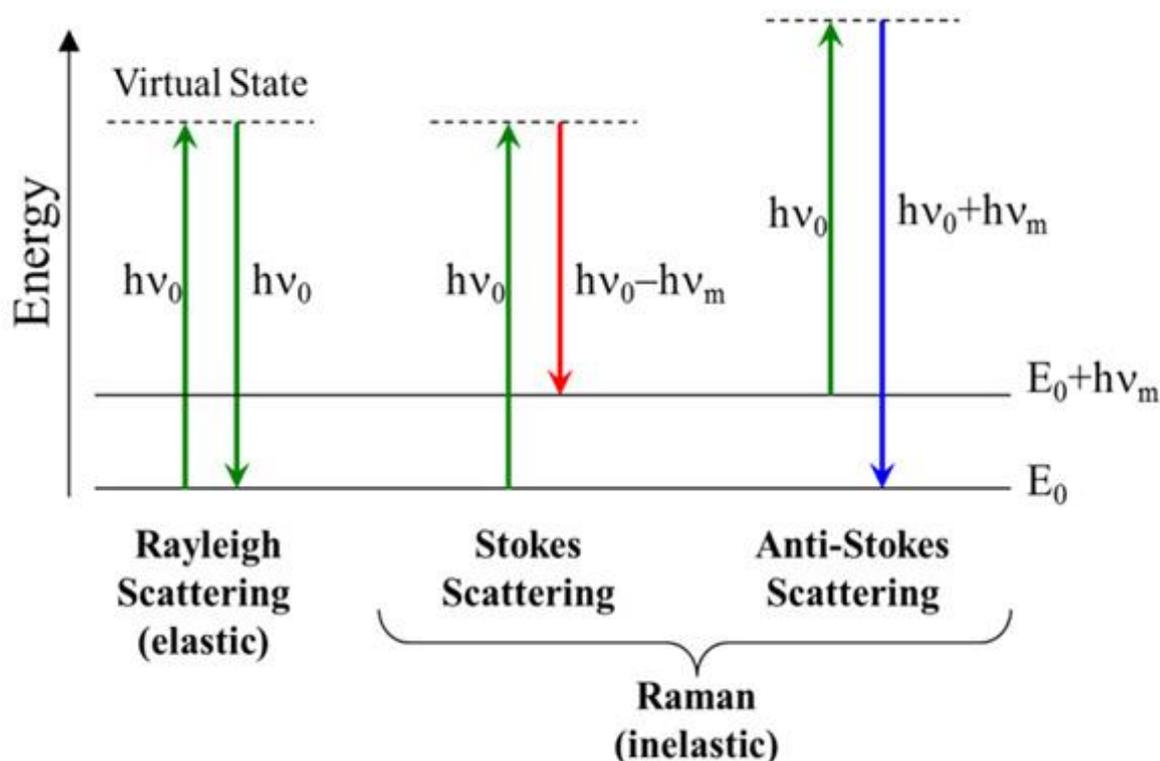
Raman spectroscopy measures the vibrational energy of bonds within a given wavelength range, showing their abundance, creating a fingerprint of the substance being examined based on its chemistry. This can be used to analyse both the contents and relative quantities of bio-chemicals in bone. Raman has the advantage of being non-destructive, non-contact and requires little to no sample preparation, and water does not dominate the spectra.

Figure 2.4 – A Jablonksi diagram of the energy transfer between molecules and photons in Raman spectroscopy (Sarkar & Daniels-Race, 2013)

A source of monochromatic light, usually a laser in the near visible, infrared or ultraviolet range, is directed onto a substance. The molecules are excited when exposed to the incident radiation from the laser light. As in figure 2.4, the molecules then either stay at the same vibrational energy level after being excited (known as Rayleigh elastic scattering), and therefore the returning light has the same energy, or they end up at a higher or lower state of energy than they started at (Raman inelastic scattering (Smith, 2005)), in which case the returning light either has more energy (anti-Stokes scattering), or less energy (Stokes scattering). This change in the energy of the photon results in a wavelength shift, which is recorded by a charge-coupled device detector, describing the vibrational modes of the molecules. Raman scattering is a rare phenomenon occurring only once out of every 1,000,000,000 interactions which is why a focussed light source and time is needed for the scans (Lewis and Edwards, 2001). The changes are measured as a frequency shift and

displayed against frequency, where each peak represents the vibrational response of a particular molecule n the system.
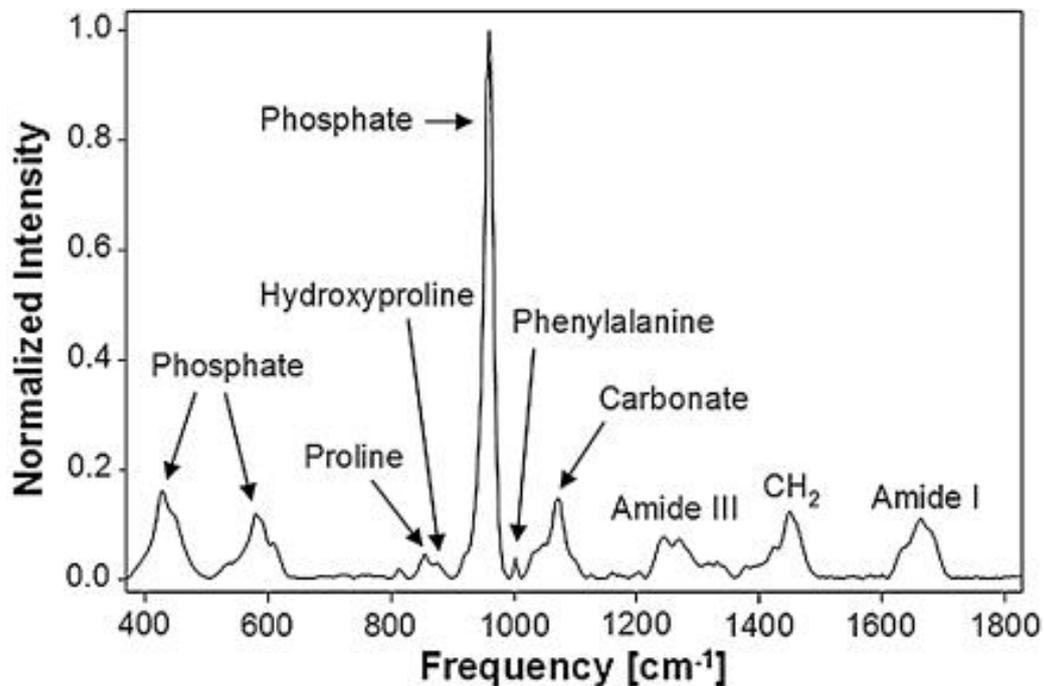
Figure 2.5 – An example Raman spectra taken from the tibia of 6 week old mouse (Morris & Mandair, 2011)

Figure 2.5 depicts several components of bone tissue:

- Phosphate is the primary component of mineral in tissue, with the main peak at 959cm^{-1}
- Proline and hydroxyproline are amino-acids in collagen at 830cm^{-1} and 880cm^{-1} respectively
- Phenylalanine at 1002cm^{-1}, another amino-acid found in collagen
- Carbonate represents the substitution of phosphate in the mineral component of bone at 1070cm^{-1} (Akkus, Adar, & Schaffler, 2004)
- The CH$_2$ band comes from methylene side chains in collagen, found at 1450cm^{-1}
- The amide I and amide III bands reflect a number of individual protein peaks which describe the organic portion of bone

Often in Raman spectral analysis individual peaks are used to create ratios for relative changes between samples, rather than be analysed in seclusion (Morris & Mandair, 2011). The phosphate to amide I (where phosphate is taken at the single highest intensity at 959 cm^{-1} and amide I uses the whole band average or absolute height of the 1616-1720 cm^{-1} region), gives an indication of the amount of mineral relative to the amount of collagen. Other bands that have been studied include amide III (1243-1320 cm^{-1} (Kavukcuoglu, Patterson-

Buckendahl, & Mann, 2009), carbonate 1070 cm^{-1} (Akkus et al., 2004) and CH$_2$ at 1450 cm^{-1} (Yeni, Yerramshetty, Akkus, Pechey, & Les, 2006).

Raman spectroscopy has been used extensively to study bone material, with several studies confirming links between mechanical properties and the ratios of mineral to organic component in bone across a range of diseases (Akkus et al., 2004; Imbert, Aurégan, Pernelle, & Hoc, 2014; Raghavan et al., 2010). It has shown directly the changes that occur with bisphosphonate treatments in osteoporotic tissue (Olejnik et al., 2014), as well as analysing bone tissue which has been altered due to certain proteins (such as osteocalcin) being deactivated through knockout mice (Kavukcuoglu, Denhardt, Guzelsu, & Mann, 2007; Seto et al., 2012). Similar to FTIR, it has been applied in-vivo with the use of fibre-optics to deliver the laser to good effect, however due to laser safety requirements to protect the patient a low power of 30mW within a 3.5mm diameter spot is used which does affect the quality of spectra acquired and any diagnosis as a result (Wang, Lee, Lui, McLean, & Zeng, 2013).

SORS (Spatially Offset Raman Spectroscopy) is a relatively new development whereby Raman scattering is collected at a depth by offsetting the excitation point and the collection of the Raman spectra, relying of the fact that most materials will allow photo migration through tissues. This has been used in in-vivo studies to look at human bone beneath soft tissue (Buckley et al., 2014). Bone is an extremely heterogeneous material and looking at it at a slight depth, of even a single millimetre, will yield different results, so care must be taken to achieve the same depth during scanning. SORS has the advantage over a traditional Raman microscope equipped machine as it has the benefit of using a non-polarised light source, and therefore isn't vulnerable to changes based on the orientation of sample (Morris & Mandair, 2011). It is also a non-ionising technique, and therefore has the potential to become a widespread diagnostic tool, as it employs relatively short scan times, is non-invasive, and carries minimal risk to patients.

2.4 Mechanical and Material Testing
Mechanical testing involves taking into account the architecture of material during assessment, as well as its composition. In the case of bone this has resulted in a myriad of instruments and methods that apply force to whole bones and observe its behaviour under stresses. The goals of this type of measurement are to assess bone in its native form, recreating how it would react if these stresses were present in nature (Donnelly, 2011). Typically a bone is placed under compression, torsion or bending, and loaded with increasing force until the material is not able to withstand the force and breaks (Van Der Meulen, Jepsen, & Mikić, 2001). The outputs of this kind of test can describe the structural

stiffness, a measure of elasticity and plastic deformation, both by measuring compression as a function of force, and observing creep effects as the material reforms following compression. It can also give a functional measure of strength through the total amount of energy required to bring the bone to fracture, or the maximum force required to do so, which can also be assessed through the amount of energy or force to bring it past the stage of elastic deformation, where permanent damage is done. This type of testing is inherently destructive and has the additional drawback of comparisons requiring contralateral counter parts in studies that seek to demonstrate the change in mechanical properties due to implants, or requires very large sample sets to overcome the natural differences in a population (Martin, Foulonneau, Turki, & Ihadjadene, 2013).

Materials testing seeks to observe the bone material at a particular location as a single entity, making it ideal for comparison to other techniques that focus on small areas, as the heterogeneous nature of bone allows its composition to differ significantly over short distances. The goal of assessing a small area of material is to either use it as a marker for whole bone strength, where the characteristics of the material can be extrapolated or associated with larger scale mechanics for clinical use (Ding et al., 2011), or to compare directly the properties of the material and local chemical composition to give insight into how chemical change impacts material properties (Chatzipanagis et al., 2016).

<u>Three Point Bending</u>

A simple application of mechanical testing comes in the form of the three point bending test, the most commonly used measure of whole-bone strength. A bone is placed on two static points to support it at either end, usually a short distance from the epiphyseal plate in long bone, and is subject to a singular compressive force from a third point directly in between the two supports as in figure 2.6 (Salim, Salleh, & Daud, 2016). This method creates the largest strain directly at the point of contact, dictating that the fracture will occur at the point of loading. An alternative method involves a four point test, whereby two prongs deliver the force, with the advantage that the load experienced by the bone is all in a single direction, whereas a single source results in shear forces (Sharir, Barak, & Shahar, 2008). However despite this advantage three point tests are more commonly used due to the geometric limitations of bone as two prongs require a level surface to interact with. While an accurate measure of ultimate bone strength, and a very good indicator of risk of fracture, due to the destructive nature of the technique it cannot have in-vivo applications. It has however provided information on a range of topics for example: the impact of micro-cracking on bone strength (Carriero, Zimmermann, Shefelbine, & Ritchie, 2014), genetic factors (van Lenthe, Voide, Boyd, & Müller, 2008), the effect of water content on bone mechanics (Libonati &

Vergani, 2014), and comparisons with other techniques looking for markers that indicate whole bone strength without the destructive element (Carriero, Bruse, et al., 2014).
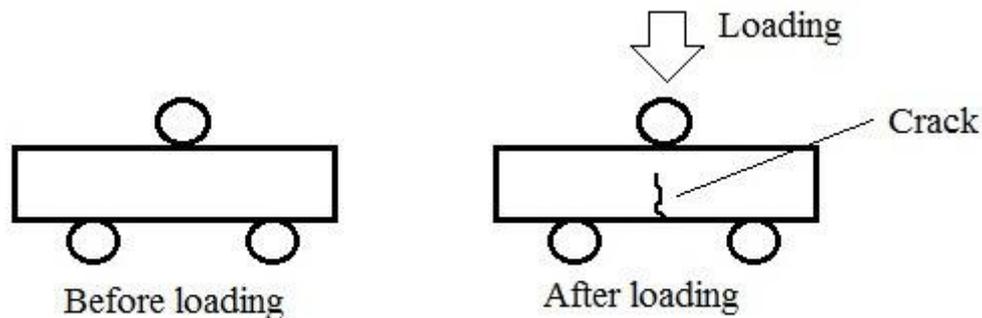
Figure 2.6 – The three point being test on a stiff object (Prabahar, Dhanya, Ramasamy, & Dhanasekar, 2017)

<u>Indentation</u>

Indentation is the use of a compressive force against a material on a flat surface whereby the only mechanical aspect being measured is the direct compression of that material. It is often coupled with the use of a strain gauge, which measures the amount of deformation that occurs in relation to how much strain the material experiences. Young's modulus is a common measurement of the relationship between stress (force applied over area), and strain (deformation) given in units of pressure. This measurement has been widely used on bone material (Currey, 2002), however requires destructive preparation to certain proportions as the bone must be flat in both the dimension the force is applied to as well as the surface it is resting on, which creates some problems in both the fact that the surface has been altered via the machining and polishing, as well as the location tested on the bone being therefore difficult to replicate (Havaldar, Pilli, & Putti, 2014). Indentation has been compared to spectroscopic measurements of bones from different species, indicating a link between the ratio of mineral to collagen as described by Raman spectroscopy, and Young's modulus calculated from indentation techniques (Buckley, 2011).

A single measurement is usually comprised of a number of indentations with the same amount of force in the same location. This is for two reasons:

- Heterogeneous material such as bone will react differently during the initial indent than subsequent indents, also achieving less depth as each additional indent has more material to compress.
- The material can be observed to have two separate values for toughness, the initial deformation from the first cycle, and the remaining distance by subsequent cycles.

The former is descriptive of the fragility of the material, while the latter is a better measure of material strength, with Young's modulus usually being calculated from one of the last cycles (figure 2.7) (Kann, Bergink, & Beyer, 2002).
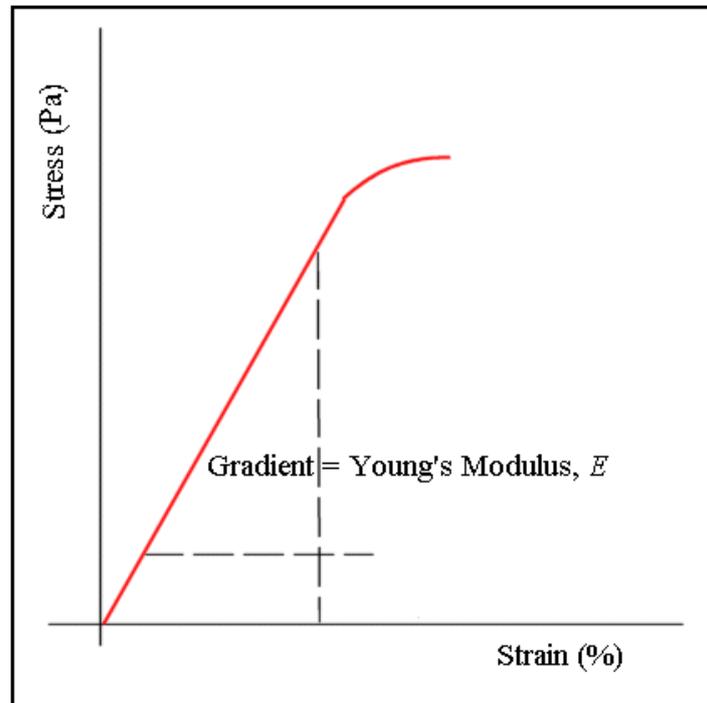
Figure 2.7 – The calculation of Young's modulus (Kann et al., 2002)

Biodent – Reference Point Indentation

Reference point indentation is a similar technique which measures bone properties on the micro-meter scale. A reference probe, which houses a test probe internally, is pressed against the surface of the object to be measured to create a reference point. Once the reference probe is against the material with sufficient pressure applied to maintain full contact, the internal test probe applies force to the bone and measures its reaction. The probe measures indentation distances over a series of cycles, testing the strength and stiffness of the material. Between cycles it can also measure elasticity as it holds position to observe creep effects (figure 2.8).
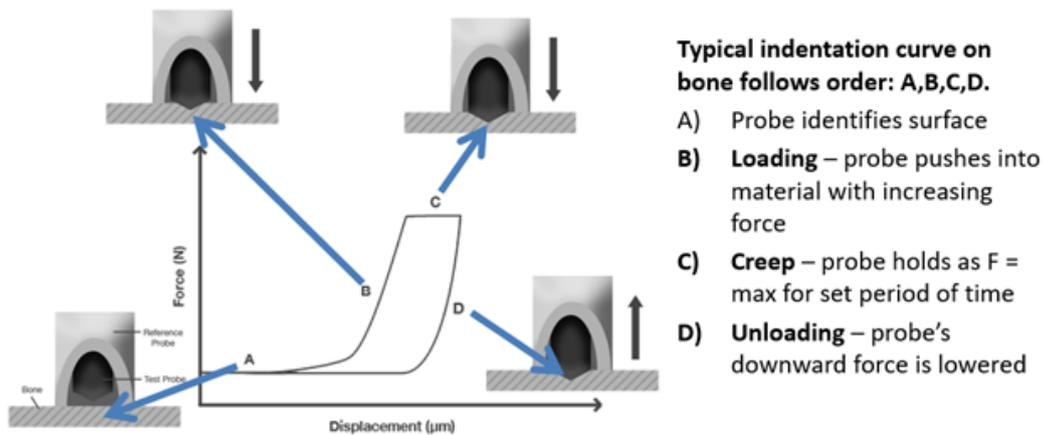
Figure 2.8 – The Biodent indentation cycle (Active Life Scientific, 2018)

The instrument collects and calculates a number of metrics based on the loading and unloading of the material being tested (figure 2.9). However only a handful have been successfully correlated with material properties in the literature. The first cycle indentation (FCI), which is demonstrated as 'Cycle 1' in figure 2.9, is a measure of hardness (Gallant et al., 2013). The indentation distance increase (IDI) is a measure of the distance indented between cycles 2 and 10, and is the most commonly cited measurement as it is inversely related to toughness, strength, and energy to failure (Active Life Scientific, 2018; Carriero, Bruse, et al., 2014). Creep is a measure of the plasticity of bone, the indenter is held for 1/3 of the duration of the indentation to observe how the material behaves after loading (Active Life Scientific, 2018). The total indentation distance is a measure of overall bone quality and its ability to resist deformation (Active Life Scientific, 2018).
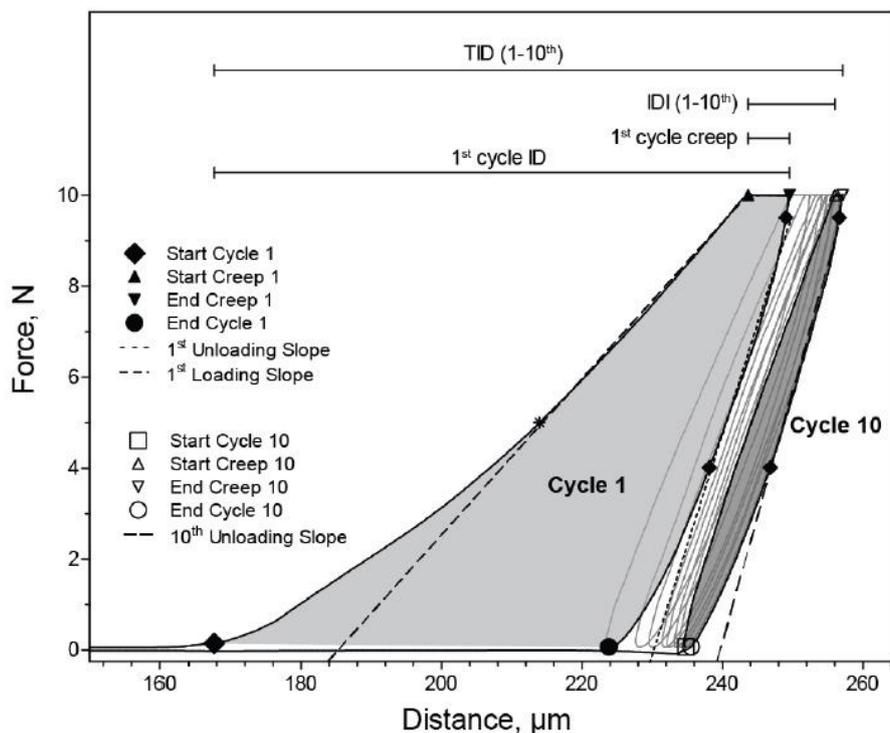
A measurement is taken by placing a sample to be tested, securely so that during indentation it will not move, and testing must occur on a surface that will not deform under the pressure of the Biodent. The surface need not be completely flat, but it ensures a firm grip on the material. The Biodent instrument is not able to be calibrated, however a known block of plastic can be indented first to ensure uniformity of measurements. The resulting measurements do not require pre-processing other than averaging a series of measurements at the same location.

Studies have confirmed links between indentation distance increase from repeated cycles and bone fragility (Ding et al., 2011). Given the nature of bone heterogeneity up to 8 repeats are needed in an area to get a reliable average (Jenkins et al., 2015). Micro-indentation provides a mostly non-destructive approach to measuring mechanical properties although micro-cracking does occur. Although comparisons to micro indentation have been made which suggest the Indentation Distance Increase is inversely comparable to Young's moduli (Gallant et al., 2013), no study has been able to compare the same exact locations on bone as both methods are destructive. Contralateral bones have been used in similar locations but as bone material varies comparisons must be approached carefully (Carriero, Bruse, et al., 2014).

2.5 Chemical Quantification

This section describes the chemical techniques used to quantify aspects of bone composition. Rather than arrange it by technique it has been ordered by the constituent they attempt to quantify.

Mineral Content

The simplest and most accurate method is derived from the process of ashing – heating bone in the presence of oxygen until only the heavy inorganic components of mineral remain which has been practised for almost 100 years (Ptáček, 2016; Shear & Kramer, 1928). However this method is entirely destructive of the sample. Therefore a variety of methodologies have evolved to estimate mineral content either without removing it, or by a gentler process of removal to allow for analysis of the organic components of bone whilst assessing mineral content through the lost weight.

Semi-destructive methodologies, most commonly used by histo-pathologists in the health service include nitric, formic and perchloric acids (Dreyer, 1965; Palma, Rocha, Valadares Filho, & Detmann, 2015). These strong acids have the advantage of very quickly removing the mineral content of bone material for fast analysis of biopsies, but the drawback of

damaging the morphology of the organic components of bone, although significant progress has been made in adapting strong-acid techniques to do as little damage to the organic phase as possible (Gupta, Jawanda, Sm, & Bharti, 2014; Sangeetha, Uma, & Chandavarkar, 2013). An alternative to strong acids are chelating agents such as Ethylenediaminetetraacetic acid (EDTA) which bind to mineral in order to remove it, without damaging the collagen structure (Kiviranta, Tammi, Lappalainen, Kuusela, & Helminen, 1980). EDTA is a much slower method of removal, and is not well suited to clinical environments that require a quick turnaround. Several studies have analysed the viability of cells for histology (not viability) and particularly genetic material following demineralisation methods and have found EDTA to preserve these features over other methods making EDTA the best choice for research methodologies (Alers, Krijtenburg, Vissers, & Van Dekken, 1999; Choi, Hong, & Yoon, 2015).

Alternative methods involve computational analysis of bone mineral density scanners. One method applies the information gained from a CT scan to estimate mineral content with a 0.97 r value of correlation (Reich et al., 1976), but has not been widely adopted due to the expense of the equipment required. However it is possibly to apply the general approach to the DEXA scan which is more commercially available, although the technique is less accurate due to the limited angles of x-rays not providing a full picture of the bone in question (Lloyd & Eggli, 1992).

<u>Collagen Content</u>

The quantity of collagen in a section of bone can be estimated once the mineral component has been removed. This is a destructive method but can be performed on a small homogenised subset of a sample. The collagen must be enzymatically digested, hydrolysed, typically in HCl over 24 hours at 110°C, and then can be assayed photometrically once tagged by a molecule of choice (Kliment, Englert, Crum, & Oury, 2011; Reddy & Enwemeka, 1996). An issue with this method is that it specifically measures the content of hydroxyproline, a common amino acid found in the repeating chain of type-1 collagen, and therefore the content of hydroxyproline found has to be multiplied by approximately 14 to estimate collagen content. Some tissues however will display different concentrations of hydroxyproline, with the potential to invalidate the estimation. Alternative methods include infrared spectroscopy which can identify a host of collagenous peaks and has been developed as an in-vivo tool for assessing skin collagen in reconstruction surgeries (Zhao et al., 2017). An alternative to infrared is Raman spectroscopy, however while this method can currently provide a ratio of mineral to collagen, it has not yet formed a connection to actual values but is likely to be a viable option in the future (Nyman et al., 2011).

Collagen Cross-linking

Cross-linking has long been a source of interest clinically, with non-invasive analysis of urinary concentrations of mature cross-links such as pyridinoline being linked to connective tissue disorders (Pasquali, Dembure, Still, & Elsas, 1994; Steinmann, Eyre, & Shao, 1995). As mature cross-links are non-reducible, when bone material is replaced they are excreted in urine and can be used as markers of turnover, or evidence of disease through the ratio of lysyl pyridinoline and hydroxylysyl pyridinoline. These cross-links can also be extracted from tissues by means of collagen digestion and hydrolysis as described in the liberation of hydroxyproline for analysis in the section above. However as the quantities of cross-links are much smaller than the most prevalent amino acid in collagen they require a different approach to quantification.

High performance liquid chromatography (HPLC) is a method by which a number of substances can be separated using different solvents and columns under high pressures, with a very sensitive detector able to address findings as low as 0.02pmol (10^{-12} moles) (Waghorn, Oliveira, Jones, Tager, & Caravan, 2017). It has been used to elucidate levels of cross-linking in a range of connective tissues, including bone (Hector, Robins, Mercer, Brittenden, & Wainwright, 2010; Yoshida et al., 2014). The constituents of a sample are separated based on weight and charge, as well as attraction to both solvents and areas of the column, the packing material of which acts as a molecular sieve, retaining certain molecules for different lengths of time. While there are other techniques that can assess these cross-links, the sensitivity offered by HPLC is necessary for accuracy (Eyre, Weis, & Wu, 2008).

Post Translational Modifications

A stratum below cross-linking are the post translational modifications of collagen (PTM), which are the immediate chemical changes made before the formation of pre-procollagen. Chief among these is lysyl hydroxylation, whereby the enzyme lysyl hydroxylase modifies lysine residues to form hydroxylysine, a critical component in collagen-cross-linking. Another important aspect is the hydroxylation of proline to hydroxyproline, critical to the folding of the collagen triple helix. These modifications are critical to the viability of collagen and its ability to become successfully mineralised and contribute to bone strength (Yamauchi & Sricholpech, 2012). In order to observe these changes bone material must first be put through the above processes, reducing it to its hydrolysed from, and then separated into its constituents where possible. Some molecules, including certain PTMs are difficult to detect, due to the nature of the molecule either not fluorescing, reacting to UV stimulation or being easily derivatised with a tagging chemical.

Mass spectrometry is a technique which relies on the bombardment of a sample with electrons to induce ionic charges in the molecules, and can also break them into smaller fragments. These charged molecules are then passed through an acceleration chamber and an electric field which attracts the charged molecules at differing rates depending on their mass to charge ratio. With this information a very accurate quantification of mass of a certain molecule in a sample can be calculated and complex structures identified. Mass spectrometry has been used to characterise the structure of thousands upon thousands of unknown molecules and plays a very large role in new drug discovery (Glish & Vachet, 2003) and analysis of the PTMs of collagen (Yang et al., 2012). The post translational modifications of collagen are not easily separated for spectral analysis, as they are not readily identifiable using HPLC without modification. However other chromatographic methods such as SDSPage on a gel allow for the separation and staining of the constituents of collagen by weight and charge, which can then be physically cut from each other for further analysis (Wu et al., 2011). Once separated techniques such as mass spectrometry can more easily identify them.

2.6 Summary

This chapter has described various techniques that have played a large role in defining the current literature on bone organisation, composition, and diagnosis of pathology, as well as techniques that are used in this thesis. It has demonstrated that the complex nature of the chemical construction of bone requires a varied approach to understand its many facets, and that an analysis of bone material from every angle is a challenging task.

