Investigating the pathogenesis of catastrophic humeral fractures in dairy heifers in New Zealand

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Alvaro Sebastian Wehrle Martinez

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Abstract

Catastrophic outbreaks of spontaneous humeral fractures in dairy heifers in New Zealand have given rise to animal welfare problems and resulted in significant economic losses to the New Zealand dairy industry. Preliminary small sample size studies have identified potential causes and/or factors associated with the occurrence of humeral fractures including periods of protein-calorie malnutrition, increased osteoclastic bone resorption related to lactation, and low liver and/or serum copper concentration (suggestive of periods of copper deficiency). Nevertheless, outbreaks of humeral fractures still occur throughout New Zealand with devastating consequences. Therefore, the main objective of this thesis was to investigate the likely causes and/or major risk factors associated with the occurrence of spontaneous humeral fractures in dairy heifers in New Zealand and propose a likely pathogenesis of the condition.

For this, a large cohort of bone samples (humerus and ribs), as well as liver and blood/serum samples were collected from 2-year-old dairy heifers that suffered spontaneous humeral fracture post calving (affected heifers) for comparison with age-matched post-calving heifers with no bone fractures (control heifers). Blood/serum samples, used for the determination of biochemical profile in affected heifers, showed increased β-hydroxybutyrate and decreased creatinine concentration indicative of negative energy balance and/or periods of undernutrition. Bone samples were used for gross, histologic, histomorphometric, Raman and Fourier transform infrared spectroscopic analysis as well as for the measurement of the collagen and collagen crosslink content in

bones from affected heifers compared with control heifers. Histologically affected humeri had osteoporosis (reduction in trabecular volume with abnormal trabecular architecture, thicker growth plates with abnormal architecture, increased resorption in the distal humerus, and a thinner cortex with increased and abnormal resorption. Abnormal cortical resorption is associated with an increased probability of fracture 54.2 times and reduced trabecular density 249.5 times. Spectroscopic analysis indicated decreased bone quality in the humeri from affected heifers with a reduced amount of bone organic and mineral components, lower mineralisation, lower carbonate substitutions, increased bone remodelling, and reduced mineral crystallinity. Analysis of collagen content and collagen crosslinking using liquid chromatography indicated reduced total collagen content and increased collagen crosslinking in the humeri from affected heifers. Finally, a survey was conducted using farms that have and have not had a case of humeral fractures showed Holstein-Friesian Jersey cross breed was a possible risk factor.

The likely causes and/or major risk factors associated with the occurrence of spontaneous humeral fractures in dairy heifers in New Zealand include breed, protein-calorie undernutrition during important bone growth periods (which significantly affected the bone chemical composition and architecture) and increased abnormal bone resorption. These factors have significantly compromised bone mechanical strength and led to the spontaneous humeral fracture.

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Abbreviations

PO_4^{3-}	Phosphate
PTH	Parathyroid hormone
1,25-$(OH)_2D_3$	1.25-dihydroxyvitamin D
ALP	Alkaline phosphatase
IGF-1	Insulin-like growth factor 1
LOX	Lysyl oxidase
deH-DHLNL	Dehydrodihydroxylysinonorleucine
deH-HLNL	Dehydrohydroxylysinonorleucine
PYD	Pyridinoline
DPD	Deoxypyridinoline
PYL	Pyrrololine
DPL	Deoxypyrrololine
FB	Fodder beet
BCS	Body condition score
DMI	Dry matter intake
P	Probability value (statistical significance)
NEFA	Non-esterified fatty acids
BHB	β-hydroxybutyrate
SCK	Subclinical ketosis
LiCu	Liver copper
HFxJ	Holstein-Friesian Jersey crossbreed
MS	Milk solids
CCJ	Costochondral junction
HE	Haematoxylin and eosin
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
CO_3^2	Carbonate
HYP	Hydroxyproline
DHLNL	Dihydroxylysinonorleucine
HLNL	Hydroxylysinonorleucine
DPD	Deoxypyridinoline
PYD	Pyridinoline

General introduction

While bone fractures in livestock occur sporadically, since 2008 an increased incidence of spontaneous humeral fractures has been reported as occurring in first calving dairy heifers throughout New Zealand.[3,11] Preliminary studies of a small number of cases suggested humeral fractures may be the result of unbalanced nutrition during critical growth periods in the first two years of the cows' life, marked osteoclastic bone resorption associated with lactation, and periods of copper deficiency.[4] The increased incidence of humeral fractures has led to animal welfare issues, economic losses for the dairy industry of New Zealand, impacting farmers and veterinarians mental health and wellbeing. As such studies aimed at identifying factors affecting bone strength in heifers with humeral fracture are imperative.