The following five chapters lay out the experimental work undertaken in this thesis, using a host of techniques described here, in the pursuit of gaining a better understanding of bone material and contributing to the pool of knowledge which enables the development of in-vivo techniques and treatments.

Chapter 3 – The Relationship between the Mineral to Collagen Ratio and Bone properties from a Single Species

3.1 Introduction

Bone Composition

It is well known that the properties of healthy bone tissue vary according to the proportion of collagen to mineral present. This being essential in nature due to the different forces acting on them creating unique and specific mechanical requirements (figure 3.1).
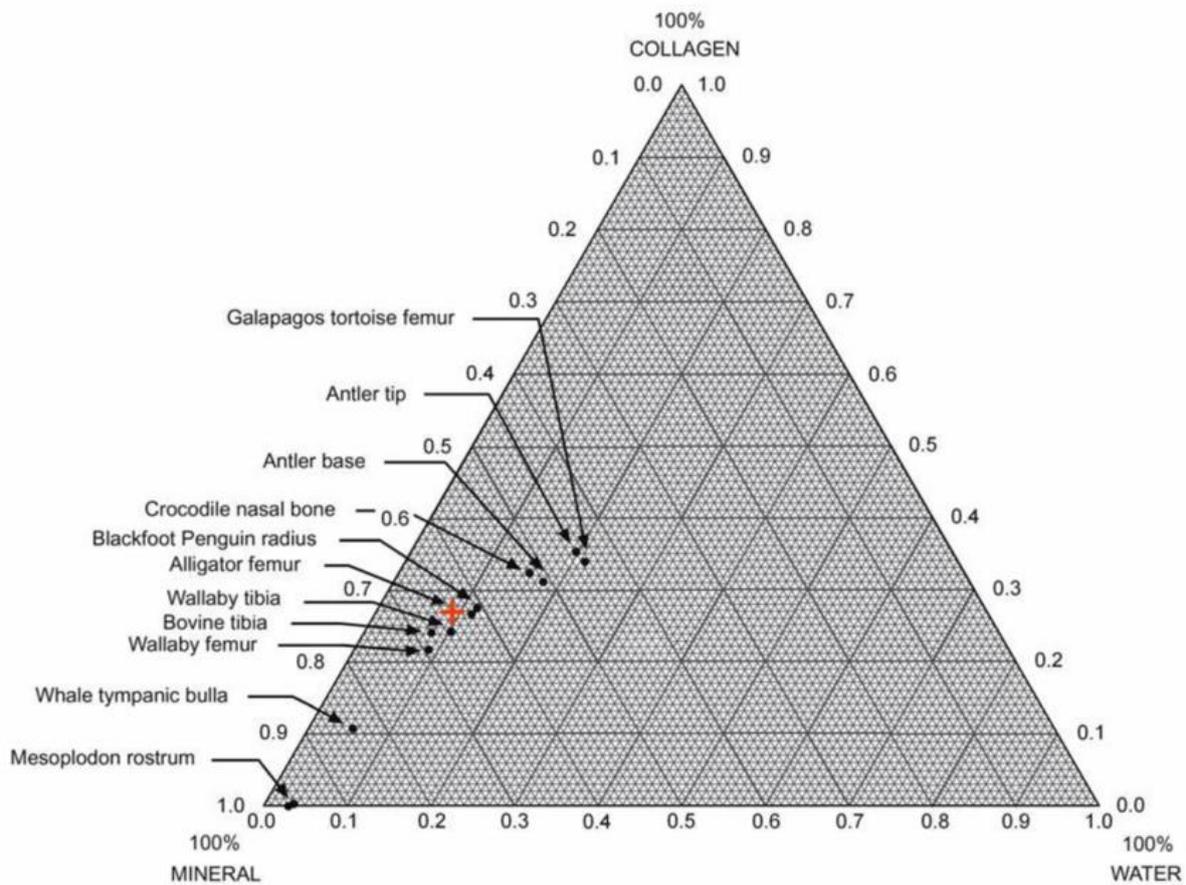
Figure 3.1 – The relative proportions of collagen, mineral and water in a range of species (red cross is a human femur) (Turner-Walker, 2012) adapted from (Zioupos, Currey, & Casinos, 2000)

There is a body of literature that has shown that mechanical properties of bone, either in the form of Young's modulus (a measurement of the ability of a material to withstand changes in length when under compression) or force required to fracture, correlate with the ratio of mineral to collagen, or with pure mineral content (Buckley, Matousek, Parker, & Goodship, 2012; Dalén, Hellström, & Jacobson, 1976; Kotha & Guzelsu, 2003; Leichter et al., 1982). However it is also known that the collagen component of bone is essential to a healthy functioning material due to both the effects of diseases which affect collagen formation genetically such as osteogenesis imperfecta (Shapiro, 2013), as well as diseases such as Bruck's syndrome which arise from disrupted cross-linking (Bank et al., 1999).There are also

significant changes to collagen cross-linking with regards to age-related osteoporosis and osteoarthritis, in which the cross-link pentosidine is found in much higher levels and associated with fracture risk (Vaculík, Braun, Dungl, Pavelka, & Stepan, 2016). In one study, it was shown that the ratio of HP:LP, two pyridinoline cross-links of collagen, correlated strongly with material properties independent of bone mineral density (Banse, Sims, & Bailey, 2002), while in another this ratio did not produce the same relationship (Follet et al., 2011). While the evidence is somewhat conflicting, it stands to reason that since large-scale changes in collagen cross-linking result in diseased tissue with weakened mechanical properties, more subtle alterations should impact these properties.

Materials Testing

As outlined in chapter 1, the bone mineral density of a patient is not an accurate predictor of mechanical properties, therefore this section aims to explore the concept that bone properties are affected by subtle modifications to the chemistry of type-1 collagen. To properly assess the impact of chemically different collagen, it is ideal to have a spread of material composition, i.e. bone with a large proportion of collagen, and highly mineralised bone. It is also desirable to obtain all bones from a single species, to rule out differences associated with the use of material adapted for slightly varied function, and genetic features unique to species and age related differences. Therefore this chapter utilises three distinct bone types from a single species of deer, described further in the methods section. Table 3.2 (Martin, 2003) depicts comparable materials and their respective properties, which validates the selection of materials chosen. This study is less concerned with the energy to fracture, a metric which measures the toughness of bone over a three-point bending test (Oksztulska-Kolanek, Znorko, Michalowska, & Pawlak, 2015), as it takes into account the architecture of the material. To effectively compare the chemical and material properties, macro-scale indentation techniques provides us with the Young's modulus of elasticity, a gauge of stiffness measuring stress and proportional deformation (Truesdell, 1960).

Properties	Antler	Femur	Bulla
Young's modulus of elasticity	7.4 GPa	13.5 GPa	31.3 GPa
Energy to fracture	6190 J m^{-2}	2800 J m^{-2}	20 J m^{-2}

Table 3.2 - data taken from Martin (2003) showing the material and mechanical properties of bones in red deer adapted for different function comparable to those used in this study

Young's modulus has been shown to correlate with the ratio of mineral to collagen across a range of species and extremes of bone material (Buckley, Matousek et al., 2012), however macroscopic indentation techniques have the major drawback of operating on a large area, typically on a polished or cut surface as it must be performed on a flat section. This means the surface has been altered from its natural state and that the technique is less suitable for

testing small or heavily curved materials such as the bulla of the red deer. It also means comparisons have to be made across larger areas, due to the size of the indenter, whereas it is known that the organic composition of collagen can differ at least down to the millimetre scale (Buckley et al., 2014). To better understand how the composition of bone provides the necessary adaptation we must look at alternative means of measuring its properties.

Micro-indentation mimics traditional mechanical testing on the micron scale, allowing local chemical changes to be compared directly to the bone properties in the same small area. Studies using micro-indentation have shown that it is capable of discerning between controls and osteoporotic patients (Adolfo Diez-Perez et al., 2010; Ding et al., 2011), and indentation values have been significantly correlated with fracture toughness separately in both mice femora and vertebrae (Gallant et al., 2013), however, in isolation it is not yet a predictor of mechanical properties (Allen, McNerny, Organ, & Wallace, 2015; Carriero, Bruse, et al., 2014). Micro-indentation was also selected as it has in-vivo applications and furthers the goal that this research contribute to better use of diagnostic tools in medicine (Herrera & Diez-Perez, 2017). This study utilises both indentation methods to firstly confirm that the bone material is typical of that found in the literature by comparing the Young's modulus of the antler and metacarpal to similar bones, and micro-indentation to collect various metrics on a scale more easily comparable to the other techniques used.

Mineralisation

As much as is known about the mineralisation process has been described in the introduction, yet some aspects of exactly how mineral is distributed and nucleates is unclear. However it is known that, for example, osteoporosis is associated with high pentosidine concentrations, and involves the loss of bone mineral density. This could implicate the cross-link pentosidine in the mineralisation process, but it is unknown whether or not it is a part of a greater mechanism that alters collagen chemistry or simply a by-product of another process. One set of modelled theories on the initial deposition of mineral rely on the electronic arrangement of the collagen scaffold, with attracting charges on the triple helix enabling the fibrillar nucleation of hydroxyapatite in gap regions (Boivin et al., 2009; Habraken et al., 2013). This would suggest that modifications to the charges on a collagen fibril, potentially by cross-linking, would alter the number of available nucleation sites for mineral, thus modulating the rate of mineralisation. Therefore one aspect this chapter will focus on is whether there are any links between levels of mineralisation, and specific modifications to type-1 collagen.

Hypothesis

Bone with different mechanical requirements shows variation in the collagen to mineral ratio and related changes in organisation of the mineral within the bone tissue and chemical characteristics of the collagen component.

Objectives

1. To determine the physical properties of bones selected from the deer to represent a diverse range of stiffness requirements (antler, metacarpal and bulla).
2. To quantify the collagen and mineral content in the deer antler, metacarpal and bulla and examine the relationship with material stiffness and toughness.
3. To examine the distribution of mineral within the collagen scaffold of deer antler, metacarpal and bulla bone.
4. To explore the collagen chemistry in the antler, metacarpal and bulla bone using Raman spectroscopy.

Biologically Adapted Bone

For this study bones from the red deer (*Cervus elephus)* are used, these provide different biological function and are expected to have a large difference in chemical composition. Each sample came from a male deer, of approximately 2-3 years of age, with no known health defects having been slaughtered for their meat.

They are as follows:

- Metacarpal – Unlike in humans, the metacarpal of the red deer is a single long bone adapted to provide load bearing and stability, providing an elongated limb ideal for high speed locomotion. This type of sample provides the most commonly conceived 'standard' bone, with a thick outer cortical layer. Not only must it be tough enough to support the deer during running speeds, where considerably more stress is put on the bone, without fracture, but it must also be stiff enough to resist permanent deformation (Currey, 1979).
- Antler – Primarily used for fighting, the antlers of a deer must be extremely tough and flexible to resist fracturing during high-force impacts (Currey et al., 2009). This has led to a material with a lower mineral content than 'standard' bone to better absorb shock without fracture (Kierdorf, Kierdorf, & Boyde, 2000). Antler also provide another observable angle in that they are grown and shed yearly, as some known alterations to collagen occur with the age of the material, rather than age of the animal, such as accumulation of pentosidine (Paul & Bailey, 1996).
- Bulla – This is a skull bone designed for acoustical vibration, to augment the sound by creating an echo chamber with presumably highly mineralised walls as found in

whale bulla (Currey, 1999). To achieve its function, bones of this type are optimised to receive and transmit auditory vibrations, leading to a very brittle structure. The bulla is located on the base of the skull, directly below the entrance to the auditory canal.

These bones from this animal have been chosen for this study as they represent a variety of levels of mineralisation, likely to be among the highest within a single species due to the uniqueness of antler material. Therefore, the bulla and antler are used as extremes of the mineral spectrum, both of which also have very niche biological functions to enhance the discussion of their chemical makeup. The metacarpal was chosen as an example of standard load bearing bone due to its size, availability (as the femur is sold with the meat of the deer), and previous successful use in a Raman study (Sowoidnich et al., 2017).

3.2 Materials and Methods

The bulla, metacarpal and antler were acquired from a farm (Welsh Venison Centre). The bullae (n=10, 2 from each skull, left and right) were excised from the heads using a diamond blade saw, soft tissues were removed by scalpel. The metacarpals (n=6) were cut at the joints and soft tissue removed. Antlers (n=6) were cleaned of any tissues and debris. All samples were stored at -80°C and were subject to a maximum of 3 freeze-thaw cycles during the analysis process to avoid artefacts from repeated freeze/thawing (McElderry, Kole, & Morris, 2011). The bulla measurements were split into two groups; the bulb, and the base where it connects to the skull, as the locations appeared to differ in morphology. Deer samples were collected from a venison farm from animals euthanized at a commercial abattoir. Under the Animal (Scientific Procedures) Act 1986, Schedule 2, these samples are not deemed as scientific procedure and are not subject to additional ethical processes. However, the use of animal by-products was registered with the Animal and Plant Health Agency.

Chemical Analysis

A representative section of material, approximately 2cm x 2cm x 1cm, was taken from each sample at the locations scanned and indented, three per antler and metacarpal, two per bulla (total 18 antler, 18 metacarpal, 20 bulla sites), weighed, then freeze dried and weighed again to determine water content (expressed as a % of wet weight). The samples were then cryo-milled to a fine powder for further analysis. 10% EDTA at pH 7.5 at 37°C was used to demineralise the samples over two weeks with the solution being removed by centrifuge, and replaced every two days, with approximately 10ml of EDTA per gram of bone powder. The resulting dry weight after washing with distilled water and freeze-drying the samples gave an approximation of the amount of mineral present (expressed as % of dry weight).

5mg of the demineralised powder bone was used in a modified hydroxyproline assay to assess the amount of collagen present (Reddy & Enwemeka, 1996; Stegemann & Stalder, 1967). The powder was subjected to 6M HCL at 110°C for 24 hours to release the collagen chains, which is then removed via vacuum centrifugation. Hydroxyproline is assessed through an oxidation process using choloroamine T, and then coupled with dimethylaminobenzaldehyde (DMBA) to form a coloured product that can be measured on a plate reader at 550nm. It is then compared against a known standards of hydroxyproline concentrations. Hydroxyproline appears in approximately 14% of collagen, and therefore the quantity assessed is multiplied by 7.14 to estimate the collagen present as a function of dry weight.

<u>Micro-Indentation</u>

Micro-indentation was performed using a Biodent instrument (ActiveLife Scientific, CA, USA) with a BP2 probe assembly. Samples (metacarpal n=6, antler n=6, bulla n=10, three locations per sample for antler and metacarpal, two for bulla) were thawed and during measurements were kept in phosphate buffered saline to retain hydration. Indentation was carried out using 10 cycles at 2Hz under a 10N load with eight repeats as recommended to account for natural variation (Jenkins et al., 2015). The indentations were made within a 2mm diameter of the locations measured by the Raman spectrometer, with care taken not to overlap the indentation sites. The bulla bulb was unsuitable for testing as it could not withstand the minimum pressure required for indentation. The first cycle indentation (FCI), indentation distance increase (IDI – the distance penetrated between cycles 2 and 10) and creep (permanent deformation) were chosen for analysis from the outputs offered by the Biodent as they have been previously used successfully in the literature to separate populations (Allen et al., 2015; Adolfo Diez-Perez et al., 2010; Ding et al., 2011) with IDI being considered the closest metric to bone strength (Gallant et al., 2013).

<u>Mechanical Testing</u>

The Young's modulus of the antler and metacarpal were calculated using an ElectroPulse E3000 (Instron, MA, USA). Samples (metacarpal n=3, antler n=4, three locations per sample) were cut and milled to a flat surface on six faces to create an oblong with dimensions 100mm x 15mm x 15mm, leaving as much surface material as possible. The material was allowed to thaw to room temperature and kept hydrated using phosphate buffered saline. A load of 20N was applied using a diamond tipped Vicker's indenter equipped with a strain gauge. Calculation of Young's modulus was performed using the unloading slope of the first cycle and averaging across the three locations, taken at 25%, 50% and 75% of the length of the beams. Young's modulus was calculated using

methodology derived from Oliver and Pharr (2004), where YM = Stress / Strain. Stress is defined as the force from the indenter (F), divided by the square root of the area (A) of the indenter (stress = $F\sqrt{A}$). Strain is defined as the square root of Pi divided by twice the proportion of the length of the material elongated under force (change in length (CL) divided by length (L)) (strain = $\sqrt{\pi}/2(CL/L)$).

Quantitative Computer Tomography

A QCT (XCT 3000, Stratec, Germany) was used to visualise the mineral density of the antler, metacarpal and bulla groups (n=2 per group). Scans were made at 5mm intervals (2mm for the bulla) and an average taken using approximately 50 images per sample. Density is reported in milligrams per cubic centimetre, at a voxel-size of 0.2mm.

Scanning Electron Microscopy

Images were taken using a JEOL JSM-6610 (MA, USA) using a 9mm working distance, spot size of $60d_p$ at 20kV using secondary electron imaging. Samples were broken off into chunks weighing less than 50mg, freeze dried and demineralised in 10% EDTA pH 7.5 for intervals of 0, 7, 14, and 21 days at room temperature under agitation. Samples were washed with distilled water, and freeze dried prior to being sputter-coated with a layer of gold.

Transmission Electron Microscopy

Samples were prepared using a method derived from Glauert & Lewis (1998). Samples were fixed in a 10% neutral buffered formalin solution for 48 hours at room temperature, samples were then washed with deionised water 5 times, before being subjected to multiple dehydration steps of 30, 50, 70, 90 and 100% acetone at 15 minutes each (with 3 repeats at 100%). The samples were then submerged in a mixture of propylene oxide and resin at a ratio of 2:1, for one hour, then a ratio of 1:1 for an hour, and finally a ratio of 1:2 for a final hour. Following this samples were placed in 100% resin to complete infiltration for 12 hours. A polymerising agent was added to fresh resin to set the material. The now resin embedded samples (3 per group) were sliced to 60nm thickness using an Ion Mill (Fischione 1050, USA), before being fixed onto a slide for viewing. Samples were imaged without staining (as staining in these samples lowered quality) at 60kv, at 20-40,000x magnification. The length of the collagen banding, and the fibril widths were measured using ImageJ. 1,000 measurements were taken and averaged per sample. This was done manually by selecting collaged fibrils that were visible without cross-over from other fibrils, using ImageJ's measuring tool a line was drawn across the diameter or length of a full band and a value obtained for the distance measured which was then adjusted for the magnification used.

Raman Spectra Collection

Samples were thawed at room temperature before measurements were taken. Care was taken to remove all soft tissue and periosteum from scanning locations.

The bones were analysed by a custom built Spatially Offset Raman spectrometer (SORS) from Cobalt Light Systems (Oxfordshire, UK). An 830nm near-infrared laser delivered 240mW at 80% of maximum power, to avoid damage to specimen, at a 2mm spot size. Three locations (two for bulla) were measured per sample (metacarpal n=6, antler n=6, bulla n=10), at 300 accumulations of 0.1s, repeated three times, totalling 168 spectra. The antler and metacarpal were measured at 25, 50 and 75% along the length of the anterior surface and marked adjacent in pencil for mechanical testing. The bulla was measured at the base of the structure as well as the thin protruding bulb. Unlike other Raman instruments, the Cobalt system is not susceptible to issues regarding orientation or polarisation (Buckley, Matousek et al., 2012; Chen, Stokes, & McKittrick, 2009) due to the large spot size, use of optical fibres, and lack of a polariser.

Analysis of Raman Data

All spectra were baseline corrected using a third order polynomial function. The spectra were then normalised to the phosphate peak ($961cm^{-1}$). The mineral to collagen ratio was derived by dividing the height of the phosphate peak by the average height of the Amide III peak. Other analysis was performed using the Amide III, Amide I, and carbonate bands. Inverse of full width at half the height of the phosphate peak was used to determine crystallinity (Morris & Mandair, 2011). Raman data was also analysed by principle component analysis (PCA), looking for the first 3 principle components over the spectral range of $600-1800cm^{-1}$ to visualise the loading plots and provide a scatter plot of their separation (Abdi & Williams, 2010).

Statistical Analysis

All significance analysis was performed using multiple one-way ANOVA with post-hoc Tukey's using Origin Pro (Originlab, Northampton). This was used to assess the differences between the bone groups, to see which factors separate them as materials from each other.

3.3 Results

Chemical Analysis

Bone	Mineral Mean ± Standard Deviation	Collagen Mean ± Standard Deviation	Water Mean ± Standard Deviation
Metacarpal	74.86 ± 3.84*	13.69 ± 0.95*	11.45 ± 4.20
Antler	62.96 ± 3.32*	19.05 ± 4.09*	17.99 ± 4.09
Bulla	80.18 ± 3.98*	6.08 ± 1.40*	13.75 ± 2.65

Table 3.3 – The mineral, collagen and water contents for each bone type, normalised to 100% *indicates statistical significance P<0.05, showing that all bone types have significantly different composition in terms of mineral and collagen content, but not water

Table 3.3 shows the mineral, collagen and water contents of each bone type, normalised to 100%. The collagen and mineral content for each bone was significantly separated from each other group. The bulla has the most mineral and least collagen, while the antler has the least mineral and most collagen, with the metacarpal in between the two. These values are as expected for these bones and confirm that they are suitable material for further analytical techniques.

Micro-indentation and Mechanical Testing

Statistically no metric was individually capable of separating the three material groups from each other using micro-indentation. The antler was most easily penetrated in both the initial and subsequent cycles but also recovered the largest amount of deformation. The metacarpal and bulla groups had very similar values despite having a large difference in mineral and collagen content (table 3.4).

Bone	FCI (μm) ± Standard Deviation	IDI (μm) ± Standard Deviation	Creep (μm) ± Standard Deviation
Metacarpal	72.15 ± 4.60	8.47 ± 0.94	1.90 ± 0.21
Antler	109.74 ± 15.00*	14.45 ± 2.51*	3.23 ± 0.58*
Bulla	73.76 ± 8.53	9.45 ± 1.30	1.85 ± 0.25

Table 3.4 – The micro-indentation metrics for each bone type, *indicates statistical significance of separation from other groups P<0.05

Young's modulus was calculated for the antler (7.83 ± 2.32) and metacarpal (14.86 ± 4.55).

<u>Comparison of Composition and Bone properties</u>

Figure 3.5 below shows the relationship between the average IDI and average mineral content taken from the same tissues. As demonstrated in table 3.3, the mineral contents of the antler, metacarpal and bulla groups are significantly separated, however the indentation distance increase average for metacarpal and bulla have a large degree of overlap.
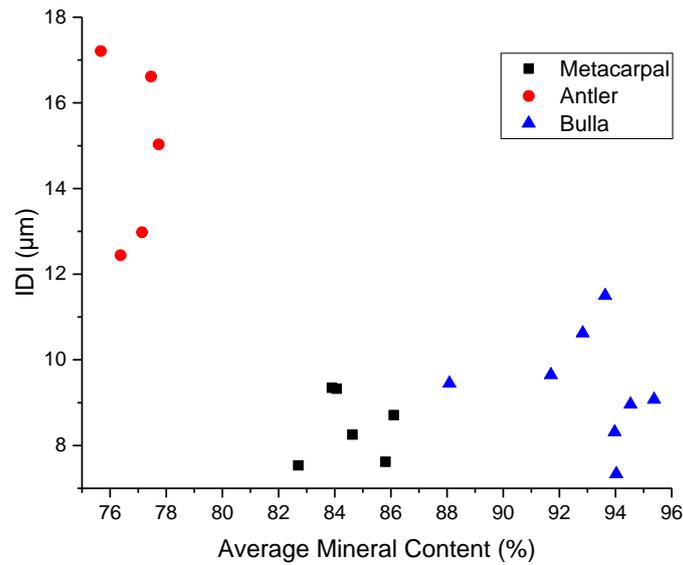
Figure 3.5 – Dry mineral content plotted against the IDI micro-indentation value

Using Raman data taken at each point of indentation we see a large overlap between the metacarpal and bulla groups in terms of the mineral to collagen ratio not represented in the chemical analysis, but observe the same trend in indentation distance increase (figure 3.6).
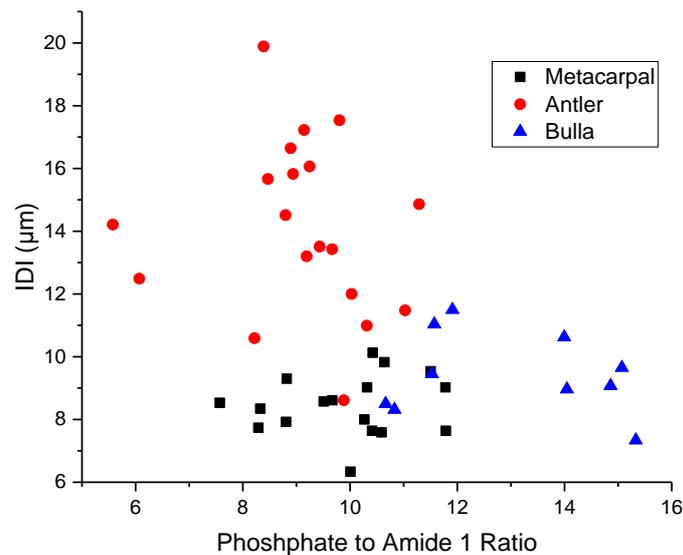
Figure 3.6 – The mineral to collagen ratio against indentation distance increase for all samples

It is also clear that the antler group has a very large vertical variation in indentation distance without a corresponding change in the mineral to collagen ratio.

Quantitative Computer Tomography

	Antler1	Antler 2	Bulla 1	Bulla 2	Metacarpal 1	Metacarpal 2
Mean	962.6	814.7	1003.8	1257.0	1090.5	1122.0
Standard Deviation	87.0	69.8	150.0	114.4	205.9	200.3

Table 3.7 – Reported average densities of each bone in mg/cm^3

QCT revealed that both the metacarpal and bulla groups have a similar average mineral density (table 3.7). The antler was the least dense of the group and contained multiple concentric circles of differing density, suggesting a manner of organisation with clearly defined start and end points for differing levels of mineralisation. Due to the voxel-size of the QCT operating on the millimetre scale, it is not possible to directly compare the density to indentation undertaken on the micro-scale.

Scanning Electron Microscopy

Figure 3.8 shows three typical images taken from each location at two to three weeks of demineralisation.
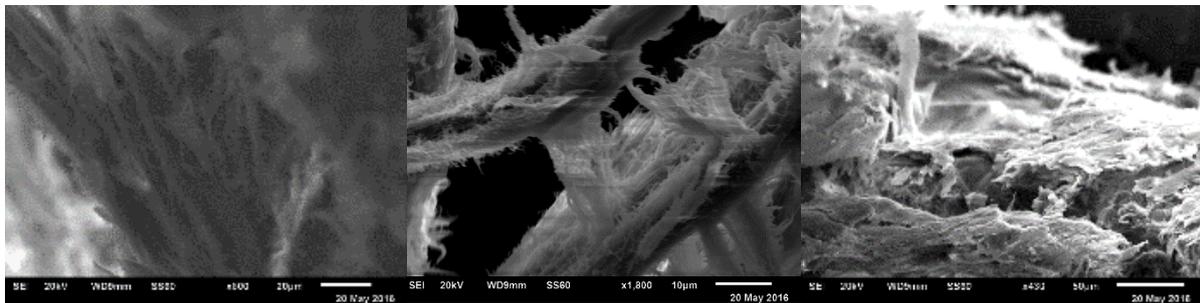
Figure 3.8 – The antler (left) has highly aligned fibres while the metacarpal (middle) is less ordered, the bulla (right) is chaotically ordered where collagen was visible.