Bone is a dynamic tissue, with numerous factors influencing bone growth and health including genetic variation, nutrition, sex, reproductive status, bodyweight, and age.[1,7,8] There is a considerable window of time for different factors to affect bone growth and strength. Acquisition of bone mass begins in the uterus and achievement of peak bone mass occurs later (associated with increased muscle mass, secretion of hormones, and growth factors).[7] Although in dairy cattle in New Zealand, considerable growth is observed until 20-22 months.[6] More importantly, humeri from cows have a longer growth period compared with the metacarpus, meaning there is an increased period of susceptibility to growth checks for the humerus.[5]

Difficulties in attaining peak bone mass and/or increased bone loss predispose individuals to bone disease.[7,9] Metabolic bone disease or osteodystrophies are an important group of diseases, with osteoporosis being the most common in humans and animals.[7,10,2] Understanding the numerous events and factors that regulate bone growth and response to injury are essential when studying bone diseases and maintenance of bone health.[7]

This thesis aimed to identify potential risk factors and/or causes associated with the increased incidence of spontaneous humeral fractures in dairy heifers in New Zealand. The main objectives of this thesis were:

- Surveying farms that have and have not had cases of spontaneous humeral fractures in dairy heifers to determine possible risk factors related to farm management and nutrition of cows. Results presented in chapter 2.
- Describe changes in several biochemical analytes associated with mineral and energy metabolism. Results presented in chapter 3.
- Describe histological and histomorphometric changes in bones from a large cohort of heifers affected by spontaneous humeral fractures and compare with age-matched controls. Additionally, to determine if the main feed cows grazed on during winter (fodder beet or pasture) or liver Cu concentration at the time of euthanasia (adequate or low liver Cu concentration) altered the histological and histomorphometric changes. Results presented in chapter 4.

- Determine if there are any differences in the chemical composition of the humerus from affected and control heifers using Raman and FTIR spectroscopy. Results presented in chapter 5.
- Determine if there were any differences in the collagen content and collagen crosslink content in humeri from affected and control heifers based on liver Cu concentration at time of euthanasia. Results presented in chapter 6.

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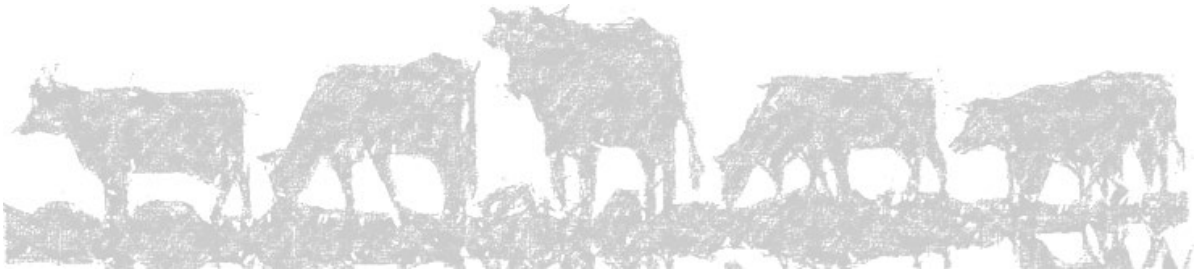
CHAPTER 1

REVIEW OF THE LITERATURE



1.1 Bone formation

The mesenchymal cells that form the skeleton originate from three different lineages, depending on the region of the body.[118,56] The sclerotomal portion of the somites provide the vertebral or axial skeleton; the limbs originate from the lateral plate mesoderm and lastly, the craniofacial bones originate from the cranial neural crest, except for the bones forming the roof and base of the skull which have a mesodermal origin.[56]