Metacarpal displays perpendicular branching of collagen fibres regularly throughout the structure which both the antler and bulla lack. Images from day 0 and day 7 (amount of time exposed to EDTA) showed too much surface mineral to observe any visual differences in collagen. Samples from the 14 and 21 day groups contained fully visualised collagen, however much of it appeared still semi-plated in mineral (figure 3.9).
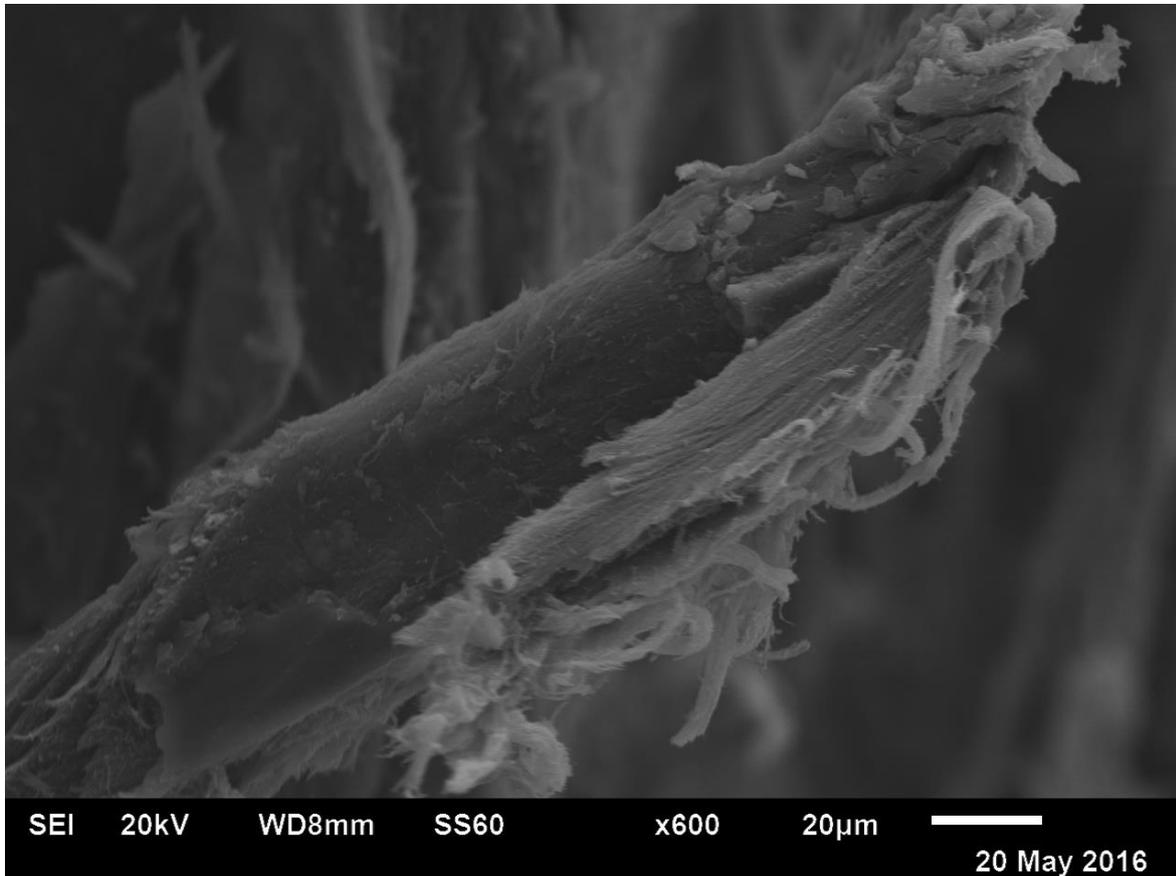
Figure 3.9 – Partially mineralised collagen fibre

Transition Electron Microscopy

Bone	Mean Fibril Width (nm)	Standard Deviation	Mean Band Length (nm)	Standard Deviation
Bulla	106.7	11.1	55.4	17.5
Antler	105.1	6.9	58.7	12.1
Metacarpal	117.6*	16.6	46.7	25.3

Table 3.10 – Mean values for fibril length and diameter *indicates statistically significant separation from other groups using one way ANOVA $P<0.05$

TEM revealed that the average fibril diameter of the metacarpal group was significantly wider than the bulla and antler groups (table 3.10), as well as the average length of the banding region being shorter, although there were large amounts of variation across all groups in both categories. Figure 3.11 depicts a typical image of an antler from which measurements were taken using ImageJ (National Institute of Health).

Sample 2 11.tif
2 Ant
Print Mag: 38900x @ 7.0 in
12:37:21 19/05/17

500 nm
HV=60.0kV
Direct Mag: 19500x
EASTMAN EM LAB
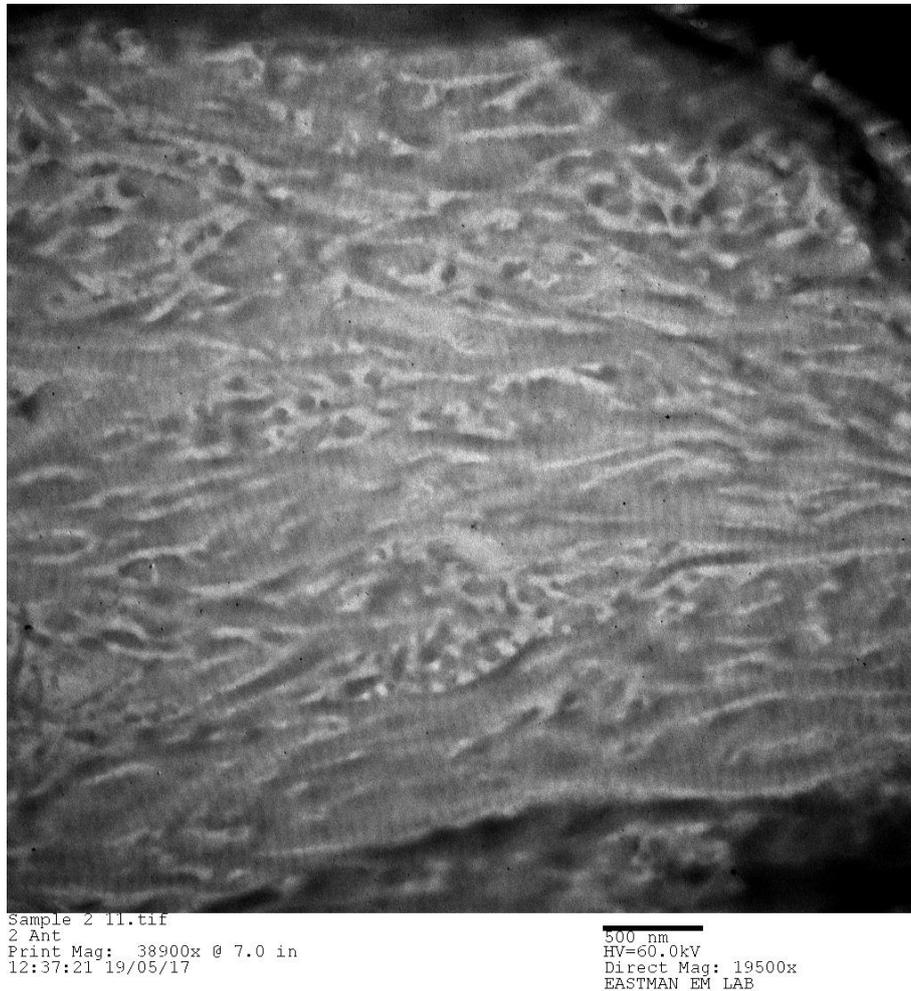
Figure 3.11 – A TEM image of antler material

Raman Spectroscopy
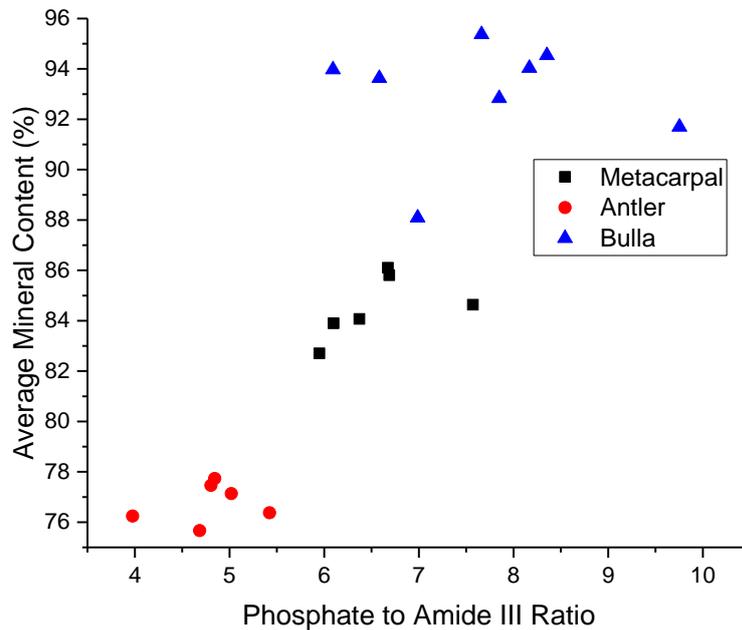
Figure 3.12 – The mineral to collagen ratio for each sample as compared to actual mineral content

Figure 3.12 displays the relationship between the mineral to collagen ratio as ascertained by Raman spectroscopy compared to the actual dry weight percentage of mineral. The mineral to collagen ratio showed a good correlation with the percentage mineral content measured biochemically. This was measured to confirm that the mineral to collagen ratio is representative of the actual quantities of mineral in the bone materials, and was calculated using the average measurement of each sample.
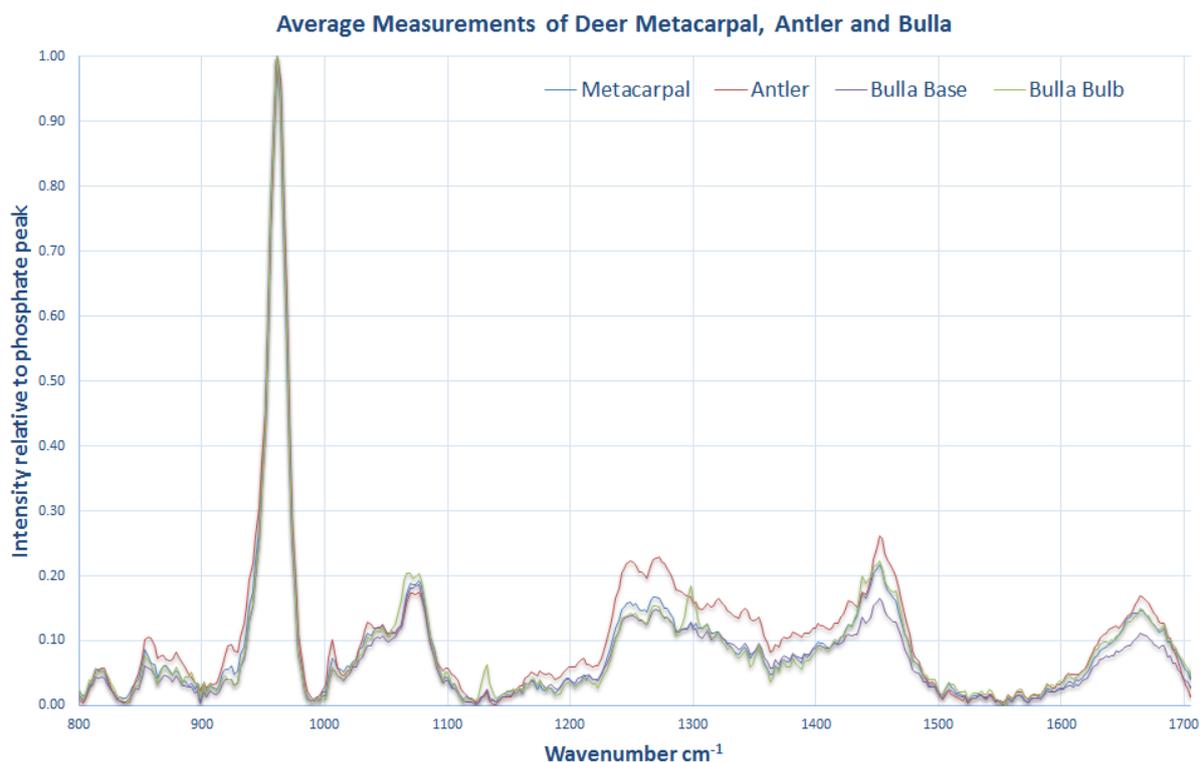
Figure 3.13 – Average spectra for each group

Figure 3.13 displays the average Raman spectra across each group, including the bulla bulb. Spectra from each material display differences in the amide I and amide III regions. This is not only due to the amount of collagen present, as the spectral bands vary in shape, suggesting that they differ chemically as well. The metacarpal in particular has a low amide III value but a much higher value at amide I whereas the antler and bulla vary less.

The ratios of amide III:I, random coil to alpha helical coil, and the FWHM were calculated for each group below (table 3.14).

Bone Type	Amide III:I Ratio ± Standard Deviation	Random Coil to Alpha Helical ± Standard Deviation	Phosphate FWHM ± Standard Deviation
Metacarpal	0.73 ± 0.12	1.09 ± 0.09	17.25 ± 0.20
Antler	1.00 ± 0.08*	1.02 ± 0.03*	17.94 ± 0.27*
Bulla	0.79 ± 0.27	1.12 ± 0.05	17.33 ± 0.22

Table 3.14 – Mean Raman ratios calculated for each bone group, * indicates statistical separation from each other group using one way ANOVA P<0.05

PCA analysis, a technique which identifies the main principle areas of the data set responsible for any observed variation and separates the data into points for each component. In this instance, a three-dimensional plot, for the three main components, grouping them with a 95% confidence ellipse is displayed for the Raman data (figure 3.15), this showed that the antler group (black) was clearly separated from both types of bulla, and the metacarpal group, primarily using PC1, which represents the amide-3 group as shown on the loadings plot (figure 3.15). The metacarpal and bulla groups were highly overlapping in each component.
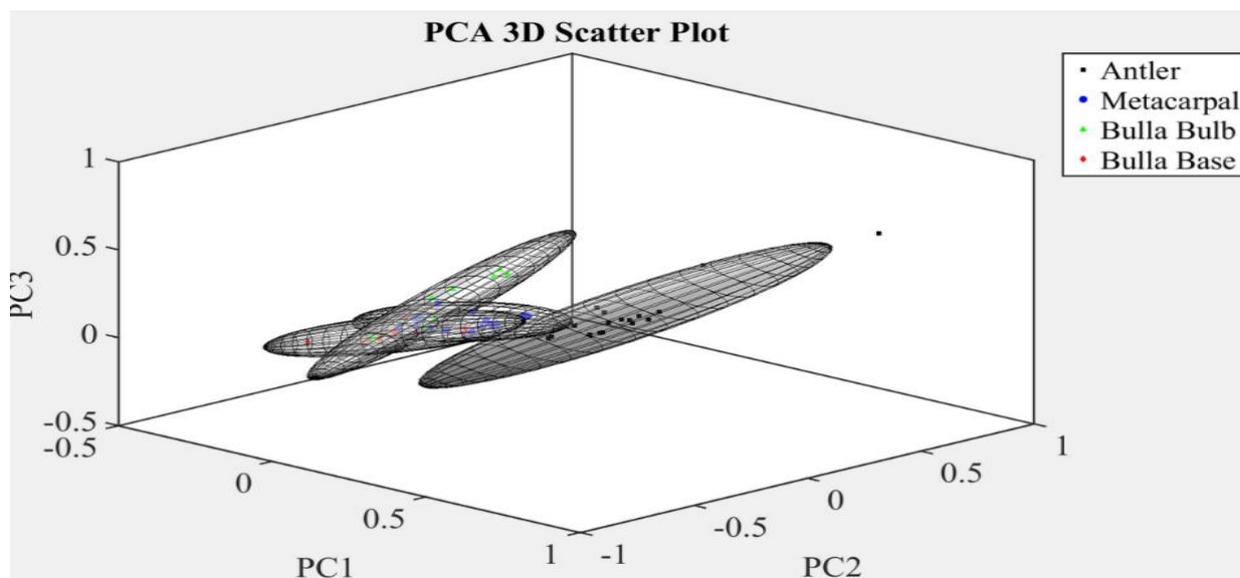
Figure 3.15 – A PCA scatter plot for each group with ellipses displaying 95% confidence areas

Figure 3.16 describes the loading plot of the PCA analysis, indicating that the areas of the spectra which are responsible for differentiating between the groups are the CH_2 wag, and amides I and III, implying that the majority of the differences between these materials are found in the collagenous regions of the spectra.
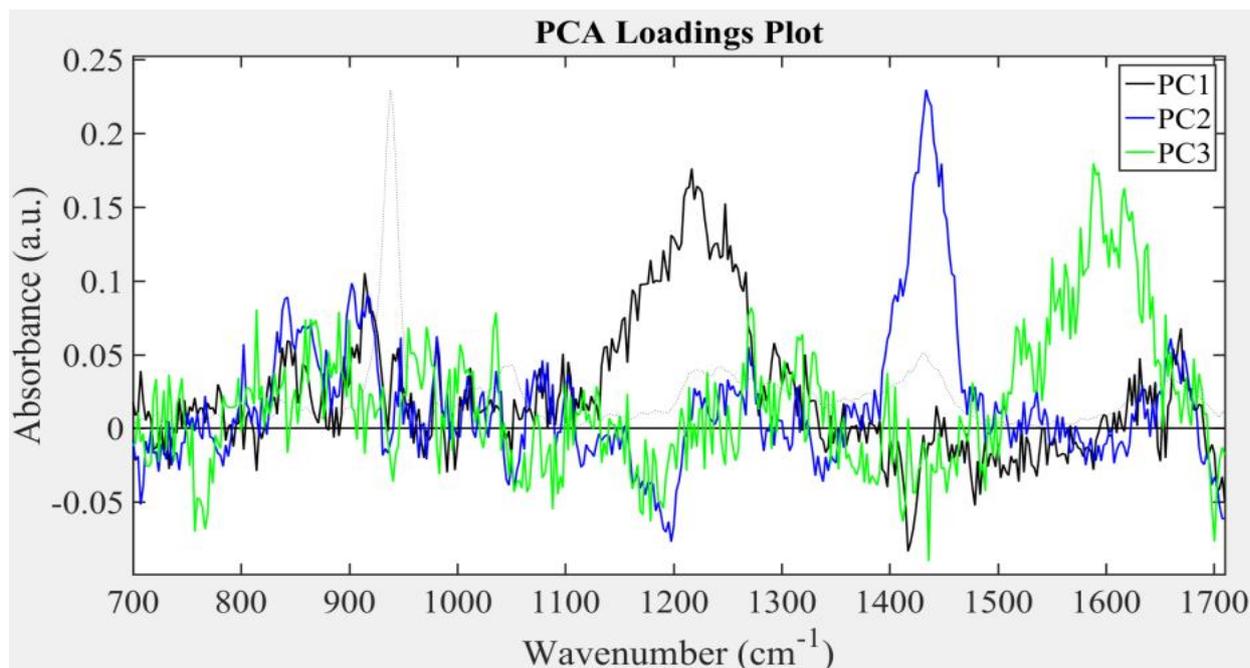
Figure 3.16 – The corresponding loading plot to figure 3.15, showing three principle components most responsible for the observed separation of materials

3.4 Discussion

Mineral and collagen content as derived both by biochemical techniques and Raman spectroscopy confirm that the antler, bulla, and metacarpal bones tested here have significantly different composition and are ideal for investigating the relationship between compositional and mechanical properties in bone. The samples observed in this study are similar to those taken from different species (figure 3.17) (Currey, 2002; Turner-Walker, 2012), but display a larger proportion of water content and more mineral than the observed trend. This is likely a difference due to the various species used as each would have different selection pressures and needs in nature. Even within the same species there can be variations between anatomical location, age, diet etc. as antler for example, all from red deer, have been reported to vary significantly in terms of composition (e.g. 45-50% mineral in Currey (2002), 56% in Dobrowolska (2002), 63% in the present study). However it should also be taken into account differences in methodology and storage may be responsible for some of this variation.
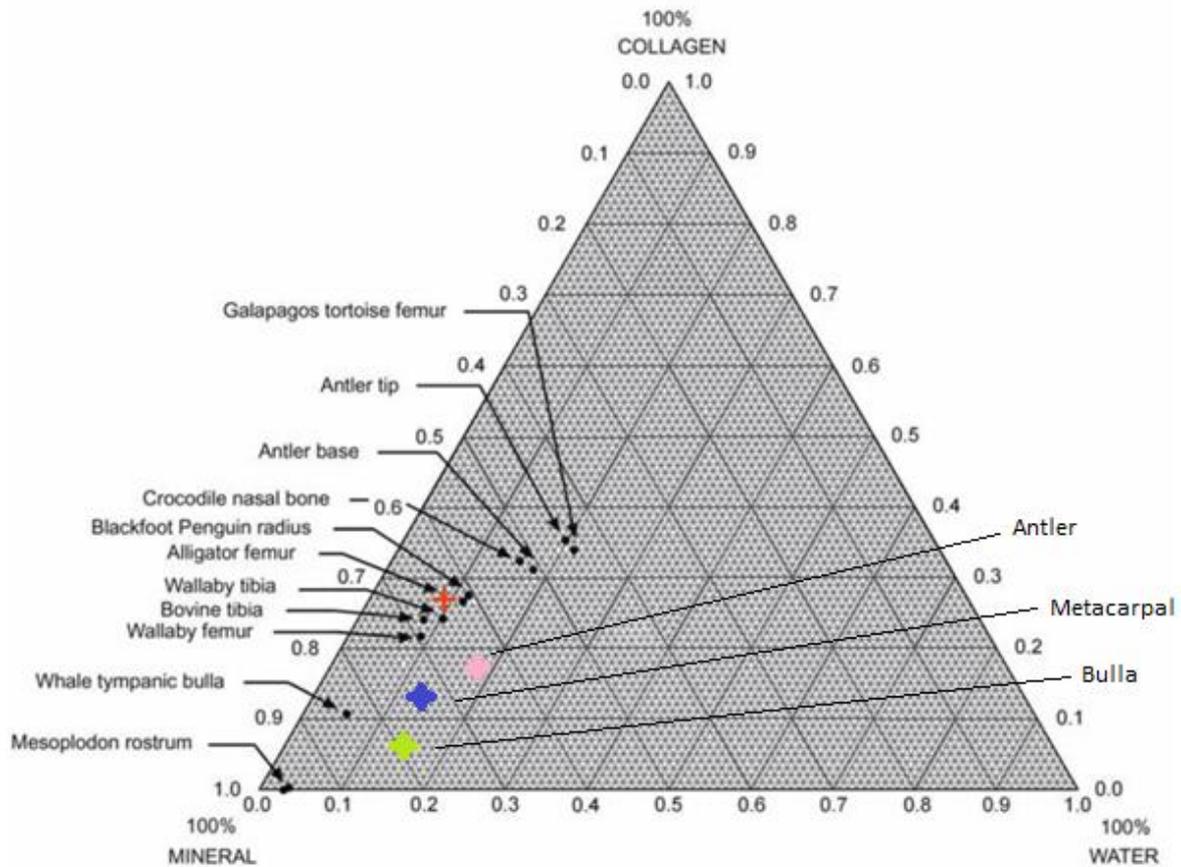
Figure 3.17 – A percentage graph indicating the composition of various animals with the antler, metacarpal and bulla included from the present study (right hand side) (Zioupos et al., 2000)

Study	Antler	Load Bearing Bone
Young's modulus of elasticity in Currey (2002)	7.4 GPa	13.5 GPa
Young's modulus of elasticity in this study	7.83 GPa	14.86 GPa

Table 3.18 – Comparison of the Young's modulus obtained in this study and Currey (2002), with load bearing bone representing a femur in Currey (2002) and a metacarpal in the present study both from the same species

Notably, both the metacarpal and antler groups proved to be similar in terms of measurements of Young's modulus in comparison to previous work (Currey, 2002) despite the differences in mineral content (figure 3.17, table 3.18). Indeed micro-indentation in this study has shown that the composition of bone as measured using the ratio of mineral to collagen from Raman spectroscopy, does not have a strong relationship with resistance to indentation. This raises the question of why these findings are not consistent with literature that has so strongly correlated material properties in the form of Young's modulus with the ratio of mineral to collagen (r^2 = 0.93 (Buckley, Matousek et al., 2012) with data taken from

(Currey, 2002; Kreith & Goswami, 2005; Les, Stover, Keyak, Taylor, & Willits, 1997)). A possible reason lies in the heterogeneity of bone tissue, as the data referenced is an average of bones taken from previous works compared against new spectral measurements whereas the present study compared indentation to Raman data on select points, ensuring that the outputs of both techniques were directly comparable, rather than a general overview. The present study has also shown that values for indentation and chemical measurements have a large variation within the same bone type (figures 3.4 and 3.3 respectively) as well as there being much available evidence of bone heterogeneity (Buckley et al., 2014; Parfitt, 2013). Therefore while an overarching relationship may exist between bone properties and the mineral to collagen ratio, one aspect cannot necessarily be used to predict the other.

The discord between bone properties and the mineral to collagen ratio, and mineral and collagen separately as measured chemically, suggest that another aspect of bone composition is responsible for the observed differences between the bone groups used in this study. Density levels acquired via QCT (figure 3.7) overlapped between each of the groups, suggesting the DEXA-style approach to bone mineral density as a surrogate for material properties does not provide an adequate explanation. Water content had high variation within the test groups and the amount of water present in bone has not been linked to material properties (Boskey, 2013). Therefore it is most likely that the responsible component of in these samples is within the organic collagenous phase.

Raman spectroscopy revealed differences between the antler, bulla and metacarpal groups in the amide I and amide III regions, areas which are sensitive to alterations in collagen chemistry (figure 3.13). The amide III to amide I ratio (figure 3.14), previously shown to increase with increasing mineral content and describe collagen alignment in turkey tendon (Kerns et al., 2016), found antler to have the highest value despite having the lowest mineral content. The ratio of random coil protein to alpha-helical protein (calculated using amide III bands 1268:1244 cm-1) (Chi, Chen, Holtz, & Asher, 1998) was lower in antler than the bulla and metacarpal which had similar levels. This change in alignment and organisation could explain the differences seen when comparing the bone properties of the groups, as metacarpal and bulla have very different mineral content, but display similar resistance to penetration and similar levels of collagen alignment and secondary protein organisation. This is aided by SEM visualisation showing perpendicular branching in metacarpal fibres not present in bulla. The alignment and organisation of collagen in bone has not been well explored, however there has been a study on type-1 collagen on the sclera of the human eye in which the increased alignment of collagen fibrils results in increased mechanical strength (Coudrillier et al., 2015). This is of particular interest to this study as it observes

collagen in the absence of mineral, in a manner that highlights the changing material strength of collagen, however it is not clear how much of an impact this change in collagen strength would affect a mineralised structure.

Using the phosphate peak at $961cm^{-1}$ the full-width half-maximal (FWHM) can be calculated by fitting a Gaussian curve (de Mul et al., 1986; Morris & Mandair, 2011). Lower values indicate a less crystalline environment suggesting that the hydroxyapatite in antler is less ordered (Morris & Mandair, 2011), however as antler are shed yearly in this species this may not be a deliberate defining factor in its organisational strength as a material, but rather a by-product of a young age that does not allow time for the crystal structure to become refined.

In terms of mineralisation of bone, there is nothing to suggest from the present study that collagen alignment or its secondary structure mediate mineral levels. There is experimental evidence in turkey tendons that shows that collagen becomes more aligned in regions that will be mineralised, pointing to a potential facilitation mechanism (Kerns et al., 2016). If this were a universal phenomenon we would expect to see heightened alignment in the bulla as it contains the largest amount of mineral which did not occur. The antler group displayed a wider phosphate peak calculated using the FWHM which suggests it has a less crystalline structure which is typically associated with young bone formation, as crystallinity increases with time as the mineral component becomes refined (Morris & Mandair, 2011). This could indicate the reason why antler are shed in nature, if the less crystalline structure is crucial to the bone properties required for a flexible fighting bone, further mineralisation, or refinement of mineralisation, would result in a more brittle antler unsuited to its role. Few studies have commented on the FWHM of the phosphate peak as a marker of bone quality, but it has been experimentally correlated with mechanical properties in MMP-2 (Matrix Metalloproteinase, important to healthy bone formation) knockout mice compared with controls showing that a more crystalline structure increases the amount of load a bone can withstand before failure (Bi et al., 2009). This would indicate that a lesser amount of crystallinity would enable more elastic properties.

In addition to Raman evidence of chemical alterations to collagen, SEM and TEM found visual confirmation of altered collagen appearance between the bone groups. The metacarpal cohort had two striking differences. Firstly, the diameter of the collagen fibrils were statistically wider than those of the antler and bulla, which has been shown in leathers to increase resistance to tearing (Wells et al., 2013), as well as association with a rise in tensile strength and intrafibrillar cross-links (Moeller, Bosch, & Decker, 1995). Secondly, perpendicular fibre bundles were observed in metacarpal samples to branch between fibres which was absent in other groups. This arrangement has been seen in the cortical bone of

humans (Pannarale, Braidotti, d'Alba, & Gaudio, 1994), and an increase in perpendicular fibres has been experimentally associated as a response to mechanical loading in dental implants (Traini, Degidi, Strocchi, Caputi, & Piattelli, 2005). These factors would help explain why the metacarpal can provide such resistance to indentation despite a lower mineral content relative to the bulla, as it is constantly under heavy loading while the bulla and antler experience relatively little. However we cannot provide evidence as to whether these are chemical changes to the collagen itself, or if this is purely an organisational response to loading, highlighting the need to explore the collagen chemistry of these samples further.

3.5 Conclusions

There is evidence to support the hypothesis that changes to the organisation of collagen has an impact on the behaviour of bone material and led to the following conclusions on this group of materials:

- Bone properties as measured by micro-indentation cannot be satisfactorily explained by the mineral to collagen ratio or mineral content.
- There are observed differences in the chemical and visual composition, alignment and secondary structure of type-1 collagen between the antler, metacarpal and bulla groups.

It is unclear at this point whether or not the observed alterations to collagen bear responsibility for the shift in bone properties, or levels of mineralisation. The organisation and chemical nature of type-1 collagen in these materials warrants further investigation as potential means of assessing bone quality if they can be shown to be linked directly to changes in bone properties.

Chapter 4 – The Impact of Collagen Cross-linking on Bone properties and Mineralisation Levels of Bone within a Single Species

4.1 Introduction

Type-1 collagen polypeptides are identical when first translated, however they are modified by a number of post translational modifications which result in differences in chemical composition which can impact upon the properties of the final bone material. This has been well characterised in cases in which the resulting cross-links form incorrectly, causing disease such as Bruck syndrome due to the lack of or improper gene expression (Van der Slot et al., 2003). However few studies have examined the effects of minor modifications on healthy tissues.

Chapter 3 demonstrated that the amount of mineral relative to collagen is not a good predictor of bone properties, and noted several differences in the organisation and composition of collagen across bones with differing mineral and collagen content. This supports the hypothesis that collagen chemistry plays a large role in the properties of the material. This chapter explores cross-linking and aspartic acid content of the same samples (used in the previous chapter).