Around the fourth week of gestation in cows (third week in sheep) limb development begins with the formation of the limb bud.[56] Bones that will form part of the skeleton develop initially by two mechanisms known as intramembranous ossification, and endochondral ossification.[100,56] Bone modelling and remodelling occur later to replace the bone initially formed.[56,100,73]

1.1.1 Intramembranous ossification

Intramembranous ossification occurs in the flat bones of the skull and part of the mandible where neural-crest-derived mesenchymal cells proliferate and adhere to form aggregates.[56] With time and the influence of by various growth factors, these mesenchymal cells differentiate directly into osteoprogenitor cells.[117,56,100,29]

The site where osteoblasts are proliferating becomes vascularised, and osteoblasts produce the various components of the extracellular matrix known as osteoid.[100] When osteoblasts surround themselves with mineralised osteoid, they differentiate into osteocytes.[100] Other mesenchymal cells wrap the outer region of the aggregates and form the periosteum.[56] These cells can also transform into

osteoblasts hence intramembranous ossification also occurs at the periosteal surfaces of all long bones during growth.[25]

1.1.2 Endochondral ossification

Endochondral ossification occurs in the vertebrae, pelvis, part of the mandible, and the long bones.[100,118] Mesenchymal cells condensate and transform first into chondroblasts and then into chondrocytes.[100,117] These cells produce a hyaline cartilage model with the shape of a long bone.[100,25,117] The central part or diaphysis of this crude cartilage model is surrounded by the perichondrium, and this zone is known as the bone collar.[117] With time chondrocytes in the bone collar hypertrophy and there is matrix mineralisation.[70,25,117] Later, blood vessels penetrate the area carrying haematopoietic stem cells, chondroclasts that remove the mineralised matrix, and osteoblasts that produce bone, establishing the primary ossification centre and allowing the formation of the marrow cavity.[56,100,25] Secondary ossification centres develop later in the extremities of the bone forming the epiphyses.[25]

As the marrow cavity grows, two sets of cartilage remain in each epiphysis. One will form the articular cartilage and the second will form the physis or growth plate.[114,100] Corresponding to the shape, size, and activity of chondrocytes in the growth plate four distinct zones can be recognised histologically:

1. Zone of reserve cartilage, also known as germinal or resting zone, is the closest to the epiphyseal end of the bone and provides stem cells.[114]
2. Proliferative zone: here chondrocytes multiply, arranged in columns, and secrete cartilage matrix, between the columns of chondrocytes.[100,114]

3. Hypertrophic zone: contains large chondrocytes and the matrix between cells becomes thinner.[117,114] Chondrocytes also produce matrix vesicles that will result in the next zone.
4. Zone of cartilage ossification: matrix mineralisation occurs here, wherein chondrocytes die by apoptosis and the mineralised matrix serves as a scaffold for bone deposition.[117] Terminal differentiation and apoptosis of chondrocytes appear to be regulated by the increase in extracellular inorganic phosphate [75,74], annexin-mediated Ca (that activates proteases, lipases, and nucleases) [119], retinoic acid, and vitamin D.[114] Another study suggests that rather than apoptosis, autophagy is induced in hypertrophic chondrocytes with vascularisation and oxygenation triggering oxidative stress and cell death.[102]

Later, osteoblasts from the bone marrow lay down osteoid on the remnants of mineralised cartilage matrix.[100,25,70,114] Osteoclasts resorb the mineralised cartilage spicules, and the newly formed bone is called primary spongy bone or the primary spongiosa.[100] This area will later be reorganised through osteoclastic activity and osteoblastic addition of new bone to form the secondary spongiosa.[100]

The activity of the proliferative zone (increasing the numbers of chondrocytes) and the hypertrophic zone (increase in chondrocyte size moving the growth plate away from the bone marrow) are responsible for the growth of bone in length.[56,100,70] Peripherally the growth plate is surrounded by the perichondrial ossification groove of Ranvier and the ring of LaCroix, both containing

chondroprogenitor cells responsible for the circumferential growth of cartilage.[60] Increase in bone width is due to appositional growth from the periosteum.[100]

On completion of bone formation, a long bone is composed of a central hollow shaft or diaphysis, a cone-shaped metaphysis, the physes, and above these in both extremities the epiphysis (Figure 1.1).[18] Architecturally the bone is formed of a compact or cortical bone with numerous longitudinally arranged osteons or Haversian systems and spongy, cancellous, or trabecular bone which is arranged according to stress or weight-bearing lines, producing a crisscrossed pattern (lattice or sponge-like pattern).[6]

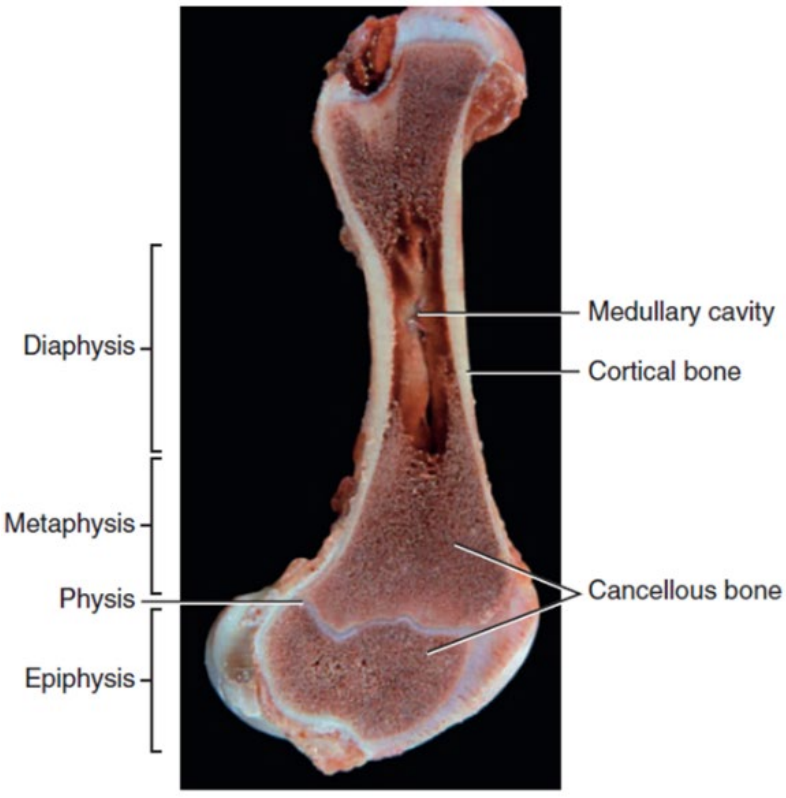


Figure 1.1 Topographic portions of a long bone after formation. Central portion or diaphysis, a transition zone or metaphysis, the physis or growth plate, and the bone extremity or epiphysis. The picture also shows the compact or cortical bone and the trabecular or cancellous bone. *Craig et al., 2016*

1.2 Bone modelling and remodelling

Bone modelling (growth) refers to changes in bone shape (longitudinal and diametric growth) that occur during the first years of bone growth and is specific to bone location and function.[73,58,118,6] Bone modelling leads to the acquisition of peak bone mass and does not require bone resorption to precede bone formation.[57,58,103] Additionally, modelling can occur on different bone surfaces, is continuous with a fast apposition rate (2-10 μm/day), active bone surfaces are larger, and there is a net increase in bone mass.[57,58] Finally, the term osseous or modelling drift refers to the uneven periosteal bone formation and endosteal bone resorption by which long bones can alter their curvature or vertical orientation in response to mechanical stressors, muscle insertions, or joint movement.[73]

Bone remodelling occurs through iterative cycles of bone formation and resorption.[118] and is necessary for microdamage repair, maintenance of biomechanical competence, and Ca and phosphorus homeostasis.[70,85,18]

Understanding the differences in the rate and site of remodelling between cortical and trabecular bone is important for determining bone quality.[118] Cortical bone has a slow remodelling rate achieved by so-called osteonal remodelling, in which activated osteoclasts form a cone-like space with an advancing tip or "cutting cone" where osteoclast resorb bone followed by a "closing cone" where osteoblasts deposit bone.[70,83] In contrast, trabecular remodelling is more active

and takes place on the surface of the trabeculae.[118,70] Figure 1.2 compares both types of resorptions.