Pyridinoline Cross-linking

Pyridinoline cross-linking was first isolated and characterised in 1977 in bovine tendon (Fujimoto, Akiba, & Nakamura, 1977), and is found in bone and cartilage, but not present in newly synthesised collagen (Uchiyama, Inoue, & Fujimoto, 1981). Pyridinoline is a maturation product of hydroxylysyl residues from the telopeptide of a collagen helix (Burr & Akkus, 2013), this form is known as Hydroxylysylpyridinoline (HP), whereas if it is formed through a hydroxylysyl residue and a lysine residue it is known as lysylpyridinoline (LP) (Figure 4.1). The process is initiated by lysyl-oxidase, but the subsequent steps are spontaneous, albeit time dependent (Szulc & Bauer, 2013). These non-reducible cross-links also serve as markers of bone turnover, being released into urine during osteoclastic bone resorption (Chapurlat & Genant, 2016). Recent research also points to the ratio of HP:LP as a diagnostic tool for bone tumours (Behrens, Bruns, Ullrich, Açil, & Gille, 2003), and osteogenesis imperfecta among others (Lindert et al., 2015).
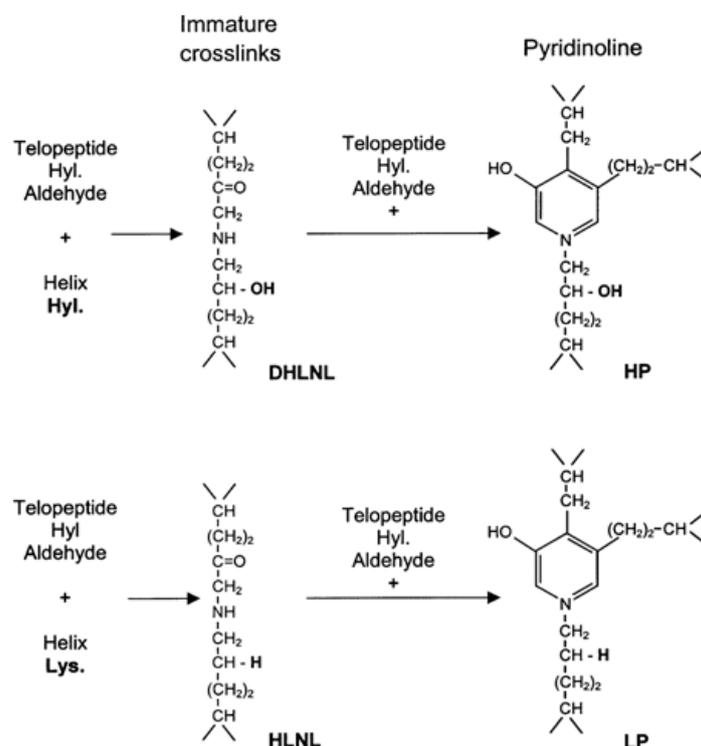
Figure 4.1 – The formation of pyridinoline cross-links (Banse, Sims, et al., 2002)

Increased amounts of exercise and activity is associated with an increase in pyridinoline cross-linking (McNerny, Gardinier, & Kohn, 2015), and this type of cross-linking has been shown to be a key factor in the mechanical strength of engineered cartilage (Yan et al., 2009). HP is also a predictor of architectural properties in trabecular bone, suggesting it has an influence on broader organisation (Banse, Devogelaer, et al., 2002). The ratio of HP:LP in healthy tissues has been shown to be an indicator of stiffness and strength (where the amount of HP is higher), as well as the overall concentration correlating to an increase in strength (Banse, Sims, et al., 2002). Therefore it is hypothesised that metacarpals would have a greater amount of pyridinoline cross-linking and a higher HP:LP ratio, which would help to explain their bone properties compared to the denser, more mineralised bulla, despite the compositional differences. Antler would be expected to contain a lower level of both total pyridinoline and HP relative to LP.

Pentosidine Cross-linking

Pentosidine is an advanced glycation end product (AGE), which forms in proteins that have become glycated as a result of exposure to sugars (Vistoli et al., 2013). It is formed through a series of reactions between the arginine and lysine residues in collagen with glucose, referred to as glycation (Miyata et al., 1998), depicted in figure 4.2.

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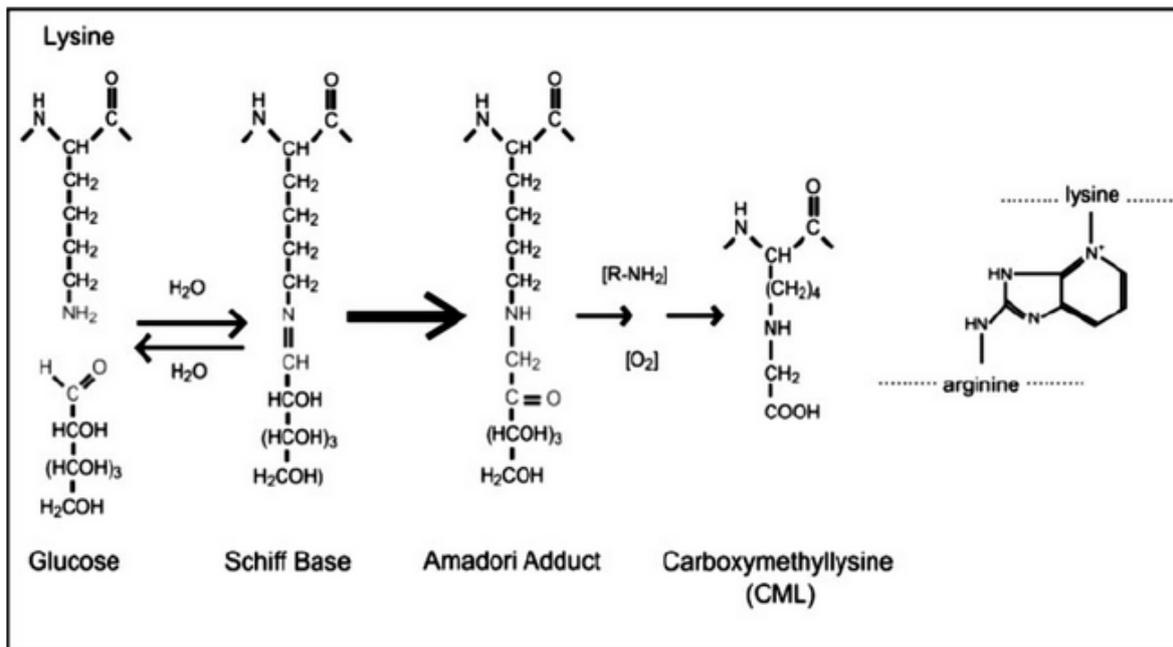
Figure 4.2 – The reaction pathway to pentosidine (far right) (Bohlender, 2005)

Many tissues, including bone, have an increase in AGE content with ageing (Chellan & Nagaraj, 2001; Takahashi et al., 1994), but are also present in pathologies such as diabetes (Andreassen, Seyer-Hansen, & Bailey, 1981), likely due to hyperglycaemia. Collagen is particularly susceptible to these post translational modifications due to the longevity of the molecule, which has a particularly slow turnover rate in bone of 2-4 years (Snedeker & Gautieri, 2014).

Pentosidine content is inversely correlated with bone formation and mineralisation (Mitome et al., 2011) as well as being associated with increased tissue stiffness (Bailey, Paul, & Knott, 1998; Reddy, 2004) leading to an increase in brittleness and fragility (Fox et al., 2011). As pentosidine is usually associated with ageing the expectation is that the deer tissues from chapter 4 will display very small quantities due to being harvested at a young age. However it is possible that pentosidine is involved in the regulation of mineralisation as it is linked to lower levels of mineralisation. Although mineralisation happens rapidly during formation, and pentosidine accumulates slowly, the presence of high levels during normal bone turnover may impact new bone formation. An increase in collagen stiffness can lead to brittleness and an increased rate of fragility, although in a bone with a relatively lower mineral content, such as antler, the brittle effect of higher levels of pentosidine may be mitigated on the whole bone level.

Aspartic Acid

In addition to collagen cross-linking, the aspartic acid content is of particular interest, acting as a marker of tissue turnover through its isomers. This isomeric transformation is common

in proteins, and the conversion of aspartic acid is one of the fastest found in nature (it would still take 15,000 years for the ratio of D to L to reach 0.333, however the next fastest isomerisation of isoleucine would take 100,000 years (Bada, Schroedert, Protscht, & Bergert, 1974)), making it ideal to study. There are two forms of aspartic acid, the naturally synthesised L-form, and its D-form counterpart. D-form aspartic acid can be converted by aspartate racemase (Liu et al., 2002), and has an effect on the pituitary and testes, increasing the amount of luteinising hormone and testosterone released in humans which have been associated with bone fracture rates (Topo, Soricelli, D'Aniello, Ronsini, & D'Aniello, 2009; Townsend, Sanders, Northway, & Graham, 1997). However it is also formed through spontaneous racemisation, as opposed to being produced directly or through enzyme action (Ritz-Timme & Collins, 2002). As aspartic acid ages in tissue, it is converted to the D-form from the L-form, therefore the higher the amount of D-form aspartic acid, the slower the rate of turnover for that particular tissue, as D-form accumulation occurs when the material is not replaced (Ohtani, Yamamoto, Iimura, Takahashi, & Kinoshita, 2008). This has been shown to be a reliable marker for age estimation of the collagen chains in tendons, leading to the theory that as the age of the animal increases, turnover rates slow, implying that there is a lowered ability to remove older damaged or degraded material, resulting in a weaker material (Thorpe et al., 2010).

In this study the bone tissue originates from young deer, approximately 2 years of age, and so we would expect to see a very low concentration of aspartic acid, especially in the antler, a material which is designed to be shed yearly. Regardless, the rate of bone turnover could give an insight into whether stronger materials are remodelled at a different speed. It is also hypothesised that aspartic acid directly contributes to mineralisation, acting as a nucleator for the process (Sarig, 2004). If this were the case, we could expect to find a relationship between the amount of aspartic acid and mineral content if levels of aspartic acid are manipulated through enzymatic pathways during bone formation.

Hypothesis

Post-translational modifications of type I collagen in bone, as expressed by differences in lysyl oxidase mediated cross-linking, and the rate of bone turnover differs in bone with different bone properties.

Objectives

1. To quantify the levels of lysylpyridinoline and hydroxylysylpyridinoline in antler, metacarpal and bulla bone from deer.
2. To quantify the levels of pentosidine and ratio of D:L aspartic acid to give a relative measure of bone turnover rate in antler, metacarpal and bulla bone from deer.

3. To examine the relationship between cross-link levels and bone turnover marker levels with bone properties (as measured in chapter 3).

4.2 Materials and Methods

Bone Sample Preparation

Representative samples of cortical bone (taken from metacarpal n=4, antler n=5 and bulla n=7 of red deer (*Cervus elephus*)) were excised using a diamond-blade saw and cut to a weight of approximately 3 grams. The bone segments were then freeze dried, and subsequently powdered in a cryo-mill (SPEX SamplePrep (6675), UK) under liquid nitrogen. The bone powder was then subject to EDTA (ethylenediaminetetraacetic acid) (10% 0.27M, pH 7.5), at 37°C in a water bath for two weeks with the solution being changed every 2 days (Choi et al., 2015). Once demineralised, 50mg of bone powder was hydrolysed in 5ml of 6M HCL for 24 hours at 110°C, and then dried under vacuum in a speed vac concentrator to remove the HCL. The powder was then re-suspended in water at 15mg/ml (method 1) or 5mg/ml (method 2).

Pyridinoline and Pentosidine Quantification (Method 1)

PYD (HP and LP) and PEN were separated on a Shimadzu HPLC system with a fluorescent detector. The cross-links were separated using a hypercarb, reverse phase column, with a guard column attached. The column flow rate was at 1ml/min, at a temperature of 25°C, with an injection volume of 50μl. The solvents used for separation were 1% trifluroacetic acid (TFA) in water (solution A) and 1% TFA in acetonitrile (solution B). The column was equilibrated with the starting ratio of A:B at 85:15 for 10 minutes and then molecules were separated over a changing gradient as shown below (table 4.3). Samples were injected with 1% TFA. The separation of PYD (both HP and LP) occurred during the first 15 minutes, where the detector was set to 295nm excitation, 405nm emission. The separation of PEN occurred around the 22 minute mark, where the detector was set to 335nm excitation, 385nm emission. After 25 minutes the column was washed with Acetonitrile at 2ml/min for 5 minutes before equilibration with the starting values.

Time (minutes)	A concentration (%)	B concentration (%)	Flow rate (ml/minute)
0	85	15	1
5	80	20	1
10	75	25	1
15	65	35	1
18	45	55	1
20	85	15	1
25	85	15	1

Table 4.3 – The HPLC settings for method 1

Quantification of Aspartic Acid Racemers (Method 2)

Sample Preparation

Prior to separation by HPLC, aspartic acid was derivatised using NDA (2,3-Naphthalenedicarboxaldehyde. An aliquot (100 µl) of the 5mg/ml of re-suspended hydrolysed bone solution was mixed with 400µl methanol, 200µl NDA,, 100µl Boc lysine, 100 µl KCN (potassium cyanide) and 100 µl borate buffer, pH 9.. The derivatisation mixture was vortexed and allowed to react for 30 minutes in a darkened environment due to the photosensitivity of NDA. 100µl of the derivatised sample was mixed with 400µl of 50mM CH_3C00- (pH 6), 5mM cyclodextrin in water (solution A) prior to HPLC separation.

HPLC Running

Aspartic acid was separated and quantified on a Shimadzu HPLC system using a fluorescence detector set to 325 emission and 434 excitation, on a C8 column (Novapack), at 35°C. Separation of D and L forms of aspartic acid was achieved using a gradient of solution A and solution B (100mM CH_3C00- (pH 6) in 20% water, 80% solvent consisting of a ratio of 9:1 methanol:acetone (solution B) as shown in the table 4.4 below. Following column equilibration, 20µl of sample was injected. An internal standard of Boc-Lys at 1ppm was also included in this method to ensure repeatability, which was eluted after both forms of aspartic acid. The flow rate was varied to speed up the process whilst retaining separation of the L and D forms of aspartic acid, at the concentrations listed in the below table 4.4:

Time (minutes)	A concentration (%)	B concentration (%)	Flow rate (ml/minute)
0	80	20	1.2
3	77	23	1.2
6	75	25	1
8	35	65	0.9
15	35	65	0.9
17	0	100	0.9
19	0	100	0.9
25	80	20	1.2

Table 4.4 – The HPLC settings for method 2

Calculations

Both methods were calibrated using known concentrations of the pure analytes at 7 points (0-50ug/L in aspartic acid for D and L, 0-15µmoles/L in PYD (HP and LP) and PEN) to establish a linear relationship between quantity and detection, and to create an equation for each using the gradient of the line to calculate absolute values. All R-values were greater than .99, indicating an accurate, repeatable methodology (e.g. figure 4.5), and all analytes fell within the expected ranges.
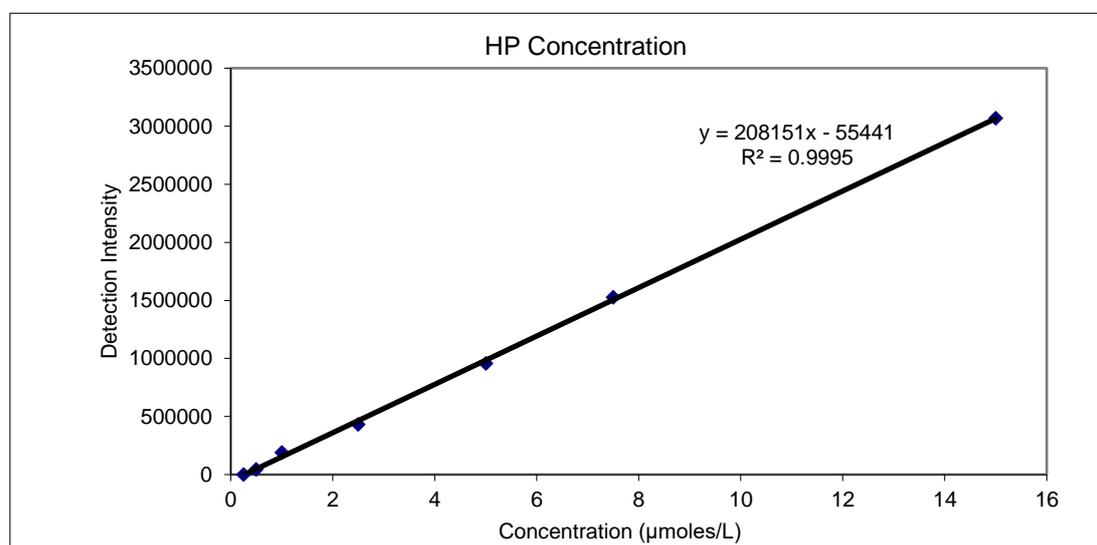
Figure 4.5 – A calibration graph for the quantity of HP

PYD and PEN are reported in µmoles/L per amount of collagen as determined by hydroxyproline assay in the previous chapter, with the HP:LP ratio of PYD being expressed without units. As the amount of collagen varies between materials, absolute values have to be corrected to take into account the quantity present, so they can be compared as materials. All values are reported in µmoles/L/ % of collagen. That is to say the values are

the quantity in µmoles/L in 1% of the collagen. This creates a relative value for comparison across the cohort.

Statistical Analysis

Multiple one-way ANOVA with post-hoc Tukey's was used to test if the groups were significantly different from each other in terms of cross-link and aspartic acid. Partial correlations were used to observe the relationship between variables without interference from covariates, and multiple linear regressions to assess the overall relationship to mineral content and bone properties. Pearson's linear correlation was used to examine the ability of Raman ratios to represent aspects of collagen chemistry.

4.3 Results
Pyridinoline Cross-links

The total amount of HP and LP relative to the amount of collagen (figure 4.6) found in each tissue appears to differ. Metacarpal (MC) on average displayed the highest quantities of both types of PYD cross-linking, with the bulla (DB) material in the middle, albeit with overlapping values with the metacarpal samples as well as low LP levels similar to antler (AN). Antler was found to occupy the lowest total values. When the HP:LP ratio was observed (table 4.7), metacarpal and antler are similar in their quantities, whereas the bulla group shows a much higher ratio.
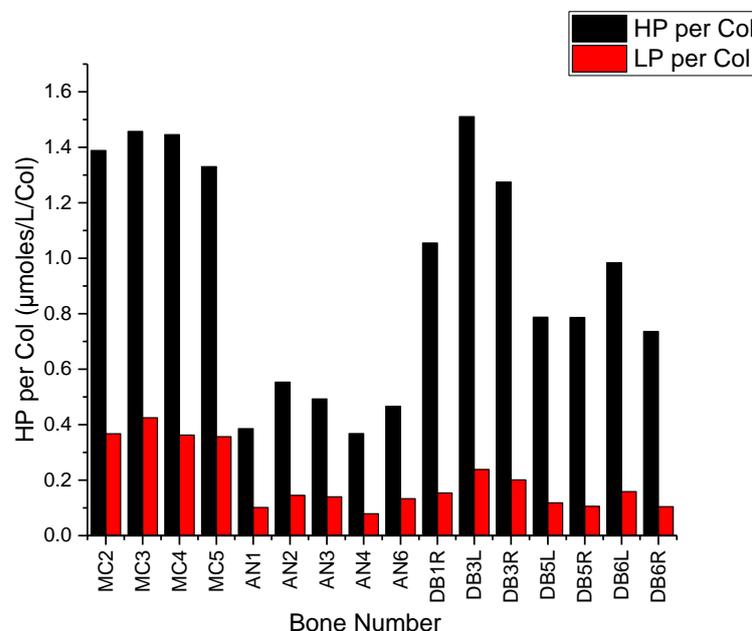
Figure 4.6 – The concentrations of HP and LP adjusted for collagen content

HP/LP Ratio	Average	Standard Deviation
Metacarpal	3.73	0.23
Antler	3.86	0.47
Bulla	6.70	0.45

Table 4.7 – The HP:LP ratio average of each bone group

Pentosidine Cross-links
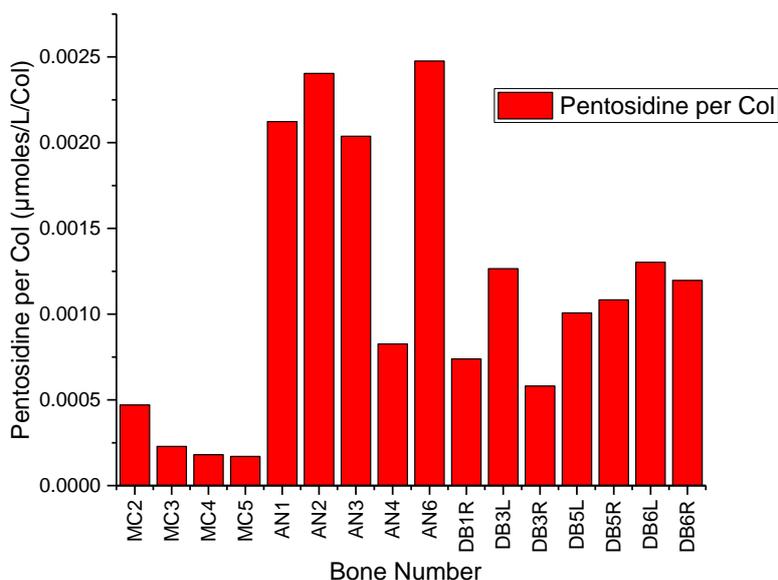
Figure 4.8 – Pentosidine concentration adjusted for collagen content

The quantity of pentosidine in these tissues is extremely low, at less than 0.0005 µmoles/L per percent of collagen (figure 4.8). This is expected due to the age of the deer as it is known that this cross-link accumulates with age. However the different bone materials split into distinct groups based on quantity of pentosidine present. Metacarpal displayed the least amount of the AGE cross-link, with the antler group containing up to 12 times this amount. The bulla occupied the middle region, overlapping only with one of the antler samples (AN4).

Aspartic Acid

Figure 4.9 shows that on average the bulla experience the slowest rate of turnover, with metacarpal being more frequently replaced than both the antler and the bulla. The tissue types are relatively distinct, with the difference between the D:L ratio of aspartic acid (MC6 at 1.86%) and the slowest (3.04% in DB1R) being less than double.
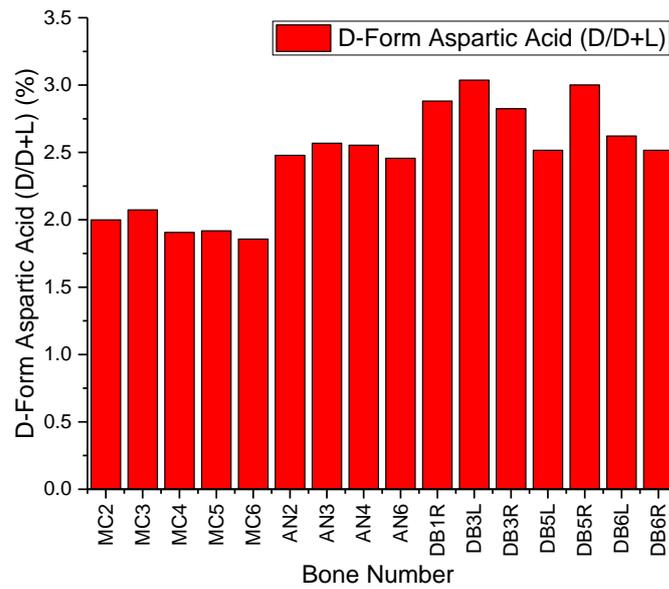
Figure 4.9 – The percentage of D-form aspartic acid relative to L-form

Bone	HP Mean ± Standard Deviation	LP Mean ± Standard Deviation	PEN Mean ± Standard Deviation	Aspartic Ratio Mean ± Standard Deviation
Metacarpal	1.20 ± 0.46	0.32 ± 0.13*	0.0006 ± 0.0008	1.95 ± 0.08*
Antler	0.59 ± 0.27	0.13 ± 0.03	0.0017 ± 0.0008	2.58 ± 0.17
Bulla	1.01 ± 0.31	0.15 ± 0.05	0.0010 ± 0.0002	2.75 ± 0.24

Figure 4.10 – The means and standard deviations for each HPLC determined value – a * represents statistical separation from all other groups as determined by one way ANOVA

Relationship between Pyridinoline Cross-link Levels, Bone Turnover Markers and Bone Properties

The IDI has been compared to the HP:LP ratio, total HP, LP, PEN and D:L ratio of aspartic, with the table below (table 4.11) describing their partial correlation coefficients.

Comparison	Partial Correlation
HP concentration and IDI	-0.15
LP concentration and IDI	-0.0068
HP:LP Ratio and IDI	-0.30
Pentosidine concentration and IDI	-0.13
D-form Aspartic Acid and IDI	0.75*

Table 4.11 – Partial correlation between various collagen component concentrations and IDI, with each other factor removed, * indicates statistical significance using a two-tailed test at $p < 0.05$

The only significant relationship appears to be with the D-form of aspartic acid, implying that the turnover of bone material is an important factor in physical bone properties (figure 4.11).
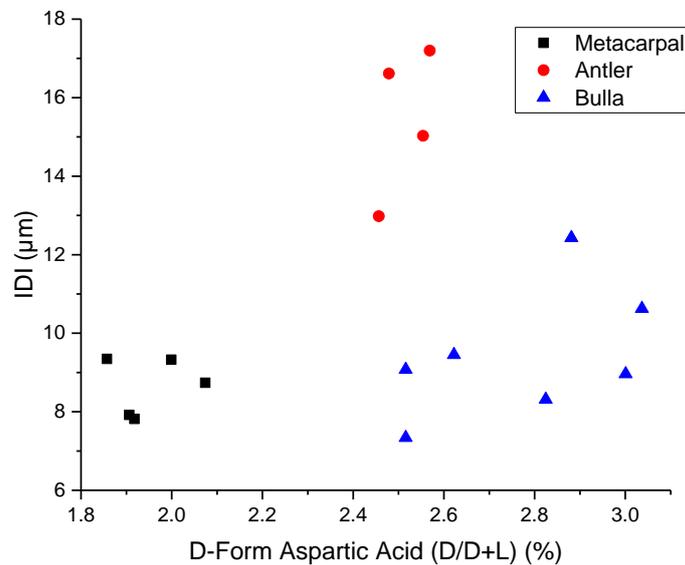
Figure 4.11 – A scatter plot of the percentage of D-form aspartic acid concentration vs IDI

The scatterplot for HP and IDI is shown below (figure 4.12), and shows a general increase of IDI with decreasing HP content, however this is only the overall trend and it does not appear that individual bones show a corresponding change.
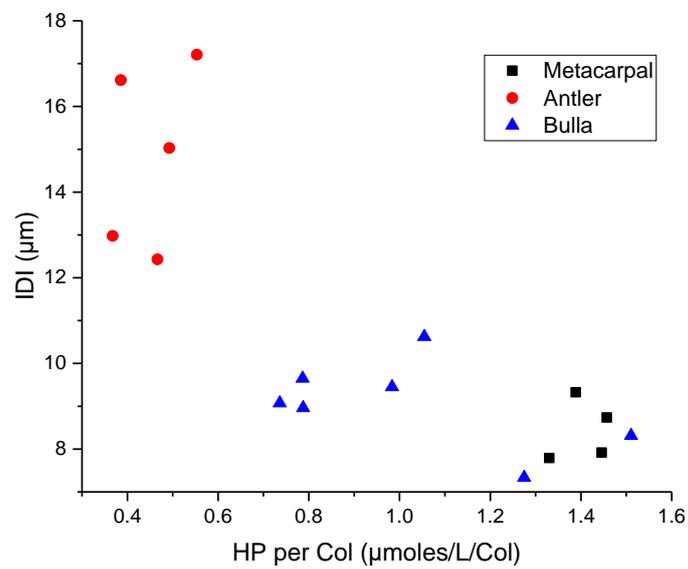
Figure 4.12 – A scatter plot of HP concentration vs IDI

Figure 4.13 depicts the correlation between pentosidine levels and IDI which again shows a general trend but not a strong relationship.
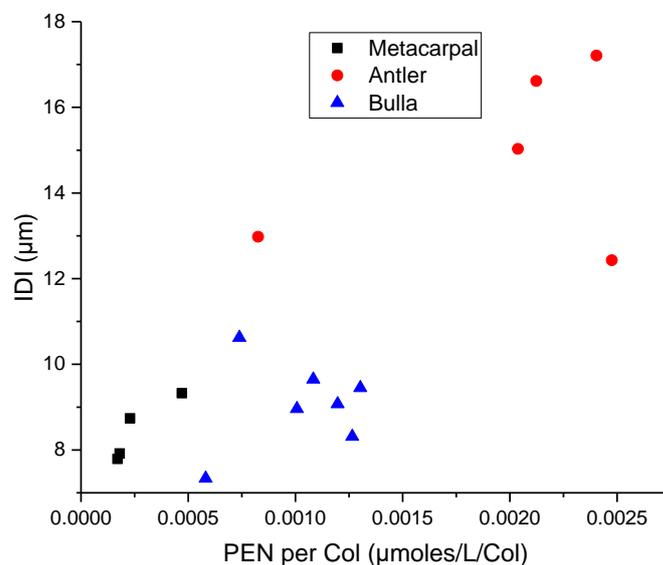
Figure 4.13 – Correlation between the concentration of pentosidine and IDI

Mineralisation

Mineral quantities were quantified for each bone in the previous chapter from a representative sample; the level of mineralisation correlated positively with the HP:LP ratio with high significance shown in the table below (table 4.14).