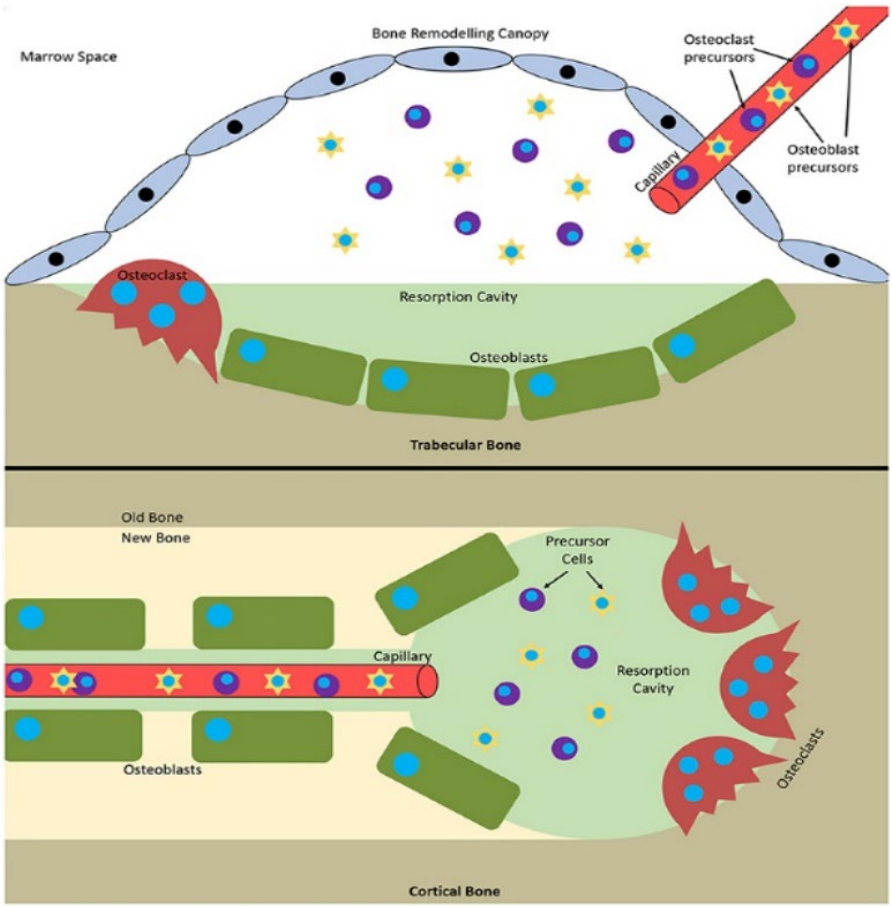


Figure 1.2 Schematic representation of trabecular (upper) and cortical bone remodelling (lower). Reprinted from *Owen & Reilly, 2018* with permission.

The process of remodelling can be initiated either by a systemic signal (parathyroid hormone (PTH) secretion due to hypocalcaemia) or locally after osteocytes embedded in the mineralised matrix experience strain-induced interstitial fluid flow and fluid shear stress resulting in activation of signalling pathways.[118,57] The bone resorption-formation cycle is overseen by the bone remodelling unit or basic multicellular unit (BMU) that consists of bone-resorbing cells (osteoclasts), bone matrix synthesising cells (osteoblasts), osteocytes, and endosteal lining cells.[18,85] These units are required for the preservation of bone composition, and dysfunction of the BMU is crucial in the

pathogenesis of osteoporosis and other bone metabolic disorders.[85,6] The characteristics and function of each cell of the BMU are described below.

Bone resorption occurs through the action of lysosomal enzymes and acid secretion from osteoclasts.[18] Osteoclast can resorb a volume of bone formed by 100 to 1000 osteoblasts.[70] Acid and lysosomal enzymes are secreted in the osteoclasts ruffled border which seals to the bone by a peripheral clear zone creating an acidic compartment that dissolves the mineral and protein components.[70,18]