Comparison	Partial Correlation
HP concentration and mineral content	0.12
LP concentration and mineral content	0.23
HP:LP and mineral content	0.71*
Pentosidine concentration and mineral content	0.15
D-form Aspartic Acid and mineral content	-0.10

Table 4.14 – Partial correlation between various collagen component concentrations and mineralisation, with each other factor removed, * indicates statistical significance using a two-tailed test

The graph below shows the relationship between the HP:LP ratio and mineral content (figure 4.15).
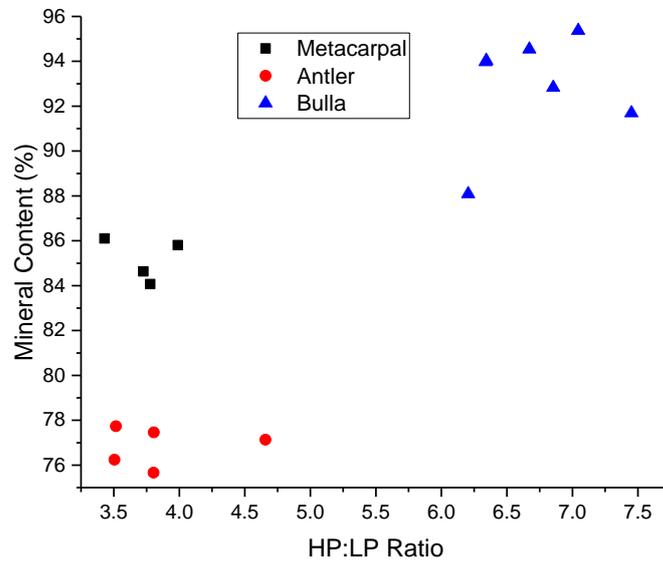
Figure 4.15 – Relationship between the HP:LP ratio and mineral content

Comparisons to Raman Spectroscopy

Raman ratios collected in the previous chapter compared to cross-link concentration (table 4.16)

Raman Ratio	Test	HP:LP Ratio	HP Concentration	LP Concentration	Pentosidine Concentration
Amide III:I Ratio	Pearson Corr.	0.04	-0.44	-0.41	0.49
	p-value	0.87	0.075	0.10	0.047*
Random Coil to Alpha Helical	Pearson Corr.	-0.58	-0.11	0.12	0.24
	p-value	0.02*	0.67	0.63	0.34

Table 4.16 – Correlation and statistical significance using Pearson's between Raman ratios and collagen biochemistry across all bone types

Multiple Linear Regressions

Table 4.17 below shows the regression with mineral content as the dependent variable, with only the HP:LP ratio approaching significance as a predictor of mineral content. Interestingly no physical bone property has a relationship with mineral content.

		Value	Standard Error	t-Value	Prob>|t|
Mineral Content	Intercept	81.28695	25.77197	3.15408	0.01971
	"HP per Col"	5.06849	8.22235	0.61643	0.56026
	"LP per Col"	5.86269	38.03841	0.15413	0.88256
	"Pentosidine per Col"	1672.29377	2223.06128	0.75225	0.48036
	"D-Form Aspartic Acid (D/D+L)"	-4.41364	6.87218	-0.64225	0.54446
	"HP:LP Ratio"	4.03814	1.82489	2.21281	0.06888
	"IDI"	0.07266	0.68549	0.106	0.91904
	"Creep"	-2.71542	2.5237	-1.07597	0.32329
	"Amide 3:1 Ratio"	2.21513	3.96536	0.55862	0.59664
	"Random : Alpha"	-5.62096	7.7775	-0.72272	0.49704

Table 4.17 – Multiple linear regression of all bone factors with mineral content as the dependent variable, the adjusted r^2 value is 0.90

When only the relationship of chemical aspects of collagen chemistry are examined the link between the HP:LP ratio and mineral content becomes clearer (table 4.18)

		Value	Standard Error	t-Value	Prob>|t|
Mineral Content	Intercept	57.96573	9.32636	6.21526	9.94525E-5
	"HP per Col"	3.26401	6.71239	0.48627	0.63726
	"LP per Col"	23.63093	30.8653	0.76561	0.46159
	"Pentosidine per Col"	799.67175	1379.01482	0.57989	0.57482
	"D-Form Aspartic Acid (D/D+L)"	-3.15746	2.576	-1.22572	0.24839
	"HP:LP Ratio"	5.33595	1.19176	4.47737	0.00118

Table 4.18– Multiple linear regression of all chemical factors with mineral content as the dependent variable, the adjusted r^2 value is 0.92

Table 4.19 shows the IDI as the dependent variable with other physical bone properties excluded as they correlated highly. The quantity of D-form aspartic acid has the only significant relationship to the IDI, as in table 4.18 where only chemical aspects of bone are considered.

		Value	Standard Error	t-Value	Prob>|t|
IDI	Intercept	18.0398	22.91783	0.78715	0.45701
	"HP per Col"	-2.572	5.79995	-0.44345	0.67082
	"LP per Col"	4.47169	27.17627	0.16454	0.87395
	"Pentosidine per Col"	-935.35922	1162.69296	-0.80448	0.44758
	"D-Form Aspartic Acid (D/D+L)"	7.79258	2.38356	3.26931	0.01369
	"HP:LP Ratio"	-1.008	1.70757	-0.59031	0.57354
	"Amide 3:1 Ratio"	3.80392	2.48724	1.52937	0.17002
	"Random : Alpha"	-0.64437	5.65844	-0.11388	0.91253
	"Mineral Content"	-0.24308	0.26129	-0.93028	0.38319

Table 4.19– Multiple linear regression of all bone factors with IDI as the dependent variable, the adjusted r^2 value is 0.72

IDI		Value	Standard Error	t-Value	Prob>|t|
	Intercept	6.64075	7.30983	0.90847	0.385
	"HP per Col"	-2.8866	5.26105	-0.54867	0.59527
	"LP per Col"	-3.84176	24.19164	-0.15881	0.87698
	"Pentosidine per Col"	-562.83667	1080.84603	-0.52074	0.61388
	"D-Form Aspartic Acid (D/D+L)"	8.11653	2.01902	4.02004	0.00244
	"HP:LP Ratio"	-2.31193	0.93408	-2.47509	0.03282

Table 4.20 – Multiple linear regression of all chemical factors with IDI as the dependent variable, the adjusted r^2 value is 0.73

While the r^2 values in these regressions are high (the proportion of the dependent variable that can be explained by all the included factors), this data set has a small sample sizes which can inflate these values. The r^2 value is also adjusted for the error which naturally increases with each variable addition therefore it is expected that removing variables with little to no relationship with the dependent variable increases the overall strength of the model.

4.4 Discussion

The HP and LP cross-links appeared to be highest consistently in the metacarpal group, and lowest in the antler, with the bulla displaying a wide range of values in between. The quantity of HP and LP does not appear to correlate well to either bone properties in the form of IDI or mineral content, however in linear regression models, the ratio is the only metric significantly associated with mineral content. This could imply that the HP:LP ratio is important in terms of facilitating mineralisation, i.e. creating a more suitably arranged environment for the deposition of mineral into the collagen framework. The ratio of HP:LP cross-linking present would then be not only responsible for creating a strong network of fibrils for mineralisation, but also a key factor in conferring strength to bone. A study on fractures in dog tibiae confirmed that there was an observed increase in the HP:LP ratio during the early stages of calcification of new tissue, but reported that the combined amount of HP and LP did not change throughout the entire process over 18 weeks (Wassen et al., 2000). This led to the theory that the HP:LP ratio facilitates mineralisation through an increase in the amount of HP relative to LP, which is also supported by the current study. In terms of strength, the amount of HP has been shown to correlate with cervical tissue strength in mice (Yoshida et al., 2014), as well as being directly involved in the organisational structuring of tissue, with lower concentrations of HP cross-linking being related to thicker but less complex arrangements of collagen (Garnero, 2012). However it has not been directly linked to bone properties, but as a higher ratio of HP:LP appears to encourage mineralisation, which in turn alters bone properties through an increase in stiffness, it contributes to further evidence of the influence of HP over the mineralisation process.

It was expected that the levels of pentosidine would be extremely low due to the fact that the deer sampled were of a young age, particularly in the case of antler material which is shed yearly. There was an unexpectedly high amount of pentosidine cross-linking in antler. In the literature pentosidine is found in higher concentrations in the elderly, and in osteoarthritis and osteoporosis (Shimada & Miyakoshi, 2015; Vaculík et al., 2016), and is generally considered to be partly responsible for the degradation of bone strength. One study has posited that the damage may lie in the ability of pentosidine to interfere with osteoclastic activity through both surface protein recognition and attachment in the resorption process, but also in discouraging the differentiation of osteoclasts (Valcourt et al., 2007). However in the present study levels of D-form aspartic acid would suggest that the bulla turns over the slowest as a material, but does not contain high levels of pentosidine, disagreeing with their hypothesis.

In the literature, pentosidine has not been reported in quantities relative to mineralisation other than as a factor in disease or accumulation over time. It appears in this study that antler display at the highest quantity, a 15 fold increase in pentosidine concentration over standard load bearing bone (i.e. metacarpal), and also have a markedly lower mineral content. This could suggest that pentosidine is incorporated into antler material deliberately to inhibit mineralisation, to create a more flexible material. Although not an enzymatic reaction, as pentosidine is able to accumulate faster in antler there may be an unknown mechanism for its control. Perhaps due to the fact that antler is not long-lived in nature, accumulation of these cross-links does not produce the long-term decline of bone properties as in other pathologies.

Bone Turnover

The D-form concentration of aspartic acid was found to be highest in the bulla, indicating it is remodelled the slowest and lowest in metacarpal, which would turn over the fastest. However, it did not correlate with levels of mineralisation. It also failed to agree with pentosidine levels, which can also function as a marker of turnover. This would suggest that perhaps the rate of turnover is not necessarily linked to factors affecting bone strength. Instead it is more likely that the rate of turnover is influenced by the amount of use and development of micro-cracks which encourage resorption (Rumpler et al., 2012). Although antler are specifically designed for fighting, it is likely that the metacarpal experiences the most active remodelling, due to the fact that it bears the weight of the animal on a daily basis and is put under great stress during running. This would agree with the fact that the bulla would be the least active in terms of turnover, as it is a purely internal structure that does not bear loads and is unlikely to suffer any damage. The D-form of aspartic acid did however

show a significant statistical relationship to bone properties in the form of IDI, indicating that a slower rate of turnover leads to diminished capability of bone to withstand repeated indentations.

Raman ratios (table 4.14) collected in the previous chapter correlated with the collagen cross-link data. Both ratios are descriptive of the collagen amide regions. The amide III:I ratio, shown to describe increased collagen alignment (Kerns et al., 2016), in this case correlated significantly with pentosidine, and near significance with HP concentration. In addition to this, the value for comparing the amount of random coil to alpha helical had a very strong negative association with the ratio of HP:LP across the three tissue types. These findings would suggest that Raman spectroscopy is capable of potentially identifying and describing very specific changes to collagen chemistry. Recently, a study has used Raman spectroscopy to monitor pyridinoline cross-links, using a similar combination of HPLC and Raman instrumentation in mineralised tissues, although they were only able to link the Raman band at 1660cm^{-1} to the concentration of HP (Gamsjaeger, Robins, Tatakis, Klaushofer, & Paschalis, 2017). It is likely that due to the nature of the overlapping amide bands that true measurements of collagen will be difficult to measure without increased sensitivity, and that a much larger series of measurements would need to be taken to establish a true representative population for use by clinicians. Nevertheless the results in this thesis thus far indicate Raman spectroscopy could be used successfully to observe modifications to type-1 collagen which have been shown here to correlate with bone properties.

Bone Type Ranking

Using the data from this chapter and chapter 3, table 4.21 has been constructed to summarise each aspect of the average bone material values and chemistry.

Components	Mineral Content	Collagen Content	Water Content		
Antler	3	1	1		
Metacarpal	2	2	3		
Bulla	1	3	2		
Bone Properties	FCI	IDI	Creep	Density	Young's modulus
Antler	1	1	1	3	2
Metacarpal	2	2	2	2	1
Bulla	2	2	2	1	-
Collagen and Mineral Properties	Fibril Diameter	Fibril Band Length	Amide III:I Ratio	Random Coil: Alpha Helical Ratio	Crystallinity
Antler	2	1	1	2	2
Metacarpal	1	1	2	1	1
Bulla	2	1	2	1	1
HPLC Analyses	Pentosidine Concentration	HP Concentration	LP Concentration	Aspartic Acid Ratio	
Antler	1	3	3	2	
Metacarpal	3	1	1	3	
Bulla	2	2	2	1	

Table 4.21 – Ranking of bone attributes where '1' is the highest value, where populations could not be separated according to ANOVA bones have been assigned the same rank. A higher ranking in aspartic acid ratio indicates a slower turnover rate.

From table 4.15 it is clear that despite differences in composition, the bulla and metacarpal share physical bone properties. These two bone groups do share similarities in the properties of the collagen in terms of organisation and composition through the amide III:I ratio, and amount of random coil relative to alpha helical organisation as well as the crystallinity of the mineral.

4.5 Conclusions

The influence of chemical modifications to type-1 collagen, in terms of cross-linking, appears to play a large role in the organisation of material and the amount of mineralisation as well as the overall quality of bone material. While it was already known that these cross-links had an impact on bone strength they had not yet been examined in the context of bones with a differing mineral volume fraction from a single species, nor compared directly to bone properties. A limitation in drawing conclusions at this stage is that both the physical and chemical properties were taken from representative samples, and so while mitigating some of the heterogeneity of bone, in exchange they lose the sensitivity of a direct comparison between the chemical and bone properties at the exact same location (which will be addressed in the final experimental chapter). Direct measurements were not taken at this

stage as the goal of these studies was to specifically examine the different types of bones and the differences between these groups.

This chapter therefore supports the following:

- Pentosidine may have an influence as a mineralisation inhibitor in antler
- The HP:LP ratio has a strong relationship with mineralisation and bone material strength
- The Raman ratio for collagen alignment agrees with pentosidine concentration, while the ratio for collagen secondary structure correlates with the HP:LP ratio, indicating the ratios could have further in-vivo applications

This supports the overall hypothesis of this thesis that collagen plays a significant role in determining levels of mineralisation and the mechanical properties of bone material (when considering bones with different functional requirements from different anatomical locations).

Chapter 5 – Regional Variations in Collagen Cross-linking and Mineralisation along the Length of the Deer Antler

5.1 Introduction

It is well documented that bone from different anatomical locations, and in different species with unique mechanical requirements, have properties to suit their natural function. It has also been shown that within a single bone there exists a tuning of the material along the length (Buckley et al., 2014). The antler is an interesting material as the proximal and distal ends are likely subjected to very different mechanical stresses, and may provide an insight into how collagen cross-linking is modified within a single bone.

Antler is a material formed of a permanent bony outgrowth known as a pedicle, from which bone material is formed initially as mineralised cartilage from the tip which is converted into bone over time, often with cartilage remnants found in the central shaft (Kierdorf, Flohr, Gomez, Landete-Castillejos, & Kierdorf, 2013). It grows at the fastest rate of any mammalian bone, up to a quarter of an inch per day, causing a temporary reversible case of osteoporosis in the rest of the skeleton due to its consumption of resources (Baxter, Andrews, & Barrell, 1999). It is comprised of a core of cancellous bone material, with an outer cortical layer, that undergoes very little remodelling and is mostly formed of primary osteons (the original deposition of bone) (Chen, Stokes, & McKittrick, 2009).

The previous chapter built on the existing literature, supporting the idea that the concentration of certain cross-links has an association with levels of mineralisation and material strength with pentosidine being associated with fragility and lower mineral content, and HP levels both in terms of concentration and relative to LP concentration being associated with increased mineral content and strength. It also contributed new knowledge in the concept of advanced glycation cross-linking not being necessarily affected by the age of collagen, but potentially as a contributor in the mineralisation process. However these studies were conducted at the whole bone level, using representative samples, mitigating the heterogeneity of bone material but not fully taking advantage of it.

Bone Heterogeneity

The heterogeneity of bone material is well documented as a phenomenon that occurs, but the mechanisms behind it have received much less attention. It is known, through techniques such as finite element modelling, that stress on bone material is not evenly distributed, and that bone can respond to stress through its network of osteocytes to regulate remodelling (Plotkin et al., 2007). In this way, the areas which are under more stress are replaced more frequently, so the accumulation of damage to the material does not result in mechanical failure. However, it is not clear how modifications to collagen are controlled for,

or even if the cells producing it react differently under physical stresses, aside from the fact that more frequent replacement of bone material halts the build-up of mature cross-links which become present over time. No studies have thus far commented on direct values as to the concentrations of cross-links in collagen, and the resulting impact on organisation and mineralisation of bone material within a single bone at different sites.

Raman spectroscopy has been used to examine the heterogeneity of bone but the fine chemical structure of collagen is difficult to discern due to the nature of overlapping bands and insufficient sensitivity. Studies have observed that within similar populations, the alignment and maturity of collagen have a significant effect on bone strength (Boskey et al., 2016; Makowski, Granke, Uppuganti, Mahadevan-Jansen, & Nyman, 2015). One study in particular differentiated between two areas of individual tendons, a mineralised and non-mineralised region specifically looking for alterations in collagen chemistry using Raman spectroscopy, and found that the alignment of collagen (as measured by the ratio of amide III: amide 1) increased with increasing mineralisation, and these collagen differences were also observed prior to mineralisation in younger turkeys. (Kerns et al., 2016). While these studies have shown that modifications to collagen is an important contributor to material strength and has an impact upon mineralisation, none have fully linked specific alterations to collagen chemistry as the root cause behind these changes.

The importance of understanding the differences in composition within a single bone, as opposed to many age and sex matched bones, is the ability to compare different regions of the same material. Variation between different bones can be ascribed to the differences between the animals in question, whereas changes on the millimetre scale of a single bone can only be due to inherent heterogeneity of the material. The variation within a single bone can be assumed to be caused by a combination of three things:

- Natural variation in the building of bone material – assuming all bone in a region is attempting to be identical, this could be caused by a lack of resource available for example.
- A designed difference – the bone organ as a whole has regions that are deliberately grown differently for purpose. This is very clearly the case in the epiphysis of long bones, where there is trabecular bone, as well as inwards from the outer cortical layers into less dense areas in the centre where there are cavities in the bone.
- An adaptive difference – the bone material responds strongly to external forces over time and its composition is affected by stresses on the cellular network causing remodelling as well as a reactive chemical change to better suit the new material for

purpose. This is observable on a larger scale in fractures, where the body tends to overcompensate for an injury with large depositions of mineral

By looking at a single bone we can draw stronger conclusions from the correlation between chemical changes and levels of mineralisation, as we are not exposed to the natural variation between different individuals.

Hypothesis

Levels of pyridinoline and pentosidine cross-links have a relationship with the level of mineralisation across a range of antler sections of differing density

Objectives

1. To quantify the levels of lysylpyridinoline and hydroxylysylpyridinoline in antler sections.
2. To quantify the levels of pentosidine and ratio of D:L aspartic acid in antler sections.
3. To examine the relationship between cross-link levels and bone turnover marker levels with levels of density and mineralisation.

5.2 Materials and Methods

Bone Sample Preparation

A single antler (referenced as AN6 in previous chapters) was examined first using a QCT (XCT 3000, Stratec, Germany), taking 2mm interval scans, 10 at the base of the antler where it attaches to the skull, 10 at a central region, and a further 10 at the tip. This was done to determine suitable locations to isolate for further chemical analysis. Regions were selected based on the density, as reported in milligrams per cubic centimetre, with areas of approximately 1cm^3 being identified as quasi-homogenous. Suitable regions (n=9, named AS1-9) were selected from the three locations based on a visible change in mineralisation, and excised using a fine bladed hacksaw (figure 5.1 displays one of the three locations and the three excised areas within it). AS1 and 2 originated from the head end, where only two bands of different levels of density were present, AS3, 4 and 5 are from 33% of the length of the antler from head to tip, AS6, 7 and 8 from 66% of the length, and AS9 is from the tip.
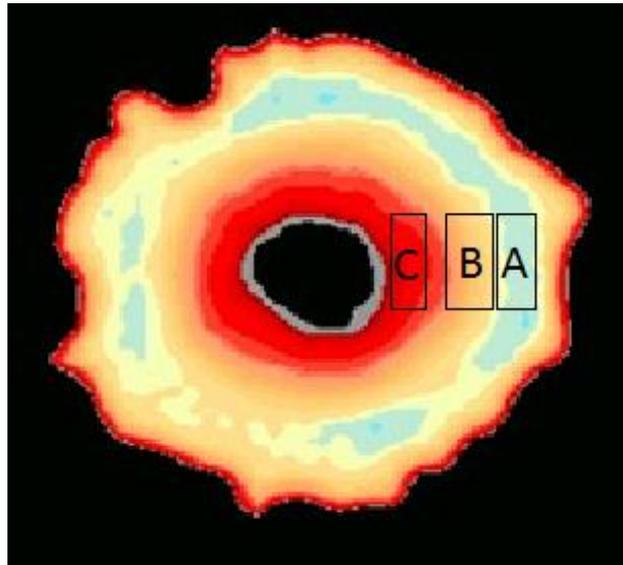
Figure 5.1 – Location from which the antler sections were taken, the banding patterns shown extended the length of the antler.

Samples were weighed and freeze-dried to determine water content, and then cryo-milled (SPEX SamplePrep (6675), UK) to a fine powder under liquid nitrogen. The antler sections (5g) were then demineralised in 20ml of 10% EDTA (pH 7.5 at 37°C) with the solution being replaced every two days over two weeks (Choi et al., 2015). The resulting powder was washed in distilled water and freeze dried before being weighed for mineral contribution. 5mg of each sample was used in a hydroxyproline assay to estimate collagen content. 50mg of bone powder was hydrolysed in 5ml of 6M HCL for 24 hours at 110°C, and dried under vacuum in a speed vac concentrator to remove the HCL. The resulting powder was then re-suspended in distilled water at 15mg/ml for determination of pyridinoline and pentosidine cross-links, and 5mg/ml for determination of aspartic acid for HPLC analysis as in the previous section. The methods used for HPLC analysis are identical to the previous chapter with the pyridinoline and pentosidine as method 1, and the aspartic acid as method 2.

Density of Antler
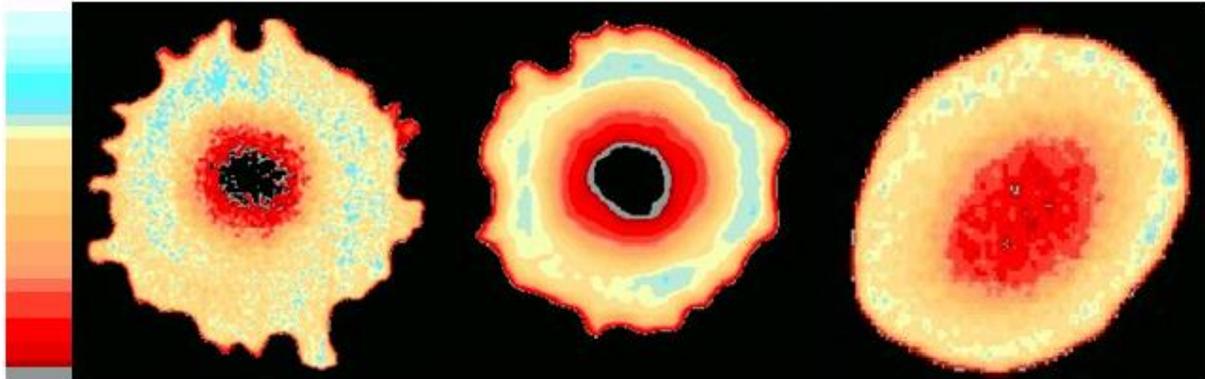
Figure 5.2 – From left to right: the head region, mid-section and tip of the AN6 antler bone, artificially coloured to indicate regions of differing density represented by the scale bar on the left where red is the least dense, and light blue is the most dense

Individual regions (figure 5.2) have distinctly banded areas of increased mineral which begin to fade from the mid-section towards the tip until they are barely present from 80% along the length of the antler. QCT data (figure 5.3) shows that total average density generally decreases from the base to the tip of the antler.
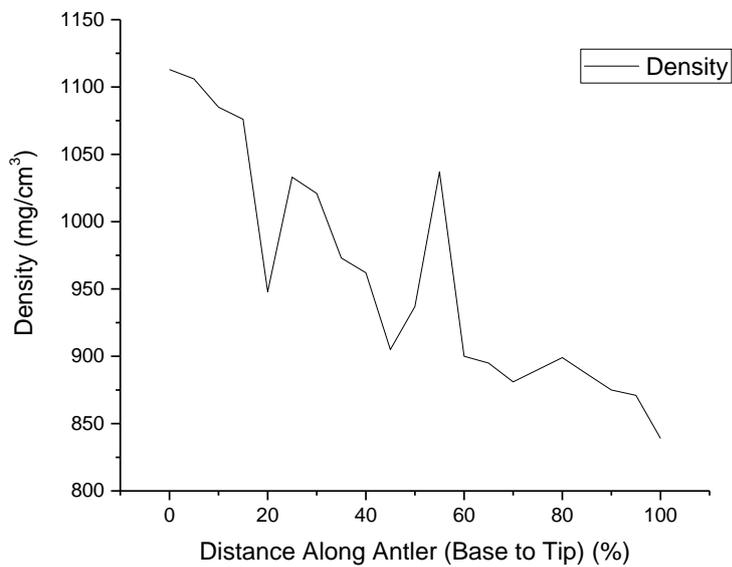
Figure 5.3 – QCT density along the length of AN6

Of interest to note is that in these samples there is no observed correlation between density and mineral content (figure 5.4). Nevertheless selecting regions of interest by density has yielded a spread of mineral content between 66% and 76% suitable for further analysis.
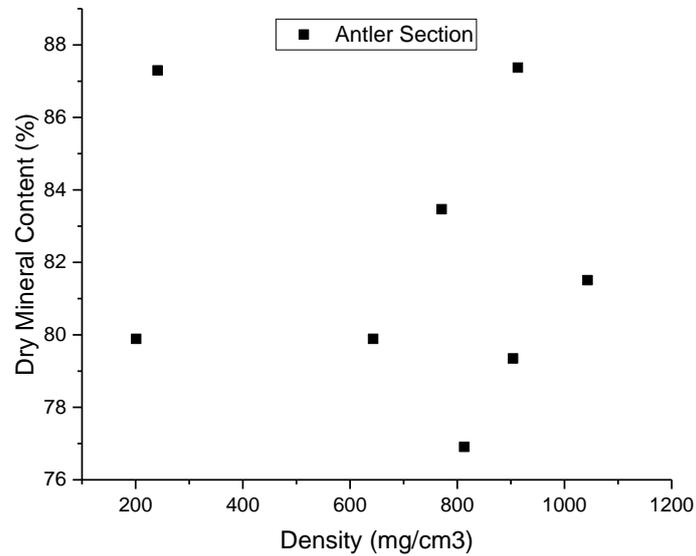
Figure 5.4 – Density and dry mineral content in antler sections

Mineralisation Levels and Cross-linking

	D Form Aspartic Acid (%)	HP (µmoles/L/collagen)	LP (µmoles/L/collagen)	PEN (µmoles/L/collagen)
AS1	2.11	6.60	1.49	0.035
AS2	2.33	5.97	1.12	0.058
AS3	2.32	8.01	1.53	0.026
AS4	2.17	10.77	2.33	0.014
AS5	2.19	8.53	1.33	0.013
AS6	1.94	10.66	2.20	0.057
AS7	2.49	9.67	1.89	0.078
AS8	2.31	6.27	0.62	0.044
AS9	2.58	11.10	3.76	0.159

Table 5.5 – Quantity of aspartic acid and collagen cross-linking in antler sections

Table 5.5 describes the quantity of collagen cross-links and aspartic acid across the antler sections. The levels of mineralisation do not appear to correlate very strongly with the concentration of HP (figure 5.6), unlike the previous chapter there is no significant relationship between the HP:LP ratio and mineral content. The concentrations of LP and pentosidine, as well as the quantity of D-form aspartic acid appear to have no significant relationship with mineralisation (table 5.7).
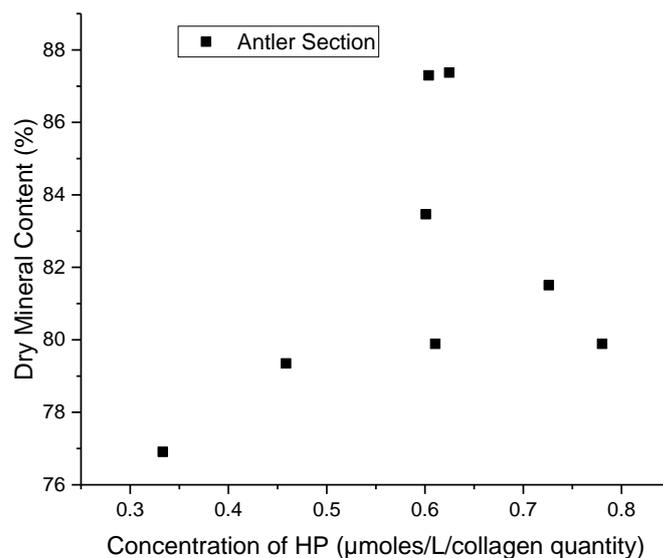
Figure 5.6 – The relationship between HP and mineralisation levels

Comparison	Partial Correlation
HP concentration and mineral content	0.76*
LP concentration and mineral content	-0.51
HP:LP Ratio and mineral content	0.34
Pentosidine concentration and mineral content	0.30
D-form Aspartic Acid and mineral content	-0.30

Table 5.7 – Partial correlations with each other factor removed for collagen chemistry and mineral content, * indicates statistical significance using a two tailed test

A subset of antler sections taken from the mid-sections of the antler are found below, with matching zones A-C as found in figure 5.1.