There are numerous pathways for recruitment of osteoclasts precursors, their differentiation, and activation.[16,10,120] The receptor activator of nuclear factor-κB (RANK)/RANK-ligand (RANKL)/osteoprotegerin (OPG) cytokine system is key.[11,16] Osteoclasts and their precursors express RANK, a receptor from the tumour necrosis factor (TNF) family, which interacts with RANKL secreted by osteoblasts.[16,11] The coupling activates TNF receptor-associated factors (TRAF) to stimulate osteoclastogenesis and activates the signalling cascade for the fusion of monocytes into mature osteoclasts.[16,10] There are many TRAF that can bind to RANK, but TRAF6 is regarded as essential for osteoclastogenesis.[10] Together, RANK and TRAF regulate osteoclast activity through many intracellular signalling pathways that can modulate the rate and frequency of osteoclast maturation and bone resorption.[120] Other factors important for osteoclast differentiation and activity include interleukin- 1 (IL-1), IL-6, granulocyte and granulocyte-macrophage colony-stimulating factor, PTH, 1.25-dihydroxyvitamin D (1,25-$(OH)_2D_3$) and calcitonin.[11,18]

Osteoprotegerin, an osteoclastogenesis inhibitory factor, acts as a decoy receptor of RANKL and is produced mainly by osteoblasts (cells in the heart, liver, and spleen can also produce OPG).[18,118,120,16] When OPG and RANK bind, RANKL is unable to bind to RANK on the immature osteoclast thus inhibiting osteoclastogenesis.[10]

The wingless integrated (Wnt)/β-catenin pathway induces differentiation of mesenchymal progenitor cells into osteoblasts and regulates bone resorption by increasing the RANKL/OPG ratio through the action of the low-density lipoprotein receptor-related protein 6 (LRP6) and LRP5 co-receptor for Wnt.[62,67] Chondrogenesis and haematopoiesis are also regulated by the Wnt system.[18] Sclerostin, dickkopfs and secreted-frizzled-related proteins are antagonists of Wnt/β-catenin signalling.[62]

Osteoblasts exist either as polygonal osteoid-producing cells or flattened lining cells.[70] They are derived from mesenchymal cells in the bone marrow.[18] The main function of osteoblasts is the production of new bone, with the matrix deposited daily by an osteoblast equivalent to the cell's own size.[70]

Osteoblasts can inhibit osteoclasts by the action of Ephrin type-B receptor 4, and downregulating expression of c-Fos and nuclear factor of activated T-cells cytoplasmic 1 expression.[16] Also osteoblasts can secrete semaphorin3A, a protein that inhibits osteoclastogenesis.[16] Osteoclasts can stimulate osteoblast differentiation through the protein Ephrin2 that inhibits guanosine triphosphate enzyme and the Ras homolog family member A gene, thereby enhancing bone formation.[16]

Osteocytes are characterized by numerous projecting cytoplasmic processes that travel within fluid-filled canaliculi and interconnect cells.[18] These cells become the mechanoreceptors of bone resulting in signalling pathways that regulate bone resorption/formation cycles.[100] Osteocyte apoptosis due to disruption of the canalicular network or lack of mechanical stimulus is hypothesized to regulate osteoclast and osteoblast function and induce remodelling of the local bone tissue.[8]

A remodelling cycle takes around 120 days and consists of six phases:

1. Quiescence phase: bone surfaces are covered by flat or endosteal lining cells over a thin collagenous membrane.[18,103]
2. The activation phase starts with the retraction of the endosteal lining cells and the collagenous membrane after osteocyte apoptosis, recruitment of preosteoclasts from the blood, and fusion of these cells to form multinucleated osteoclasts.[103,18]
3. Resorption phase: osteoclasts resorb the surface they are attached to, forming resorption pits or lacunae, this phase can last for up to 4 weeks.[18,103]
4. Reversal phase: debris is removed by macrophages and endosteal lining cells and osteoblasts precursors are recruited.[103,18]
5. Formation phase with osteoid production and secretion.[18,103] Mineralisation starts after ~ 10 days of osteoid deposition and is called primary mineralisation.[7] This phase can last up to six months.[7]

6. A phase of quiescence with osteoblasts differentiating into flat-lining cells and/or osteocytes.[6]

After the end of one cycle, a phase called secondary mineralisation occurs and consists principally of maturation of the mineral component (increased amounts of crystals and/or increased crystal size).[7,20] This phase increases the mineral content of bone with age and provides bone strength and rigidity, hence in adults the degree of mineralisation depends on the rate of remodelling.[7]

1.3 Control of bone growth

Growth plate cartilage and bone growth are dependent on numerous local and systemic genes, hormones, growth factors, environmental conditions, and adequate nutrition. Appendix A shows a list of the major factors that control bone growth.