Antler Section	Density (mg/cm^3)	Dry Mineral Content (%)	HP Concentration (µmoles/L/collagen)	Pentosidine Concentration (µmoles/L/collagen)
AS3 (A)	904	69.36	0.46	0.09
AS4 (B)	643	70.09	0.61	0.13
AS5 (C)	201	75.63	0.78	0.12
AS6 (A)	1043	74.09	0.73	0.15
AS7 (B)	771	70.95	0.60	0.12
AS8 (C)	241	68.30	0.60	0.06

Table 5.8 – Antler section chemistry

5.4 Discussion

In the previous chapter, the HP:LP ratio was a strong predictor of levels of mineralisation across three different bone types. However, in this small data series the ratio appears to have no discernible effect on mineral levels, but the HP concentration on its own demonstrates a relationship with mineral content. Therefore it may be that the levels of LP in these tissues is unrelated in terms of mineralisation, but the quantity varies making the ratio of HP:LP an ineffective method of assessing mineralisation. LP cross-linking is nearly exclusively found in calcifying tissues, which has led to an assumption that it is important to the mineralisation process (Bueno & Glowacki, 2011), however it is possible that it exists as an antagonist to HP concentrations, preventing over-mineralisation. The quantity of total HP (table 5.7), irrespective of the LP cross-links, has a linear correlation ($R^2 = 0.787$) with mineral levels. This suggests that HP cross-linking is an integral part of collagen organisation, facilitating mineralisation by creating a packing environment more suitable to the deposition and maturation of mineral crystals. One important aspect of increased cross-linking is that it prevents intermolecular slipping during deformation, which may provide a more suitable environment for tight packing of the mineral component (Uzel, 2011), thus encouraging mineralisation.

Pentosidine levels in these antler sections appears to have no discernible correlation with mineral content. This could be due to the fact that antler in general has many times the amount of pentosidine than the other tissues examined in the previous chapter, and that while indicative of strength and a factor in mineralisation levels, small variations do not have as large an impact at the concentration of HP. A study on human bone comparing type-1 diabetes mellitus and controls found that levels of pentosidine increased in diseased

patients, and was associated with an increase of mineralisation levels in trabecular bone (Farlay et al., 2016). The influx of glucose from an elevated blood sugar content is the root cause of the additional pentosidine formation in diabetic patients, leading to an elevated risk of fracture of up to 12 times the normal rate. This is contrary to the current observations, however it may be that there are other reasons for the increased levels of mineralisation associated with the pathology.

Due to the nature of isolating the bone sections and the densities involved, materials testing would have been ineffective, and so it cannot be said that pentosidine would have had a significant relationship with bone properties either. A limitation of the current chapter is that it assumes these sections based on density, are to some degree linked with different mechanical needs. An antler is strictly used for fighting, and therefore the most likely force it would encounter is a parallel blow from another deer. This would explain to some extent the design of the antler banding, a softer outer layer followed by a more rigid layer allows for a crumple-zone effect similar to that found in cars, whereby force is absorbed by a layer that is able to compress before passing to the rigid zone, which confers the strength of the material. The following inner layers becoming further reduced in density would allow for whole bone flexibility, as well as the essential cavities to provide a blood supply. Finite element models would assist in the prediction of force transmission, and help explain why certain areas of bone might have evolved to better accommodate these forces.

The thesis hypothesis expects that collagen chemistry has an impact on both mechanical properties and mineralisation levels, however the main driving force behind the design and remodelling of bone material is that it is fit for its purpose in nature. Therefore an accurate understanding of the force transmission that bone experiences is essential to understanding why the material is organised as it is, and what role chemical diversity of collagen plays in achieving this. FEM models of antler do not yet exist but are under development, and would greatly assist in piecing together a complete understanding of bone composition (de Bien et al., 2012). While FEM has drawbacks in that it is purely theoretical and based on the input of methods that do not capture every aspect of bone strength (such as X-ray techniques), it would provide a welcome additional comparison point.

A subset of the antler section data found in table 5.8 describes the density of the material in relation to the obtained collagen chemistry values. While the density follows a similar pattern, decreasing towards the centre, the levels of mineral, HP and pentosidine do not necessarily follow suit. In the proximal end (AS3, 4, 5) as density decreases the mineral content rises, whereas the opposite occurs in the mid-shaft, with the mineral content decreasing as density decreases. Likewise the concentration of HP acts the same as the

mineral concentration, and pentosidine appears to have no relation to the observed changes. This demonstrates an interesting disharmony between the organisations of bone material, i.e. the amount deposited within a given space, and the chemistry of the material itself, which lends credence to the idea that density is not an appropriate medium through which to measure bone quality.

5.5 Conclusions

This chapter focussed on the idea that regions of a single bone are independently adapted for purpose, and that collagen chemistry would change between these areas, resulting in a different cross-link profile and as a result, mineralisation levels. The following conclusions can be drawn from this chapter:

- The HP cross-link has a strong relationship with the quantity of mineral
- The LP and pentosidine cross-links do not have a relationship with the antler sections in terms of mineral content

It was found that the HP cross-link had a very strong correlation with the amount of mineral present across a range of tissues with differing mineral levels from the single bone, and that the LP cross-link varied without apparent effect on mineral levels, affecting the HP:LP ratio which was shown to have a strong statistical relationship to mineral levels in deer bone. Pentosidine, which was a strong predictor of both mineralisation and bone properties in the previous chapter, did not have a relationship with mineral content in these sections.

This supports the overall hypothesis, building on the previous chapter, by suggesting that the HP cross-link has an inherent role in controlling mineral deposition, either by means of the way in which collagen is organised due to its presence or by a chemical or nucleating function. This highlights the need to determine whether the mineralisation process is affected by the combined observations of previous chapters in changes to collagen chemistry. The next chapter aims to better understand the mineralisation process by means of artificially mineralising demineralised tissues in order to assess the impact of the observed differences in collagen chemistry.

Chapter 6 – The Impact of Collagen Chemistry on Re-mineralisation In-vitro

6.1 Introduction

Measurements in tissue samples can reveal associations between collagen cross-links and bone properties, but cannot discern cause and effect. Previous chapters have shown that HP, alone or in the form of the HP:LP ratio, has an association with mineralisation and bone properties, however it cannot be said that it is the control mechanism for these features of bone. In this chapter we explore an in-vitro model which seeks to create an artificial mineralisation process which can be used to draw conclusions from the materials which are re-mineralised.

Prior experiments in this thesis have associated levels of the HP cross-link strongly with levels of mineralisation. Therefore it is hypothesised that some aspect of this cross-link promotes mineralisation, and that a tissue with more HP (and thus a higher HP:LP ratio) cross-linking will more readily take up mineral than one with less, taking into account the starting quantity of mineral. The process of bio-mineralisation is complex and involves a number of cellular processes, and has many influences from whole body homeostasis of the necessary components to high-influence proteins in the extra-cellular matrix. This procedure is extremely difficult to replicate and while possible to some degree, involves the control of many factors outside the capabilities of this study. Therefore this experiment requires a simplified process which can imitate mineralisation and provide some insights into the relationship between mineral uptake, and collagen chemistry.

Mineralisation

The process of calcification is intricate and not yet fully understood, but has a huge clinical relevance in terms of fracture care and prevention. Inducing mineralisation and increasing bone material content in patients with osteoporosis has long been a clinical goal, with treatments such as bisphosphonates disabling the function of osteoclasts to prevent resorption of bone. Thus enabling osteoblastic activity to produce a more dense material (Drake, Clarke, & Khosla, 2008), although the cessation of resorption has a number of side effects including brittle bones due to the lack of repair and replacement caused by the lack of osteoclastic activity, it is currently the best treatment for osteoporosis. There are a significant number of alternative treatments including fluoride, hormone replacement and vitamin supplements, but none have wholly positive effects on increasing bone density without associated fragility issues (Faibish, Ott, & Boskey, 2006). Although simply increasing the quantity of bone material does not necessarily aid whole bone mechanics, it is a step towards prevention of fractures (Roschger, Paschalis, Fratzl, & Klaushofer, 2008). An important aspect of mineralisation is the concept that it happens in two major stages; during

initial formation where osteoid is deposited and immediately mineralised, and the ongoing mineralisation that can take weeks or months to complete once the material has been deposited (Boivin & Meunier, 2002). Therefore, the cessation of osteoclastic activity would not be wholly effective if it is the secondary phase of mineralisation where mineral is not being adequately deposited. Hyper-mineralisation is also a relevant issue in the pursuit of increasing bone strength through mineral levels, although this only occurs in pathologically altered organic matrix or as a result of abnormal hydroxyapatite crystal growth (Abou Neel et al., 2016).

Artificial mineralisation is of key importance in a number of fields, but particularly relevant to creating bone grafting materials, as they commonly lack the mechanical properties required to function successfully in bone (Ishihara, Okazaki, Akiyama, Akasaki, & Nakashima, 2017). Therefore a number of studies have focussed on creating a collagenous substitute that could be naturally mineralised (Ngiam et al., 2009), or re-mineralising demineralised bone material in an attempt to create a method that could be applied to graft materials, or indeed in patients. A common area of focus in the literature is on dentine (the hardest component of teeth found beneath the surface enamel), a substance very similar to bone which is extensively mineralised, where fluoride and calcium salts are the primary means of increasing mineral content (Li, Wang, Joiner, & Chang, 2014), this has been achieved by a number of methods involving pH cycling in solution and artificial saliva substitutes to facilitate remodelling (Li et al., 2014; Shetty, Hegde, & Bopanna, 2014). However re-mineralising in studies where samples were first demineralised from full their healthy starting total rarely achieved higher than 40% of initial mineral levels, although succeed in creating up to a tenfold increase in material strength compared to the demineralised state (Soicher, Christiansen, Stover, Leach, & Fyhrie, 2013).

Another important aspect of re-mineralisation is a better understanding of what factors can be modified to increase mineralisation levels. Many studies have observed that fluoride compounds of hydroxyapatite are more resistant to lactic acid action and increase levels of mineralisation, as well as concentrations of amorphous calcium phosphate among other chemicals (Skrtic, Antonucci, Eanes, & Brunworth, 2002; Skrtic, Antonucci, Eanes, Eichmiller, & Schumacher, 2000), leading to the development of dental implants, or filling materials which contain high concentrations of these additives to enhance natural mineralisation (Ferracane, 2011; Skrtic, Antonucci, McDonough, & Liu, 2004). These approaches focus very much on the mineral aspect of mineralisation, that is to say they attempt to manipulate the process through the construction of hydroxyapatite substitutes, taking advantage of its highly modifiable nature. One study has observed modified collagen ex-vivo in an attempt to create a suitable material for implantation, through the use of

polylactic-co-glycolic acid which enabled the gel to achieve higher levels of mineralisation when submerged in mineralising solution (DeVolder, Kim, Kim, & Kong, 2012). However no approaches have yet made an attempt to modify existing collagen cross-linking levels, such as HP, or observe a set of materials in which the HP concentration is known to verify its impact.

<u>Hypothesis</u>

<u>An increase in hydroxylysylpyridinoline levels in type-1 collagen increases the amount of mineral that can be taken into bone material</u>

<u>Objectives</u>

1. <u>To demineralise and re-mineralise sections of bone tissue that have a varied hydroxylysylpyridinoline content</u>
2. <u>To assess the rate of mineral uptake both in total quantity and percentage</u>
3. <u>To examine the relationship between HP cross-link levels as measured in previous chapters and amount of mineral absorbed by demineralised bone tissue</u>

<u>6.2 Materials and Methods</u>
<u>Bone Sample Preparation</u>

Samples in this experiment were taken from the same metacarpals and antlers used in previous experimental chapters. Cortical bone was excised using a diamond saw blade from the mid-shaft of each metacarpal (n=6) and each antler (n=6). The cut sections were then machined to a specification of 20x4x4mm using a model G0781 Mill (Grizzly Industrial, USA), ensuring parallel surfaces and comparable surface areas. Tissue hydration was maintained during machining to prevent burning or drying out of material using PBS. The beams were then weighed and demineralised in 20ml EDTA (10% 0.27M, pH 7.5) for 4 weeks at 37°C, with the solution being changed every 2 days. Bones are referred to in the results section with the codes AN (antler) and MC (metacarpal). The demineralised weight was then recorded before beginning re-mineralisation.

<u>Alternating Immersion</u>

Cortical beams were subjected to 200ml of 0.55M calcium chloride (10043-52-4, Merck) solution, and 0.5M sodium phosphate (7558-79-4, Merck) solution sequentially in order to form the naturally occurring calcium phosphate crystals within the bone (Soicher et al., 2013). Samples remained in each solution for 35 minutes before being transferred to the alternate solution. This was repeated for a total of 120 hours (with an expected end point of 96 hours), with the bones being removed and examined at each 24 hour step for mineral

content by freeze-drying the sample for dry weight. Each weighing step was preceded by a washing step using deionised water, and a light brushing to remove any loosely attached surface material.

Raman Spectroscopy

The beams were analysed by a Renishaw InVia microscope (Renishaw, UK) using an x50 objective, and a laser of 830nm at 100% power (approximately 10mW at sample). As polarisation effects have been noted in this particular technique and instrument, the orientation of each beam was matched to ensure comparability. Spectra were recorded at 10 second intervals, with 12 spectra collected per sample location, at 3 sites on each beam (to provide an average of the material) for a total of 360 averaged spectra per bone sample. All spectra acquired were subject to baseline correction using a third order polynomial performed in Matlab (The Mathworks, USA). Samples were observed prior to demineralisation, immediately after, and then again at the 120 hour time point.

Statistical Analysis

A student's t test was performed to assess the difference between population means.

6.3 Results

The starting weights and the total mineral content for each beam given in the table below (table 6.1).

Beam	Initial Weight (mg)	Demineralised Weight (mg)	Mineral Content (%)
AN1	87.56	22.73	74.04
AN2	64.89	18.37	71.69
AN3	124.78	36.52	70.73
AN4	107.69	31.59	70.67
AN5	81.02	23.54	70.95
AN6	82.82	24.62	70.27
MC1	148.83	34.61	76.75
MC2	250.62	57.97	76.87
MC3	154.21	34.57	77.58
MC4	207.32	57.36	72.33
MC5	144.07	32.01	77.78
MC6	156.34	35.34	77.40

Table 6.1 – Bone beam starting mineral contents

As expected the experiment reached its conclusion over the 96 hour period, with no further uptake of calcium phosphate in the 96-120 hour period. The first 24 hours displayed the smallest uptake of mineral by weight, while the 24-48 and 48-72 hour categories had varied uptake. Metacarpals continued to regain mass during the 72-96 hour period, while the antlers (with the exception of AN2) finished regaining mass during the 48-72 hour section.
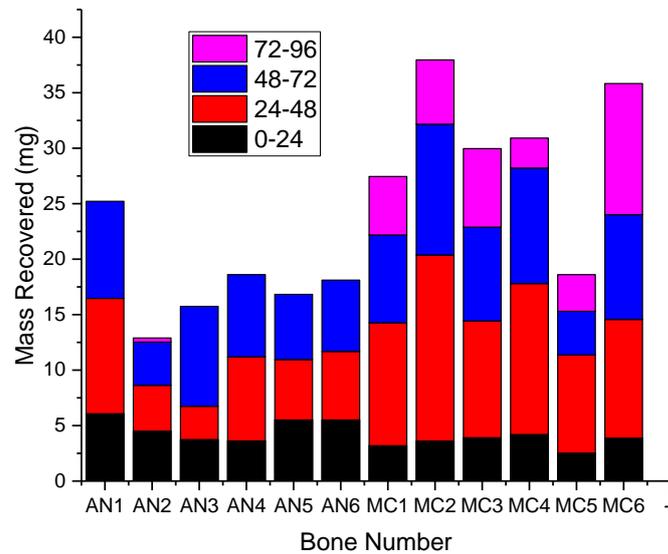
Figure 6.2 – The amount of mass recovery over the 96 hour period

When the percentage gain of mineral was observed it was clear that the antler had regained a larger proportion of its starting mineral, and had done so at a faster rate (table 6.3), confirmed with a t test for total recovery (p = 0.0055).

Percentage Mineral Regained	Antler	Metacarpal
Mean	41.30	26.97
Standard Deviation	8.62	4.60

Table 6.3 – Percentage mineral regained over the 96 hour period

The percentage of mineral regained (figure 6.4) over the time period had varied results, with uptake of mineral not being distributed in an ordered way, other than in the initial 24 hours of mineralisation for the metacarpal group, which was low in each sample.
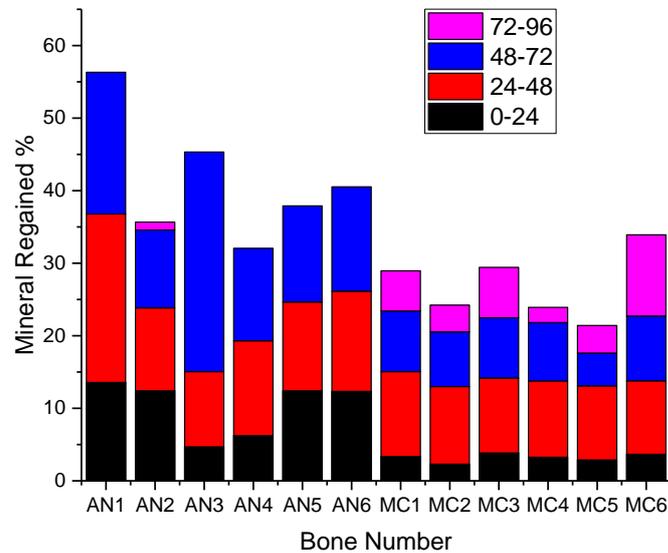
Figure 6.4 – Percentage mineral regained

Raman spectroscopy was performed on the initial beam, then again after demineralisation and a further time on the final re-mineralised sample. The only observable difference in the spectra is the phosphate peak at $959cm^{-1}$ which is present prior to demineralisation, was not observed after demineralisation, and did not reappear after re-mineralisation, suggesting that the calcium phosphate inserted did not form the same arrangement of mineral as native hydroxyapatite. No spectra reported the presence of typical bone mineral peaks after demineralisation. However as the Raman instrument used cannot penetrate to a depth greater than 1 μm it is possible that all the mineral formed was below the surface. Figure 6.5 displays the average metacarpal spectrum, with no observable differences aside from the phosphate peak, suggesting that the demineralisation and re-mineralisation process did not affect any of the organic components. The carbonate band at approximately $1070cm^{-1}$ appears to be much flatter than expected. This is due to a large amount of noise generated from these samples, which was subsequently removed obscuring the peak during polynomial curve fitting. The larger phosphate peak was unaffected as it has such a high intensity as to not be mistaken as interference.
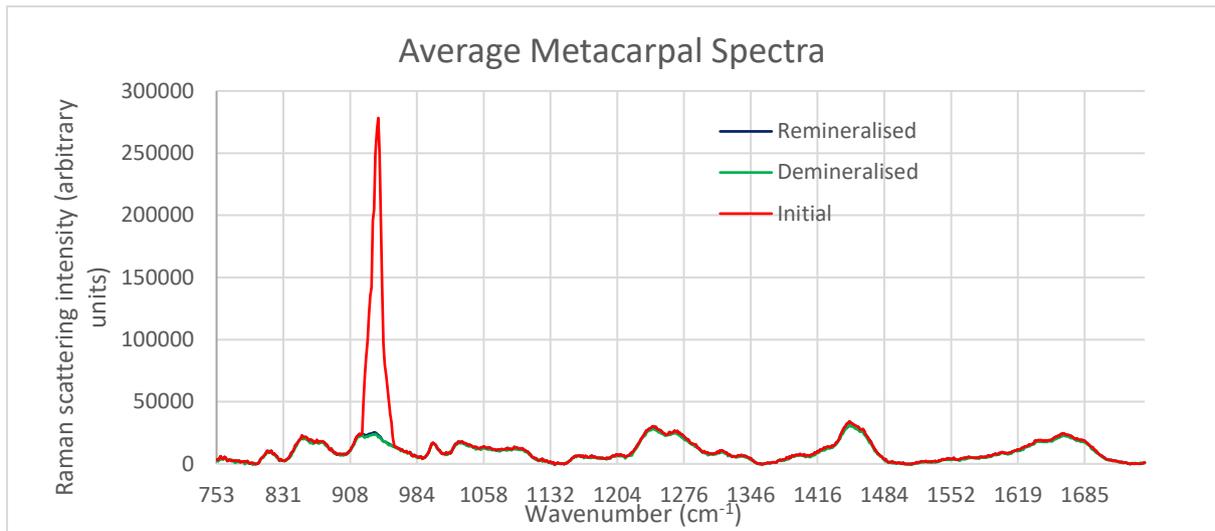
Figure 6.5 – Averaged Raman spectra of the metacarpals (n=5)

Figure 6.6 below displays the relationship between the average HP content per tissue type as described by a representative sample in earlier chapters, against the percentage of mineral regained during the present experiment.
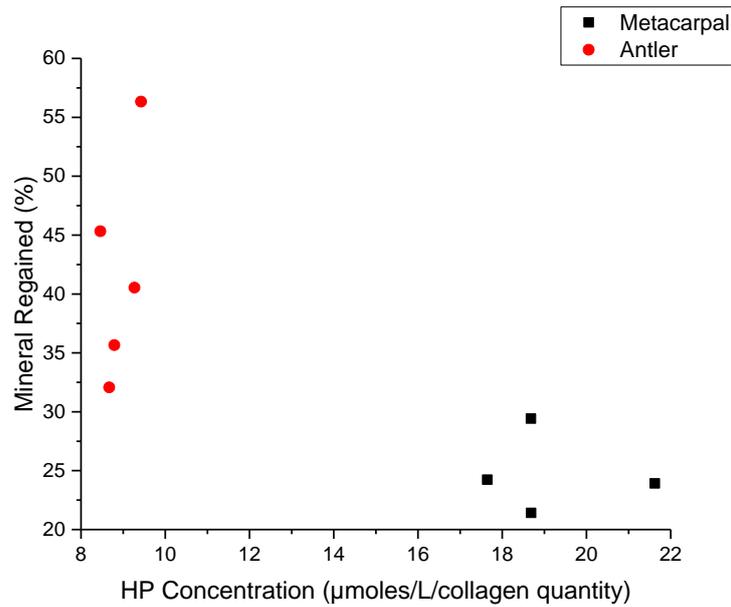
Figure 6.6 – Concentration of HP vs quantity of mineral regained

6.4 Discussion

This study compared the mineralisation recovery of two bone materials with different functions and starting mineral content. It was unable to confirm that the calcium phosphate created is similar to hydroxyapatite, but did demonstrate that the two types of bone, antler and metacarpal, uptake mineral at different rates. The experiment was based on a study by Soicher et al (2013), who showed that this method successfully mineralised bone beams that had been demineralised, and has expanded on it as a method of comparing the speed and amount of mineral recovery in different tissues.

The insertion of calcium and phosphate separately in solution would ensure that the molecules were small enough to penetrate the collagenous layers, in order to precipitate directly into spaces left by the natural mineral that was removed (Talham, 2002). If this had happened we would have expected to see both groups uptake mineral at a rate relative to the amount of space available, with a decreasing rate of uptake at subsequent time points as less space becomes available. However it appears that the less mineralised antler group regained a larger proportion of its mineral content overall, and at a faster rate than the metacarpal group. Therefore some aspect of the organisation of the material, potentially collagen chemistry, within these materials facilitated a faster uptake of mineral within antler, as well as an overall total amount regained. This is surprising as metacarpal is the naturally more mineralised material, with an established advantage in its collagen chemistry shown in previous chapters to agree with mineral levels (HP:LP ratio), while antler had high concentrations of pentosidine, associated with a loss of mineral in osteoporosis.

It is possible that the density of the materials played a role in the amount of calcium phosphate absorbed into the structure. Antler is considerably less dense, which may provide a larger surface area for the nucleation of mineral, however less total mass of mineral was taken up than the metacarpal which is denser. The precipitation of calcium phosphate in-vitro has been shown to be reliant on the banding pattern of collagen fibrils due to the relatively low concentrations found in the body (Koutsoukos & Nancollas, 1987), with the associated mineral remaining in a relationship with the collagen fibrils (Hall, 2005). Therefore it may simply be a case of the material with the most collagen being able to more readily absorb mineral, despite when existing in nature as a less mineralised material. This is likely a limitation of this study, as the method of entry of mineral in-vivo is much more carefully controlled, with ions being directly deposited where they are required. Previous measurements of collagen by TEM in these samples showed metacarpals to have a significantly wider fibrils, as well as perpendicular branching of fibres viewed under SEM which indicate a different arrangement of the collagen which could account for the decreased speed of uptake.

An interesting feature of the uptake of mineral was that while the antler group was relatively similar during each 24 hour period, the metacarpal group exhibited a uniformly slow start. Metacarpal recovered, on average, less than half the mineral of antler during the initial 24 hours (between 2 and 4%), but then increased in speed. This may be due to the lack of porosity compared to antler, with the denser metacarpal requiring more time for the individual calcium and phosphate ions to penetrate deeply enough into the structure before precipitation could occur. After some initial mineralisation, the metacarpal group increased its uptake to between 10-11% in the 24-48 hour period, suggesting that once some mineral had precipitated, it became easier for further crystallisation on the basis that the existing precipitate could be built upon.

Raman spectroscopy failed to detect the new calcium phosphate form of mineral, which has two likely explanations: If the mineral formed did not exhibit the same bonds as the native hydroxyapatite, then we would expect to observe the disappearance of the phosphate peak at approximately 959cm^{-1}. In-vivo calcium phosphate forms amorphous complexes (which could be represented by a widening of the phosphate peak, representing the lack of crystallinity) before becoming the fully formed hydroxyapatite crystal (Mahamid et al., 2008), and while hydroxyapatite is a naturally occurring crystal formed from calcium phosphates, there are many alternative end-products which form different crystal lattice arrangements (Bouyer, Gitzhofer, & Boulos, 2000; Takagi, Chow, & Ishikawa, 1998). This would explain the lack of a particular phosphate peak, and as phosphate has a number of bands across the spectrum, they may be well represented in areas outside the normal range of study (Morris & Mandair, 2011). The alternative possibility is that the surface of the beams did not become re-mineralised. This is potentially a result of the machining process that the beams underwent to create a comparable surface area. While the shaping of the cores was performed in a moist environment to prevent damage to the collagen structure, high temperatures can result from the machining, although approximately 150°C is required to denature mineralised collagen (Bozec & Odlyha, 2011). If the surface was slightly damaged, or the morphology sufficiently altered then mineralisation of the outermost layer may have been impeded. Future experiments could potentially mitigate this issue through the use of SORS technology, to access a greater depth of penetration. Raman spectroscopy was able to confirm that the demineralisation and re-mineralisation process did not significantly affect collagen chemistry.

The gap between the metacarpal with the highest regained mineral and the antler with the lowest is negligible, despite a twofold decline in the quantity of the relevant cross-link. Indeed, looking at the antler in isolation, a change in mineral uptake does not appear to vary

at all with HP concentration. This would suggest that for this particular mineralisation process, the collagen cross-link did not have a significant role.

It is important to factor in that the artificial process used in this chapter did not make use of the natural cell activity associated with mineralisation, and relied instead on a simple model of mineral deposition based on the spontaneous reversible reaction of calcium phosphate formation. Mineralisation is a much more carefully controlled process which utilises a membrane-bound vesicle deposition system to deliver ions to the extra-cellular matrix (Anderson, 1995), and the presence of a chemically different environment in terms of collagen cross-linking can impact cellular activity (Grover et al., 2012). It may be that the complexity of the mechanisms in the natural process of mineralisation are responsible for mineral deposition in such a manner that cannot be replicated by simple means.

6.5 Conclusions

The aim of this chapter was to recreate a simple version of the mineralisation process, and test whether two bones (the antler and metacarpal of the red deer) with markedly different collagen content and cross-linking profiles, to see what impact if any this had on the uptake of mineral. The results indicate the following conclusions:

- The alternating solution method of re-mineralisation does not produce native hydroxyapatite in bone material
- HP concentrations are not associated with levels of percentage re-uptake
- The process appears not to affect collagen peaks on Raman spectroscopy

However due to the drawbacks of this simplified method it is difficult to give these conclusions a strong weighting. While a correlation with the re-uptake of mineral and cross-link concentration could be drawn, it is unlikely that a factor of collagen organisation which has a significant link to mineralisation levels would act as an inhibitor in this experiment.

It is clear there is some link between the way in which collagen is organised and the uptake of mineral. The most likely explanation is the available volume within the antler was higher, and the calcium phosphate did not precipitate solely into the collagenous matrix. However it is not clear whether or not the chemistry of collagen played a role in the mineralisation process at this point, nor is it certain that this particular mineralisation strategy was able to provide an accurate substitute by which to ascertain the involvement of collagen cross-linking. Therefore this chapter does not aid the hypothesis of the central thesis, but has provided some insight into the chemical consequences of this mineralisation technique. Future iterations of this experiment would ideally aim to include the complexities of natural mineralisation into its model. For example it should be theoretically possible to simulate

living conditions for tissue in native bone, and demineralise it while keeping the cells inside the bone material alive and functioning. Then it would be possible to observe the mineralisation process, again ideally within different bone types, is a state as close to in-vivo as possible. This would require some technological developments as it would be difficult to maintain cells in a 3D environment of host tissue, whilst introducing chelating agents such as EDTA, performing spectroscopic measurements, and all without exposing the tissue to infection risks.

Chapter 7 – Bone properties and Collagen Chemistry on the Anterior Axis of the Human Tibia

7.1 Introduction

Bone material is put under many different stresses, but most commonly in the same way, i.e. a femur receiving a vertical load from the pelvic bone (comprised of body weight and gravity). Therefore one would expect it to be adapted to best carry out this task, while retaining the flexibility to perform other necessary functions (for example the femur must be strong enough to also withstand a horizontal force from a fall). In which case the bone properties and architecture of bone along the length of the material would differ based on location, providing the necessary support at different regions. Indeed it has been shown previously that the mineral content of bone varies along the length of long bones (Buckley et al., 2014), however it is unclear how this impacts upon bone properties and whether or not the observed changes are a result of a subtle tuning of collagen chemistry intended to control mineralisation and whole bone mechanical strength.

The bones of mammalian limbs have to be both stiff, to provide a structure capable of supporting huge weights, as well as being structurally resistant to fracture. The nature of these bones is to have a thick cortical shaft with less dense trabecular bone at each epiphysis. The organisation of the bone material is not such that it immediately changes its properties, but has, at the very least, millimetre scale alterations to mineral content and potentially collagen chemistry, which enable the material to withstand the complex stresses of daily use (Buckley et al., 2014). Surprisingly few studies have observed material data along the length of a heterogeneous object such as bone, although some have observed general heterogeneity across a number of similar bones to establish a baseline for expected variation (Currey, Pitchford, & Baxter, 2007). However examining a single line region enables the comparison of very minute changes to collagen chemistry and the resulting properties of the bone. While there is little chemical data available, there are a number of FEM models which predict the reaction of bone under stresses generally based on x-ray data and architecture of the material. These have been used to examine the stresses on human bones when under loading to better understand the mechanics behind stress dissipation (Havaldar et al., 2014). These models can provide a starting point for selecting locations of interest for study, but do rely on x-ray data which is not optimal at predicting bone behaviour.

An important paper to this study was conducted using pQCT technology, taking slices of multiple human tibiae at short distances to build up an understanding of the spread of bone mineral density and bone mineral content (Capozza et al., 2010). It was shown that the central cortical regions of bone material were more densely mineralised, and that this was

directly related to the bones design to perform bending tasks and articulation with the knee joint. This allows us to posit the idea that these changes are driven by small modifications to collagen chemistry, as observed in earlier chapters to have a large impact between drastically different bone types. However it is also important to address the bone properties themselves in relation to changes observed and assumptions made based on an understanding of bone mineral distribution, as we have seen in previous chapters the quantity of mineral in isolation is not a good predictor of bone properties, and the DEXA scan is not a strong predictor of fragility.

In addition to collagen chemistry, this study observes two further organic influences on bone material in the form of deoxyribonucleic acid (DNA), and glycosaminoglycans (GAGs). The total quantity of DNA within a section of bone gives an indication of cellular numbers, allowing for inferences to made on how active a portion of material is metabolically, with multiple methods available for quantification (Ewing et al., 2016; Hansen, Wiemels, Wrensch, & Wiencke, 2007; Pérez-Martínez, Pérez-Cárceles, Legaz, Prieto-Bonete, & Luna, 2017). DNA quantity is found to rise in pathological (Salvianti et al., 2017) and healing tissue (Khan et al., 2008), but to this author's knowledge no studies have attempted to look at total DNA and its relation to rate of turnover in bone as determined by aspartic acid in healthy tissues. It is likely that with increased metabolic activity, the rate of turnover of the material will be higher. GAGs are an important factor in bone morphogenesis and homeostasis, providing stability to hydroxyapatite molecules as well as affecting osteoblast differentiation and aiding in regeneration of bone material (Mathews, Mathew, Gupta, Bhonde, & Totey, 2014; Salbach et al., 2012). GAGs are a form of linear polysaccharide with a repeating base disaccharide containing a hexosamine accompanied by either a hexuronic acid or galactose molecule with various degrees of sulphate modifications (Coulson-Thomas et al., 2015). The current effects of GAGs on bone formation and repair are somewhat unknown as conflicting results have been obtained on their influence on osteogenesis (Mansouri et al., 2017; Salbach-Hirsch et al., 2014), and they have been implicated in a range of pathologies (Banka et al., 2015). The final form of GAGs are highly dependent on cellular processes, the presence of certain enzymes and the quantity of sulphate substitutions, leading to the theory that GAGs are not only unique to a cell type, but can have conflicting roles with GAGs associated with other cell types, explaining the inconsistencies in the literature (Mansouri et al., 2017).

In this study we acquired data across the cortical region on the anterior surface of three pairs of human tibia, for several reasons:

- There is a large body of comparative literature to draw on when discussing human samples that can be readily compared to our findings, including several finite element models which aid in an understanding of the force distribution in the bones observed (Dehankar & Langde, 2009; Liao, Han, & Kuang, 1998). The human tibia in particular has excellent models available which describe a change in expected forces across the central cortical region (Tarapoom & Puttapitukporn, 2016).

- A long region of cortical bone would provide an area of very similar bone material over a distance in which we could reasonably expect a change in bone properties and bone chemistry.

- Pairs of bone present an opportunity for additional comparisons between limbs, which may provide an insight into the environmental impact on collagen chemistry and bone properties as both limbs are theoretically identical.

- Human samples have much higher clinical relevance, and as this thesis aims to contribute to in-vivo applications it is appropriate that samples and methods are chosen that are compatible with these aims.

<u>Hypothesis</u>

<u>Bone properties vary along the anterior surface of the human tibia in tandem with modifications to type-1 collagen chemistry</u>

<u>Objectives</u>

1. <u>To measure the quantity of pyridinoline and pentosidine cross-links, and aspartic acid content on the anterior cortical length of the human tibia</u>
2. <u>To measure bone properties using micro-indentation along the same surface</u>
3. <u>To measure the quantity of DNA and GaGs at the same sites</u>
4. <u>To examine the relationship between the bone properties and the collected chemical information at these locations</u>

<u>7.2 Materials and Methods</u>

The tibiae used in this study are referred to by their age and siding, i.e. 86-R is from an 86 year old patient and is the right tibia, where 86-L is the contralateral tibia of the same individual. The 0% region is the distance from the centre of the bone closest to the distal end of the tibia, and conversely the 100% region is the most proximal, towards the articulation of the knee. Human bones were collected from the Vesalius Clinical Training Unit, University of Bristol with ethical approval (REC 08/H0724/34).

<u>Bone Samples and Preparation</u>

Human tibiae (n=6) were used in this study from donors provided by Bristol Royal Infirmary (Versalius centre), from three individuals aged between 62 and 86, both male and female, and used no less than 2 years after collection. The individual tibia were previously used in another study as control samples, the cause of death is unknown due to anonymity purposes but each individual did not have any known conditions which affected their skeletal health. The bones were cleaned of any soft tissue and stored in a -80°C freezer when not in use, taking care to minimize the number of freeze-thaw cycles they were exposed to in order to avoid damage to the sample (McElderry et al., 2011). The bones in this study underwent three cycles. Any periosteum on the anterior surface of the tibiae was removed and the bone stored wrapped in PBS soaked gauze to maintain hydration.

Raman Spectroscopic Analysis

The spectra were acquired using the Spatially Offset Raman Spectrometer (SORS, Cobalt Light Systems, UK) used in the first experimental chapter of this thesis. An 830nm laser achieved 300mW at 100% power onto a 2mm spot size at 3mm intervals, beginning at 33% on the total length of the tibia and completing at 66%, for between 50 and 60 data points. The tibiae examined were of different lengths and therefore percentage length was used to determine the start and end points. A mechanical platform automatically moved the specimen by 3mm for each scan to ensure accuracy controlled by Labview (USA) software. Each spectrum was acquired by 10 measurements of 30 seconds each for a total of 5 minutes of exposure time. The data was processed by baseline correction using a third order polynomial function, and peak height ratios calculated using the phosphate, carbonate, amide I and amide III peaks.

The Raman data presented in this chapter was collected, pre-processed and ratios analysed by Dr John Churchwell (IOMS, UCL) for a different investigation, currently unpublished, pertaining to the Raman measurements along multiple axis of bone. The measurements, which were taken along the anterior surface of the same tibiae, will be contrasted with the bio-chemical and material testing data gathered by the author.

Materials Testing

The tibia (n=6) were examined using a micro-indentor (Biodent, Activelife Scientific, CA, USA) with a BP2 probe assembly. Each tibia was measured on the flat surface on the anterior side, in a straight line in the centre of the bone. The start and end points for measurements were calculated from 33% to 66% of the total length, in line with the measurements taken by Raman spectroscopy. Each measurement was created through 8 individual measurements taken within a 2mm region along which the Raman instrument scanned to account for the heterogeneity of bone (Jenkins et al., 2015). Each tibia was

measured at 5 points, at 0%, 25%, 50%, 75% and 100% of the total length of the measured central region. Indentations were made with a load of 10N, with 10 repeated cycles at 2Hz. The collected metrics were the total indentation distance, indentation distance increase (taken from cycles 2-10), creep (permanent deformation), and energy dissipated.

Chemical Analysis

The water, mineral and collagen contents were calculated as in the first experimental chapter (section 3.2), through freeze drying, decalcification in EDTA and hydroxyproline assay respectively. The concentrations of pyridinoline and pentosidine cross-links, as well as the concentration of aspartic acid were ascertained through HPLC, with the method described in the second experimental chapter (section 4.2). This took place at the 0%, 25%, 50%, 75% and 100% of the length of the cortical region, at the same locations measured by micro-indentation. The material was extracted using a pillar drill (DP16SL, Scheppach, UK) equipped with a round edged 4mm cavity drill bit so as to extract small cylindrical cores at test sites. Additionally the following two assays were conducted at the same points, using the same material after the processing steps of decalcification and hydrolysis as described in the second experimental chapter:

DNA quantification was performed using a fluorescence assay using a microplate reader (Tecan, UK) (Leggate, Allain, Isaac, & Blais, 2006). Samples were papain digested (buffer Sterile PBS, 5 mM cysteine.HCl, 5mM EDTA.Na2, pH 6.0) at 60°C for 24 hours in a water bath, and compared against a series of DNA standards on a microplate reader using a Hoechst 33258 dye (Sigma) at 0.1 μg/ml (excitation 348nm, emission 457nm).

GAG content (Frazier, Roodhouse, Hourcade, & Zhang, 2008), was measured similarly, following the papain digestion steps above. Content is measured against chondroitin sulphate standards from 0-10μg/ml, absorbance is read at 525nm.

The DNA and GAGs values are expressed as μg/ml/mg of collagen.

Statistical Analysis

Partial correlations were used to observe the relationship between variables without interference from covariates, and multiple linear regressions to assess the overall relationship to mineral content and bone properties.

7.3 Results

Mineralisation
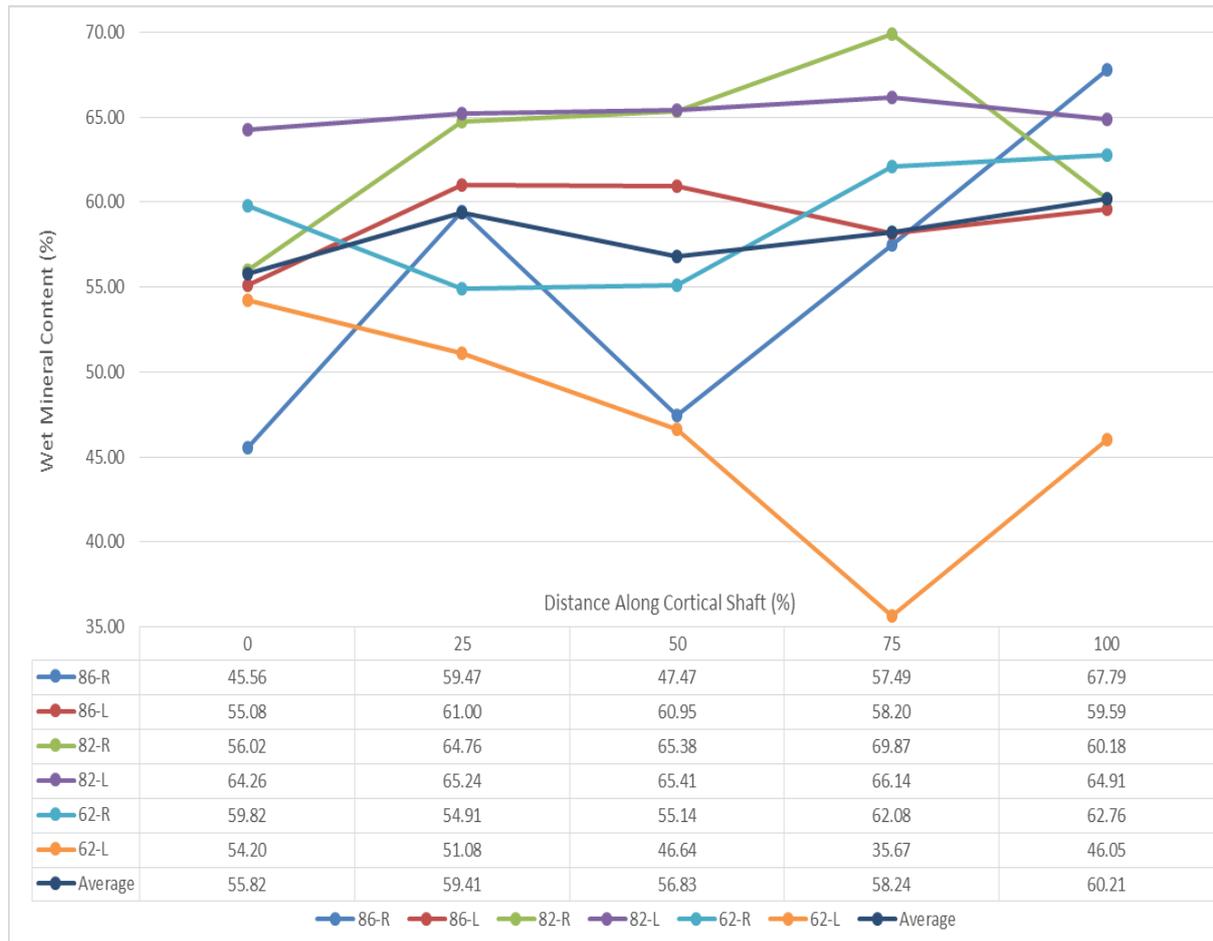
Figure 7.1 – The wet mineral content distribution of each tibia at each location

Mineral levels in ranged from 35 to 70% of the wet weight of bone (figure 7.1), indicating on the lower levels that there may be significant pathologies involved in the health of some of these individuals. There was no recognisable pattern to the amount of mineral based on relative position in the cortical shaft, nor was there a significant relationship observed between the matching limbs. At the highest point single bone variability was above 30%, and on the other end of the spectrum one bone varied by only 2 percentage points at the 5 sampled locations.

The average measurement at each of the locations along the cortical region of the tibiae indicated that across all metrics the central points were on average stiffer, with very high amounts of variation. Figures 7.1 and 7.2 below describe the total amount of indentation, and the repeated effect of indentation from the 2nd to 10th cycle of the process.
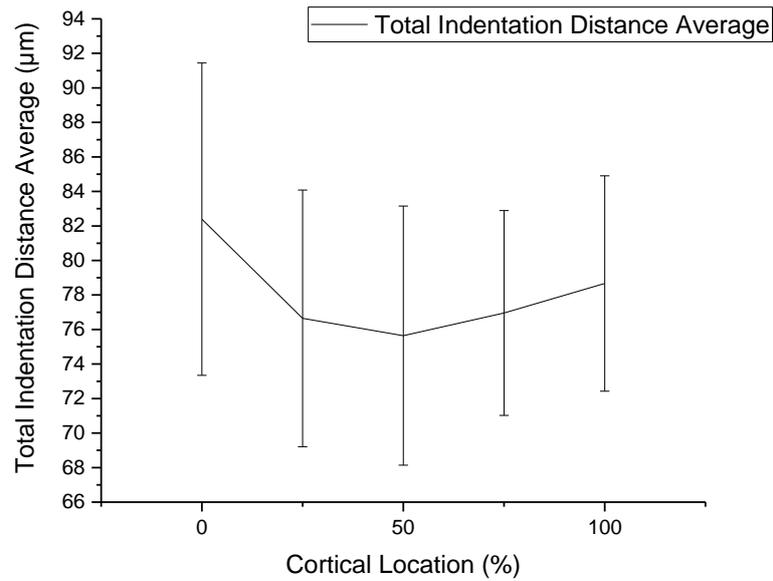
Figure 7.1 – The total indentation distance penetrated average across all tibia
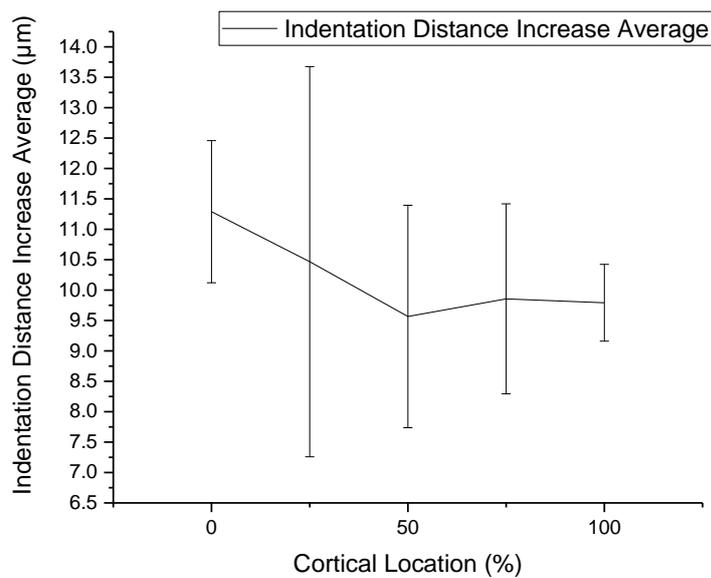
Figure 7.2 - The indentation distance increase penetrated mean across all tibia (n=6)

However the indentation distance increase, the metric more closely associated with gross mechanical properties (Gallant et al., 2013), indicates a continued resistance to penetration in the proximal region of the cortex, greater than in the opposing direction. Although this is in keeping with the general trend of total penetration, it suggests that perhaps the bone properties are not necessarily centred.

<u>Chemical Analysis</u>

In contrast with earlier experiments conducted on young deer bones, in which the HP concentration is far greater than the LP concentration of PYD cross-links at between 3 and 7 times the amount, the concentration of LP in aged human tibia had up to a 5-fold increase over the HP concentration. The graph below (figure 7.5) shows that both types of PYD cross-link follow the same averaged pattern, and that neither experienced large changes in quantity with a large variation per data point.
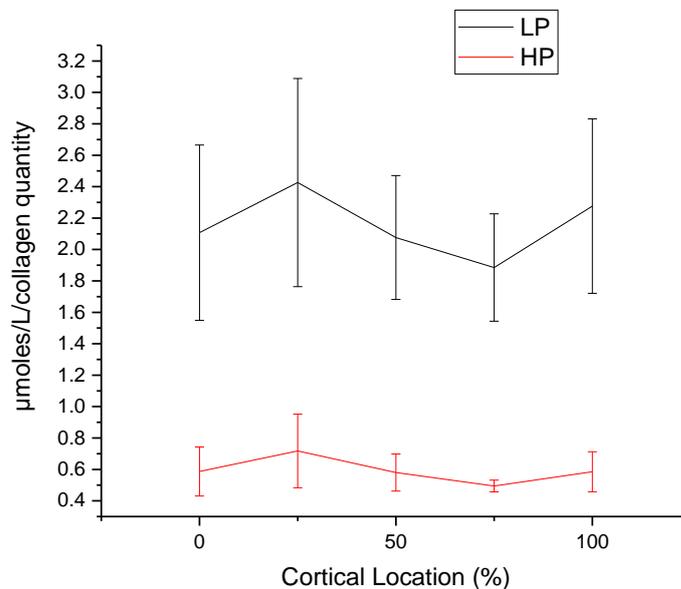
Figure 7.5 – The average concentrations of HP and LP by cortical location

The average pentosidine per collagen concentration (figure 7.6) had considerably higher variation than pyridinoline cross-links, being most present at 25% of the cortical length, towards the distal portion of the cortical region, dipping in the central region before increasing towards the proximal end of the cortical shaft. However, there was also significant variation at each data point.
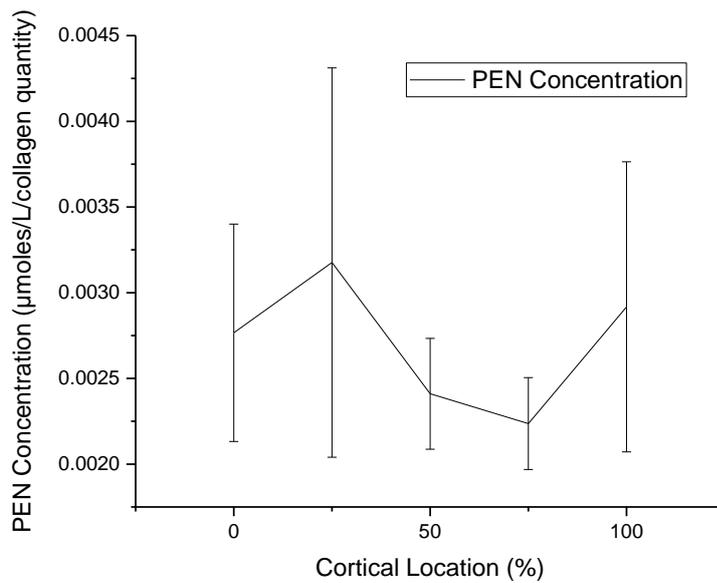
Figure 7.6 – The average concentrations of pentosidine by cortical location

The quantity of D-form aspartic acid is (figure 7.7), on average, higher in these tibia at the central regions of the cortex as well as the ends, with a dip at the 25% and 75% regions. However the standard deviation of the cortical distance points was between 0.75% and 1.49%, indicating a large spread of points for each average, making it difficult to draw conclusions about the average measurement.
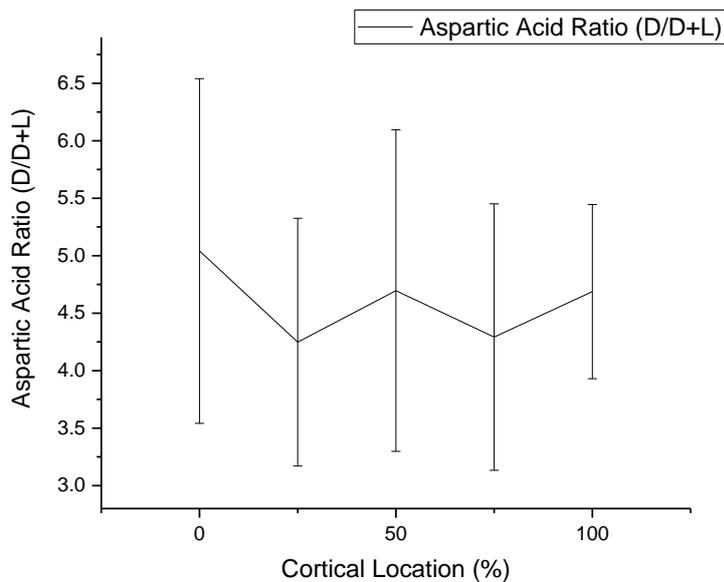
Figure 7.7 – The average concentrations of D-form aspartic acid by cortical location

<u>Raman Spectrometry</u>

Spectral measurements using the Raman ratios of bone (example in figure 7.8) revealed no distinct relationships by location, however they document heterogeneity on the millimetre scale including the large deviation in mineral content. These points cannot be compared directly to chemical analysis nor micro-indentation due to the nature of the heterogeneous material, as the Raman measurements in tandem with the data presented in this chapter indicate that a very slight change in distance can have a large bearing on both material and chemical properties.
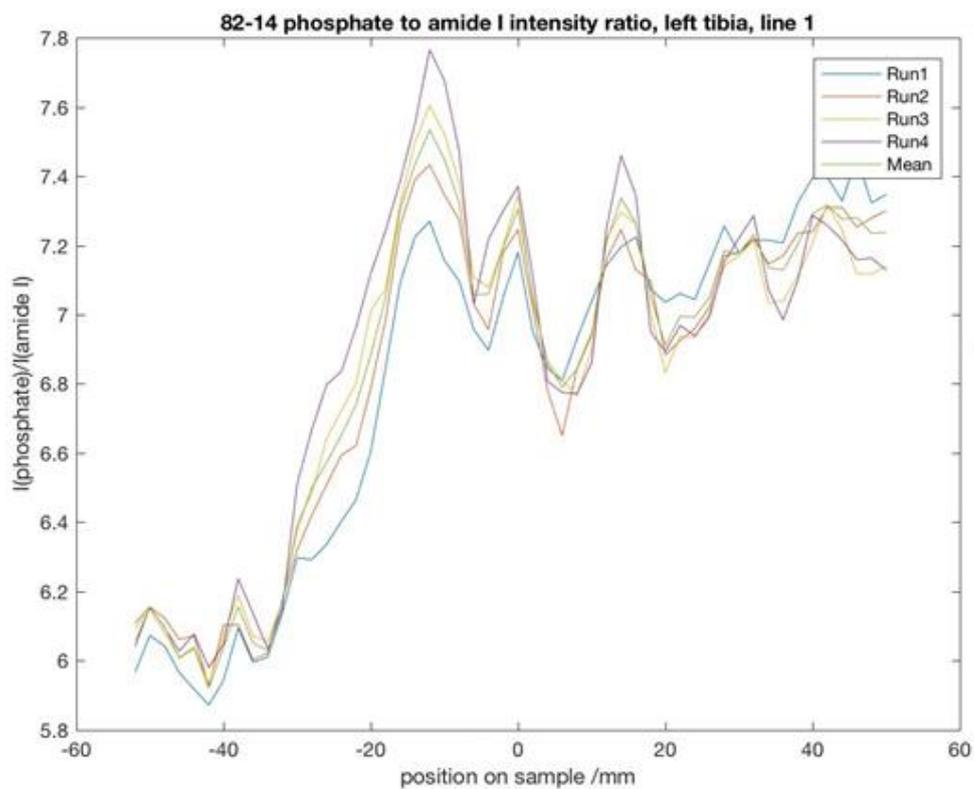
Figure 7.8 – Example Raman spectra displaying the heterogeneity of the mineral to collagen ratio (figure and analysis) provided by Dr. John Churchwell)

<u>Statistical Analysis</u>

The tables below (7.9 to 7.14) describe the relationships between the collected chemical and physical properties of the tibiae and the dependent variables of IDI and mineral content. Each r^2 value reported under the linear regressions is the value adjusted for error.

Comparison	Partial Correlation
HP concentration and IDI	0.35
LP concentration and IDI	-0.30
HP:LP Ratio and IDI	-0.34
Pentosidine concentration and IDI	-0.17
D-form Aspartic Acid and IDI	0.11
GAGs and IDI	-0.03
DNA and IDI	-0.10

Table 7.9 – Partial correlations between bone chemistry and IDI with each other factor excluded, a * indicates statistical significance using a two tailed test

|		Value	Standard Error	t-Value	Prob>|t|
IDI	Intercept	40.23256	14.92427	2.69578	0.01354
	"% D Aspartic Acid"	0.57106	0.34372	1.66141	0.11149
	"HP per Col"	60.58701	19.92859	3.04021	0.00622
	"LP per Col"	-14.61157	5.67835	-2.57321	0.01772
	"Pentosidine"	-824.57227	902.45122	-0.9137	0.37124
	"HP:LP"	-136.14707	48.21558	-2.82372	0.01017
	"GAGs"	1.84519	0.90357	2.0421	0.0539
	"DNA"	-3.60165	4.59577	-0.78369	0.44197
	"Mineral Wet"	-0.03672	0.05288	-0.6943	0.4951

Table 7.10 – Linear regression displaying the relationships of the variables with IDI (r^2 = 0.60)

		Value	Standard Error	t-Value	Prob>|t|
IDI	Intercept	37.20077	13.58964	2.73744	0.01148
	"% D Aspartic Acid"	0.45634	0.21435	2.12891	0.04372
	"HP per Col"	56.42873	18.8606	2.99188	0.00633
	"LP per Col"	-14.48985	5.44426	-2.66149	0.01366
	"HP:LP"	-128.74201	44.91146	-2.86657	0.0085
	"GAGs"	1.22949	0.67048	1.83375	0.07912

Table 7.11 – Linear regression displaying the relationships of the variables with IDI with the least relevant variables dropped (r^2 = 0.49)

Comparison	Partial Correlation
HP concentration and Mineral Content	0.24
LP concentration and Mineral Content	-0.16
HP:LP Ratio and Mineral Content	-0.31
Pentosidine concentration and Mineral Content	-0.26
D-form Aspartic Acid and Mineral Content	0.73*
GAGs and Mineral Content	0.34
DNA and Mineral Content	-0.06

Table 7.12 – Partial correlations between bone chemistry and mineral content with each other factor excluded, a * indicates statistical significance using a two tailed test

		Value	Standard Error	t-Value	Prob>|t|
Mineral Wet	Intercept	106.77215	65.50132	1.63008	0.11955
	"IDI"	-0.18102	1.27645	-0.14181	0.88872
	"Creep"	-10.20449	11.77837	-0.86637	0.39709
	"Energy Dissipated"	0.18381	0.10613	1.73199	0.09948
	"% D Aspartic Acid"	4.92385	1.05651	4.66049	1.70472E-4
	"HP per Col"	101.40496	93.42497	1.08542	0.29132
	"LP per Col"	-18.47305	25.73987	-0.71768	0.48168
	"Pentosidine"	-4311.99294	3590.136	-1.20107	0.24448
	"HP:LP"	-318.10829	216.23583	-1.47112	0.15763
	"GAGs"	6.592	4.22414	1.56055	0.13513
	"DNA"	-5.19582	19.08283	-0.27228	0.78835

Table 7.13 – Linear regression displaying the relationships of the variables with wet mineral ($r^2 = 0.51$)

		Value	Standard Error	t-Value	Prob>|t|
Mineral Wet	Intercept	70.4179	56.04886	1.25637	0.22216
	"Energy Dissipated"	0.1157	0.0881	1.31339	0.20258
	"% D Aspartic Acid"	4.41763	0.89539	4.93376	6.18576E-5
	"HP per Col"	44.9806	76.92766	0.58471	0.56469
	"LP per Col"	-4.48546	22.15984	-0.20241	0.84145
	"Pentosidine"	-3851.51532	2863.27152	-1.34514	0.19228
	"HP:LP"	-200.9566	182.6621	-1.10015	0.28316
	"GAGs"	3.45924	3.01853	1.146	0.26411

Table 7.14 – Linear regression displaying the relationships of the variables with wet mineral content with the least relevant variables dropped ($r^2 = 0.53$)

The partial correlations only revealed a visible correlation between higher levels of D-aspartic acid, indicating slower turnover of bone material, and mineral content. Linear regression analysis to examine the relationships further concluded that D-form aspartic acid was the only significant factor in predicting mineral content. Regression analysis for IDI

determined that the bone property is highly influenced by HP, LP, the HP:LP ratio as well as rate of turnover.

7.4 Discussion

A large issue with taking measurements for any clinical purpose is the known natural variation in human bone tissue. Studies have previously demonstrated that the chemical composition of bone varies along the length of the material (Buckley et al., 2014). Interestingly, the literature also supports the idea that this phenomena can be disturbed by drug treatments such as bisphosphonates which intefere with the bone degradation process, creating a less diverse matrix (Donnelly et al., 2012; Gourion-Arsiquaud et al., 2013). This suggests that these small changes in composition are important to bone health, and in-fact may play a large role in the mechanical properties of bone material. Bone may oscciate between stiffer and less stiff regions deliberately in an attempt to create a material which can better withstand force by allowing the less stiff regions to deform to disperse energy more effectively. The results in this chapter of the observed Raman spectral ratio changes indicate that this heterogeneity is present across each of the bones tested. Changes in cross-link concentrations along the length of the diaphysis may indicate that these cross-links are subtly controlling the bone properties of bone to not only develop a strong material, but to also create a deliberately varied material to better provide areas of energy release and extra flexibility. An added component to this is the rate of turnover of material, which in these tibia has a very strong correlation with mineral content, but not with any other aspect of collagen chemistry. This could imply that the amount of mineral, while potentially reliant on collagen chemistry and the presence of cross-links, is regulated somewhat by turnover, while the collagen chemistry plays a larger role in bone properties.
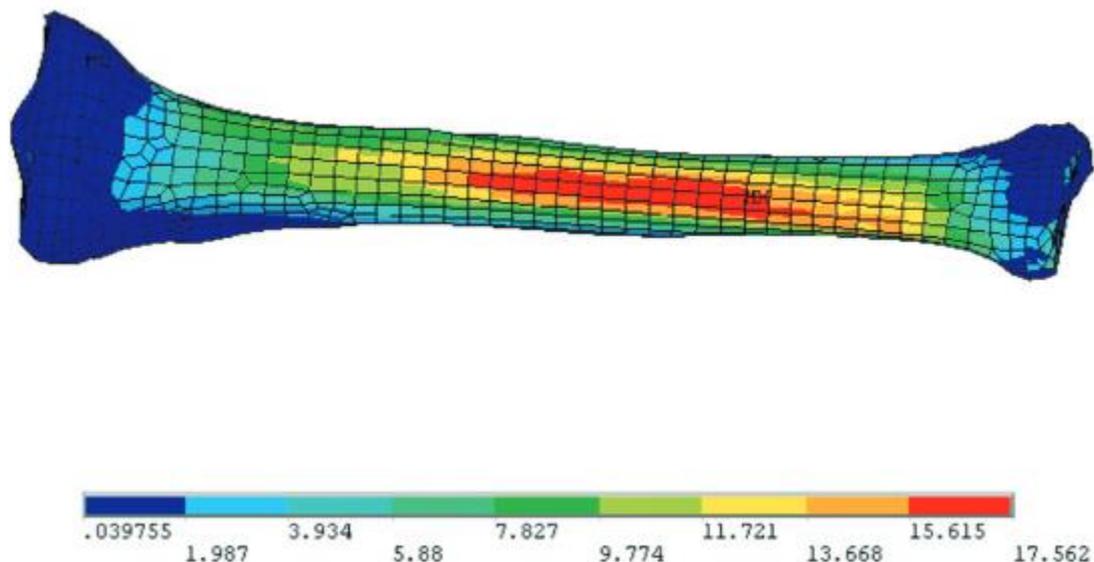

Figure 7.15 – Distribution of stress in arbitrary units, taken from (Tarniţă, Popa, Tarniţă, & Grecu, 2006)

Finite element models of the human tibia have been used to show stress distribution under normal compressive forces (originating from the proximal end, as in a standing human), creating a stress pattern as seen in figure 7.15 (Tarapoom & Puttapitukporn, 2016; Tarniţă et al., 2006). This formed some of the basis of the selection of location for this study, as seen below however, normal compressive stress is borne most in the central region, the area across which the data was collected. We would have expected to observe weaker bone properties either side, with stronger more durable bone in the centre, however this was not the case for bone properties nor the mineral to collagen ratio as calculated using Raman spectroscopy. Although the natural variation in bone makes it difficult to draw conclusions from an averaged tibial model and 6 real-life samples, it would appear that the pattern of stress does not have a bearing on bone properties. However, it is known that material stress encourages bone growth, and that the bone organs respond to stress to encourage remodelling (Nomura & Takano-Yamamoto, 2000; Robling & Turner, 2009), it would be counterintuitive to think that these reactions do not better prepare the material for these stresses. Instead it is more likely that while helpful, finite element modelling is not a sufficient estimation of the forces bone experiences, and the local variation of properties could go far beyond the millimeter scale. There also exists the possibility that bone is not shaped primarily based on the stress it experiences more frequently, and has to adapt to a variety of potential forces in order to best fulfill its functions. A bone that was optimised for walking but could not withstand a horizontal blow in a fight would be a biological failure, and therefore perhaps bones are a compromise of these needs.

Pyridinoline cross-linking, in the form of HP, correlated well with bone properties, as it has done in previous chapters on different material in terms of both species and age, both solo as HP in the case of antler material, and in the form of the HP:LP ratio across all deer material. The fact that this correlation holds true across these variables leads to the validation of the hypothesis that the HP concentration (and HP:LP ratio) directly impacts material strength irrespective of mineral content. However in this chapter, the concentration of HP and HP:LP ratio has no apparent relationship to the amount of mineral, unlike with the deer material, although this could be explained by the much lower base levels of HP present in the older tissue, as the concentrations of HP and LP are inversed between the two groups (with the human tissue having higher quantities of LP than HP). The much larger concentration of LP relative to HP saw LP contribute to the physical bone properties not seen in other chapters. This could explain the negative partial correlation between HP and bone properties. Large LP excretion concentrations have been associated with bone degradation and numerous pathologies including osteogenesis imperfecta (Shapiro & Brennen, 2014), which could explain why high levels were found in ageing bone tissue in this

study. As there is a limited number of potential sites for cross-link formation, a consequence of high LP concentrations would be reduced HP cross-linking and a lowered HP:LP ratio, which has been shown in this thesis to be strongly associated with bone properties and may be involved in creating an environment more suited to becoming mineralised.

Pentosidine concentrations are associated with ageing, where these cross-links tend to accumulate as the turnover of material slows (Chellan & Nagaraj, 2001), therefore it is unsurprising to find it in relatively large quantities, affecting bone. In this study it has no relation to mineralisation levels or bone properties however, while other studies support the theory that pentosidine inhibits the rate of mineralisation (Mitome et al., 2011). The rate of turnover in these tissues appears to be important to both mineral content and bone properties, with a slower rate of turnover being significantly associated with a higher mineral content and increased fragility through further penetration in testing.

As mentioned in the introduction to this chapter, the use of DNA and GAGs as markers of bone properties in diseased tissue have conflicting results in the literature, with both metrics having been found to both correlate with bone properties as well as not be significantly related to them (Marturano, Arena, Schiller, Georgakoudi, & Kuo, 2013; Pfeiffer, Vickers, Frank, Grodzinsky, & Spector, 2008). GAG concentration in this study was found to have an almost significant relationship with IDI of the bones tested, indicating that they may contribute to the bone properties of the material indirectly as a result of potentially inhibiting aspects of normal bone formation (Mansouri et al., 2017). GAGs are known to provide a stabilising role to hydroxyapatite, but, in this study, did not display a relationship with mineralisation levels. The DNA content of the tibiae sites had no statistically significant correlations.

Interestingly this study has revealed that while the average material and chemical properties follow an averaged pattern across the cortical region of bone, the individual displays such heterogeneity of bone composition, that it is difficult to apply this knowledge. One possibility is that this study did not analyse both small enough areas as well as not enough points along the length of the material, as well as not examining enough bones. Due to the destructive nature of testing, as well as the weight requirements for each test it is impossible with the current methodology and technology to isolate smaller regions. This study began with the knowledge that biochemical heterogeneity varies on at least the millimetre scale, however it is clear from the fact that there is an overarching average trend that is not represented in the individual that this heterogeneity is likely environmentally influences by the needs of the individual in day to day life. While percentage distances were used to establish similar points for comparison, the ability of bone material to drastically alter its composition on potentially

the micrometre scale may be the cause of the difficulty in establishing trends for both material and chemical changes along the length of the bone.

7.5 Conclusions

From the samples observed here we can draw the following conclusions:

- Bone material displays changes in bone properties on the millimetre scale
- Turnover of bone has a strong relationship with mineralisation levels and bone properties
- Pyridinoline cross-linking has a relationship with bone properties

This chapter examined chemical aspects of collagen chemistry in relation to mineralisation levels and bone properties in clinically relevant human samples. It found that, similar to previous chapters, aspects of collagen chemistry had significant relationships with bone properties. It is clear that cross-linking is integral to the strength of bone material, and may have a larger impact on bone mineralisation, although in this chapter we are only able to conclude that a low rate of turnover in these samples is the most significant contributor observed to high mineralisation levels.

The data presented here contributes to the understanding of the organisation of bone material, in terms of the distribution of its mechanical strength and aids the central aims of the thesis by supporting the theory that chemical alterations to type-1 collagen play a large role in these bone properties. It did not however add to the notion that these alterations necessarily regulate mineralisation, which is in conflict with data presented in earlier chapters, which will be discussed in more detail in the following general discussion.

Chapter 8 - Main Discussion and Conclusions

This thesis has explored the concept that modification to collagen chemistry impact both bone properties, and has a role in the mineralisation process.

8.1 Methodologies

Probing of Chemical Information Non-invasively

Raman spectroscopy is an attractive solution to in-vivo chemical analysis of bone material, with the potential to non-invasively assess patients with non-ionising radiation. It has been shown to be an effective tool in the detection of small quantities of drugs in the form of spatially-offset Raman spectroscopy (SORS) as well as pioneering use on living human patients in detecting subchondral bone (Feng, Ochoa, Maher, Awad, & Berger, 2017; Olds et al., 2011). Although in this thesis it was used ex-vivo, the principles remain the same and the related challenges to data analysis and acquiring serviceable spectra. As shown in this thesis, Raman has the potential to identify changes in collagen cross-linking chemistry through alignment and secondary structure ratios, which in turn can be used to aid assessments of the mechanical properties of patients. This widens the remit of Raman as a potential tool for assessing fragility in-vivo as well as detecting defects in collagen chemistry, such as those caused by genetic diseases as in osteogenesis imperfecta or other pathologies, such as diabetes mellitus, or indeed osteoporosis, all of which have been shown to have associated modifications in collagen (Gajko-Galicka, 2002; Saito & Marumo, 2010). Recent advances in SORS technology allow it to penetrate the epidermis and access bone material directly with little to no preparation or side-effects, allowing for the probing of chemical information quickly and effectively (Liao et al., 2017; Matousek & Stone, 2016)s. The data presented in this thesis is directly relevant, and will potentially aid in the clinical refinement of this technique.

An issue with Raman technology that needs to be highlighted is the variety of methods by which raw spectra can be analysed and assessed and the amount of interpretation involved. After raw spectral acquisition, which can be affected greatly by orientation depending on the instrument (Kazanci, Roschger, Paschalis, Klaushofer, & Fratzl, 2006), the data must be processed to anchor the unrefined data to the baseline so true peak heights can be determined. However, a multitude of methods exist for this purpose and while broadly similar, the use of different methods on the same set of data can result in differing results (Fan et al., 2016; Guo, Bocklitz, & Popp, 2016; Liu, Sun, Huang, Li, & Liu, 2015). This invariably leads to difficulty in replicating studies, especially when a number of Raman based papers will not specify the baseline correction technique used, nor where the method originated, although in many cases this is presumably to protect the intellectual property of

its creator. In addition to baseline correction there are many smoothing techniques, and methods for the eradication of noise, which can inadvertently alter information depending on the original quality of the spectra acquired. Further to this is the subjective choice of bands used for the interpretation of information. For example, the mineral to collagen ratio can be acquired through comparisons between the main phosphate peak against amide III or amide I, and additionally can be compared using a single wavenumber at the highest point in those regions, or by taking an average of the entire region (Morris & Mandair, 2011). These differences can amount to a drastic alteration of the information contained within a spectrum, and so caution must be employed when contrasting studies and selecting appropriate techniques to use during analysis so as not to manipulate the data repeatedly until the desired result is achieved.

In-vivo Micro-indentation

As discussed in the introduction, micro-indentation is a powerful tool with the capability to take direct measurements of the bone properties of bone material with minimal damage to surrounding tissues. Typically a set of indentation cycles will penetrate in the region of 60-100 microns, and if performed on a location where the bone is close to the surface of the skin, with only a small amount of invasiveness. Figure 8.1 shows a patient undergoing a micro-indentation procedure under local anaesthetic (Diez-Perez et al., 2016). A recent systematic review of all micro-indentation literature pertaining to human studies to date concluded that, although a connection with gross mechanical properties is dubious, the bone properties attained through a range of metrics have been found to correlate with various other mechanical and biological values (Arnold et al., 2017).
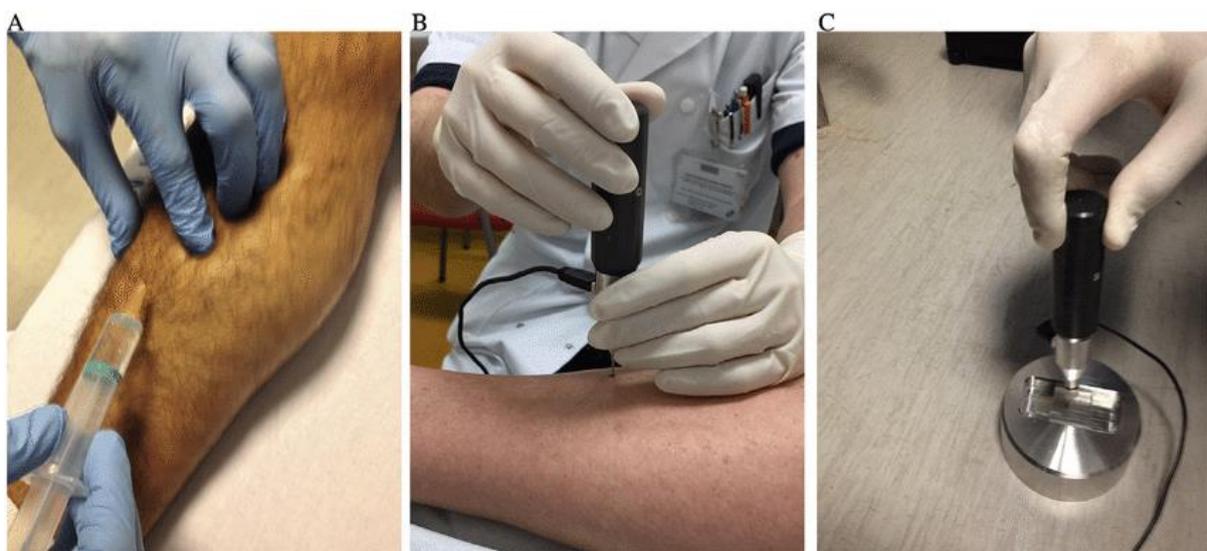

Figure 8.1 – The clinical micro-indentation process of a) anaesthetic, b) measurement, c) comparison to a reference material (A Diez-Perez et al., 2016)

Much like Raman spectroscopy, in which the mineral to collagen ratio has not, and cannot necessarily be calibrated to actual mineral and collagen values, micro-indentation cannot be easily translated into whole bone mechanical properties. Its use is therefore not to provide a surrogate for whole bone mechanical measurements, but to act as an independent technique to provide information on local bone behaviour. In the present studies, micro-indentation has been used both to separate materials based on resistance to penetration, and to observe trends along the length of bones. Values have not correlated well with mineral values, as is part of the overarching hypothesis of this thesis, but have been significantly correlated with collagen cross-linking which is essential to bone strength (Garnero, 2012). A drawback however, is that bone heterogeneity requires that a large amount of measurements (at least 8) be taken for accuracy, which makes the clinical process more invasive (Jenkins et al., 2015), it is not yet clear if the costs in terms of pain and risk of infection outweigh the benefits of this process. The combined efforts of this thesis suggests that micro-indentation is a good tool for the assessment of local bone strength, and is an indicator of the cross-link profile. Although many more studies (such as (Diez-Perez et al., 2010; Herrera & Diez-Perez, 2017; Malgo, Hamdy, Papapoulos, & Appelman-Dijkstra, 2015)) will need to be conducted for the validation of an in-vivo method, and to select suitable sites on the human body that can be easily tested and also are representative of aspects of skeletal health.

<u>Remineralisation Techniques</u>

Chapter 6 utilised a method for re-introducing mineral into a decalcified beam of cortical bone which had been proven to increase bone mass and strength (Soicher et al., 2013). However this method failed to replace the lost mineral in such a way that was consistent with the organisation of the collagen, suggesting it may have just been filling up any available cavity, and additionally did not form a complex similar to that found in naturally occurring bone as Raman spectroscopy failed to identify the expected phosphate peak. Further work in this area could focus on identifying the mineral composite formed here to establish whether it has a place in the chemical pathway towards becoming hydroxyapatite.

Remineralisation of bone tissue is of crucial importance, both from a scientific viewpoint of understanding the mineralising process, as well as from a clinical standpoint in returning lost mineral density to sufferers of osteoporosis. Many studies have created protocols for the remineralisation of tissue similar to the one employed in this thesis (Lata, Varghese, & Varughese, 2010; Qi et al., 2012; Soares, De Ataide, Fernandes, & Lambor, 2017), although there is a substantial gap in the literature pertaining to the remineralisation of bone tissue specifically, with much of the research focussed on teeth. It is clear from this study that

either native hydroxyapatite or a similar compound must be inserted into bone for it to have the desired effect of replacing the original mineral content. Approaches have attempted to facilitate the natural process through the use of saliva substitutes in the case of teeth (Reynolds, 1997), however due to the complex series of biological reactions involved in the mineralisation process, creating a suitable environment will likely prove difficult ex-vivo. Teeth have the unique advantage as a testing platform for bone remineralisation in that they are exposed and easily accessible, leading to developments based on direct applications of chemicals (Lynch, Navada, & Walia, 2004). Bone on the other hand, cannot be directly accessed in the same way and alternative approaches must be considered. The evidence in this thesis suggests that collagen organisation plays a significant role in mineral deposition in healthy tissues, which could be a potential avenue for drug treatment.

8.2 Data

Bone properties and Mineralisation

This thesis opened with the premise that mineral levels control a great deal (up to 70%) of the bone properties of bone, and observed that previous research had found very strong correlations between mineral levels and large scale materials testing using Young's modulus of elasticity (Buckley, 2011). The use of micro-indentation as an alternative means of measuring bone quality allows for an assessment of bone quality in a much larger number of small locations, providing a more suitable overview of a material as many measurements can be taken for a more representative average. It also does not require destructive sample preparation involving creating a flat surface as in larger materials testing, avoiding the pitfalls of this preparation affecting bone behaviour. This thesis found that mineral levels had no significant relation to bone properties when tested across three distinct bone types of deer with differing compositions, nor in a set of human tibia. This suggests that perhaps the original claim of mineral as a defining feature of bone strength is exaggerated, and while mineral content is vitally important to bone properties, it is not a suitable metric by which to judge mechanical strength in isolation clinically.

The level of mineralisation was found to be impacted by collagen cross-linking across the deer bone types, as well as by the rate of turnover of material in ageing human tissue. While urinary cross-links (from the degradation and replacement of bone material) have been a focus of study in determining bone quality in terms of both turnover and the ratio of HP to LP in the case of pyridinoline (Ok et al., 2017), they rely on a whole body approach to assessing collagen health, bearing in mind that pyridinoline cross-links also appear in tendon and cartilage alongside bone. Current patient care is determined by risk of fragility fractures which is assessed by DEXA. DEXA is ignorant of the organic phase of bone whilst also

posing a radiation risk. Current treatment through bisphosphonates have questionable impacts upon fragility rates despite increasing mineral content (Diab, Watts, & Miller, 2013). The research conducted in this thesis would support the idea that analysis of collagen composition is of importance to understanding and treating bone disorders, and that currently the most applicable technique to access this information in-vivo is Spatially Offset Raman Spectroscopy. As SORS is developed and its resolution and sensitivity grows, it may lead towards the identification of therapeutic targets in the future that could improve upon current bisphosphonate treatment. While this thesis cannot comment on potential avenues of research for improving bone quality, it does heavily suggest that increasing mineral content is not a suitable way of increasing material strength and that a focus should be placed on both controlling the rate of turnover of material and manipulating collagen cross-linking.

Collagen Composition and Bone properties

Many studies have commented on the importance of aspects of collagen chemistry because of their roles in disease (e.g. osteogenesis imperfecta), or known accumulation effects in ageing (Pouran et al., 2018), however few studies have effectively explored the relationship that collagen modifications have on healthy tissues with regards to bone properties. This study has shown that bone properties are highly correlated with collagen chemistry in tissues with differing mineral contents, as well as the existence of a local tuning in the same tissues.

Pentosidine is known to be highly associated negatively with bone properties. However the traditional explanation for pentosidine build-up is due to age accumulation, cited as being caused by a drop in the rate of tissue turnover and has even been used successfully to predict the age of individual animals (Chaney, Blemings, Bonner, & Klandorf, 2003). While this seems like a sensible explanation given the data, in this thesis it has been shown that pentosidine concentration has no apparent relation to mineral content or bone properties. It may be the case that pentosidine is controlled for by a mechanism associated with ageing, but that it may also be a deliberate deposition which happens to also be a by-product of the ageing process. It has also been shown here that pentosidine is found in larger quantities in materials with lower levels of mineralisation, in very young individuals. These observations suggest that pentosidine is either a controlled aspect of collagen formation which has an impact on mineralisation, or in tissues with lower levels of mineral it opportunistically forms cross-links due to the availability of unobstructed collagen. Therefore these combined studies open up the possibility that pentosidine has an inhibitory effect on mineralisation, and is intentionally incorporated into materials which require higher flexibility in young tissues. This could be tested in in-vivo cell models in which mineralising collagen gels could be

encouraged to form pentosidine cross-links to see the difference in levels of mineral formed over time.

Pyridinoline cross-linking, in the form of HP concentration, in the literature is already known to have a relationship with the strength of the material (Banse, Sims, et al., 2002), and this was successfully confirmed in experiments in this thesis in both deer and human tissue. In addition, in bones with different biological function, which had differing mineral content, it was shown that the concentration of HP in the form of the HP:LP ratio was a far greater predictor of bone properties over the mineral content, cited to be responsible for up to 70% of material strength (Ammann & Rizzoli, 2003). Many studies have confirmed higher HP than LP levels across a range of tissues (bone, tendon, ligament, heart valves) and conditions, but none have shown LP levels higher than HP as found here (Bank et al., 1999; Marturano, Xylas, Sridharan, Georgakoudi, & Kuo, 2014; McNerny et al., 2015; Takahashi et al., 1994; Uebelhart, Gineyts, Chapuy, & Delmas, 1990; van Geemen et al., 2016). This suggests that perhaps ageing tissue cross-links need to be observed in more detail, and that renal excretions may not be representative of bone cross-links.

Chapter 3 visually presented data concerning the physical appearance and organisation of collagen. It was observed that the metacarpal, the bone under the most stress in the group, had wider collagen fibrils and it was also noted that under SEM it was possible to see perpendicular collagen fibres not found in the other bone types. It is likely this confers an additional amount of strength and stiffness to the material, which is possibly why it displayed bone properties similar to that of a much more highly mineralised bone. The reasons for this change in organisation are unclear, while the metacarpal group had a differing cross-link profile and wider fibrils, a more detailed study would have to be undertaken to assess exactly why this occurs and how it impacts bone properties.

Mineralisation

Many chapters have commented on aspects of collagen chemistry, which potentially relate to mineralisation, and the strength conferred by the mineral component of bone. Only chapter 6 looked at the actual mineralisation process and attempted to create a simple replication to gain an understanding of how the organisation of collagen can facilitate mineral uptake. While the artificial method was a simple one, it did demonstrate that something other than the volume available dictated how much calcium phosphate was taken and held by the matrix which is assumed to be as a result of collagen organisation and chemistry. Models of the mineralisation process have concluded that the sequence of amino acids play a large role in creating an electro-chemical environment which aids mineral nucleation (Tavafoghi & Cerruti, 2016), therefore it is likely that the enzymatic and AGE cross-links discussed in this

thesis have an impact on the network of chemical charges which facilitate the precipitation of calcium and phosphate into the matrix. It is probable that aspects of these cross-links themselves also facilitate or inhibit mineralisation by providing binding sites for calcium or phosphate ions (George & Veis, 2008). However there is a lack of studies that have explored the mechanisms by which cross-links act on mineralisation, though this is likely related to the fact that there is not yet a consensus on the subject of mineralisation as a whole. Regardless, the data collected in the present studies suggests that the cross-linking of collagen has an impact on the mineralisation of healthy tissues, and may be used to modulate both bone properties and degree of mineralisation throughout bone.

8.3 Future Studies

There are many avenues of research in bone that have not been adequately explored. This research has raised further questions and areas of interest that should be addressed in the future:

SORS

The Raman instrumentation used in this study has proven its potential to discriminate between types of collagen cross-linking (see chapter 4, in which the pentosidine cross-link concentration correlated significantly with the amide III:I ratio and the HP:LP ratio correlated significantly with collagen secondary structure), and which in turn have been shown to be extremely relevant to bone strength. Could SORS be therefore used to not only discriminate between healthy and diseased tissue in-vivo, but also predict mechanical strength of the bones patients and therefore risk of fracture?

Micro-indentation

This study utilised a form of reference point micro-indentation, which is currently being used in clinical trials as a potential in-vivo assessment of bone quality. Initial experiments focus very much on its relation to whole bone mechanical strength, however it could be possible to further the work in this thesis by the combination of micro-indentation and chemical composition analysis to better understand exactly what contribution each component of bone makes to its total strength and which aspects are then lacking in strength-deficient material.

Artificial Mineralisation

The mineralisation process is complex and requires a multitude of active participants in terms of bodily function, and therefore is difficult to replicate for the purpose of re-mineralising tissues which lack mineral content which will be an area of study in the future. However we can also apply artificial mineralisation to cell culture studies. By creating mineralised tissues using bone cells and a collagen scaffold we could effectively modify the

quantity of chemical cross-linking on the cellular level and directly observe the impact this has on mineralisation. In addition to this there are many complexes that can be formed by calcium and phosphate, and as the native hydroxyapatite molecule is too large to effectively fit between established fibrils, further study could focus on identification and testing of a range of compounds to artificially increase bone mass.

Change along Bone Length

In this study we explored only one aspect of the length of a single bone, the human tibia. It was found that there was an interesting order to the bone properties along the length of the anterior aspect, however this would need to be validated due to both a small sample size, as well as being observed only in one aspect of one bone type. In addition to this it would be interesting to take measurements along the circumferences of the bone, as the stresses in a perpendicular direction would be very different and may be represented in how the material is chemically constructed.

In-vivo Cross-link Analysis

One aspect that the potential use of SORS and this thesis have opened up is analysing the collagen cross-linking of individuals in-vivo using Raman spectroscopy. For example it is known that bone strength in an individual is greater if they are subject to regular exercise. It is unknown whether or not this is as the result of remodelling only, or if the new bone is potentially primed for a different cross-link profile which will enable it to provide greater strength or induce a change in mineral levels. This could shed new light on the activity of the osteocyte network and its role in active remodelling.

8.4 Central Thesis Conclusions

The data presented in this thesis adds to our considerable knowledge of bone composition, bone properties and impacting factors on mineralisation, using emerging technologies with clinical applications that have been successfully used from both a scientific standpoint and have been shown to have the ability to diagnostically assess patients. The main research question of this thesis was as follows:

Are chemical changes to type-1 collagen associated with the mechanical properties of bone and levels of mineralisation?

It has been shown that there are significant relationships between modifications of type-1 collagen, in terms of cross-linking, and associated bone properties of bones and levels of mineralisation which has led to the following conclusions:

- Bone material strength is reliant on collagen cross-linking which is modified in quantity to produce the qualities required of it in nature.
- Material strength is better assessed through collagen cross-linking rather than by using bone mineral density.
- Collagen composition likely plays a significant role in determining the mineral content of bone tissue.
- Raman spectroscopy is a capable in-vivo tool which can access chemical information which correlates with the quantity of collagen cross-linking and therefore bone properties.

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