1.4 Composition of bone matrix

Bone matrix consists of an organic component (osteoid), an inorganic or mineral component, lipids, and water.[118,18] Osteoblasts secrete osteoid composed mainly of type I collagen (around 90% of the total organic material) and smaller amounts of collagen type III, V, and fibril-associated collagens (includes collagens IX, XII, XIV, XIX, XX, and XXI).[100,18,116] The remainder of osteoid (10-15%) consists of non-collagenous proteins (phospho- and glycoproteins).[18] The composition of bone matrix is not static; it varies between different animals, with health and disease status, age, and with tissue site, and contributes to the mechanical and metabolic functions of bone.[8]

1.4.1 Bone collagen

Within the bone structure collagen provides:

- A structural framework for minerals and proteins.
- Accounts for the ability of bone to resist pressure, tension, and torsion.
- Provides a substrate for cell migration, adhesion, and differentiation.[116]

There are several different types of collagen molecules as determined by the structural organization and biochemical characteristics.[115] In cartilage and bone the predominant collagen is fibril-forming with one or several collagenous triple helical domains and attached non-collagenous domains.[115] Fibril-forming collagen provides tensile strength, and torsional stability and is the site of formation of hydroxyapatite crystals.[116] In bone, collagens type I and type V assemble into quarter-staggered heterofibrils creating a banding pattern with a diameter between 25 and 400 nm.[116] Collagen type III and V control the fibril diameter of type I collagen fibrils.[96,115]

1.4.1.1 Biosynthesis of collagen molecules

Figure 1.3 shows the steps involved in collagen synthesis.[39] Collagen biosynthesis starts with the formation of polyproline polypeptide chains by ribosomes.[39] In the rough endoplasmic reticulum (RER) several post-translational modifications occur including hydroxylation, glycosylation, and folding of polyproline type II polypeptide chains into a triple helix procollagen molecule.[39,115] The presence of a repetitive amino acid sequence (GLY (glycine)-X-Y; X is usually proline and Y is usually hydroxyproline) allows triple helix formation.[81] Some proline residues are then converted by prolyl-4-hydroxylase to 4-hydroxyproline, while other proline

residues are converted to 3-hydroxyproline by prolyl-3-hydroxylase.[115] This process of hydroxylation of proline residues must be done before the triple helix is formed, and requires oxygen, Fe, and 2-oxoglutarate/ascorbic acid as cofactors.[39,115] The enzyme lysine hydroxylase is responsible for the hydroxylation of the lysine residues forming δ-hydroxylysine.[115] This last hydroxylation is important for collagen glycosylation and the formation of tissue-specific patterns of crosslinks.[116,115,101]

After hydroxylation, the procollagen molecule is secreted from the RER for transport to the Golgi apparatus, where the molecules are packaged in secretory vesicles and secreted into the extracellular space or matrix.[116] In the extracellular matrix, at the end of the procollagen molecule, *N*- and *C*- terminal domains are removed by procollagen-N-proteinase and procollagen-C-proteinase respectively, forming a helical tropocollagen molecule with short and non-helical domains.[39,96] Tropocollagen molecules are then assembled into collagen fibrils before the next step which includes the formation of collagen crosslinks.[39]

1.4.1.2 Collagen crosslinking

Collagen crosslinks are necessary for stabilisation of the newly-formed fibrils and in bone, are necessary for bone mineralisation and bone strength.[96] There are two pathways by which collagen crosslink formation can occur: enzymatic and non-enzymatic.[101] The enzymatic pathway occurs through the action of the lysyl oxidase (LOX) enzyme, which acts in the extracellular space by attaching to specific lysine or hydroxylysine residues in the telopeptide portions resulting in the formation of two aldehydes (allysine and hydroxyallysine).[101,39,115] These two

aldehydes will then spontaneously react with lysine and hydroxylysine residues on the adjacent tropocollagen chain and form divalent or immature crosslinks named dehydrodihydroxylysinonorleucine (deH-DHLNL) and dehydrohydroxylysinonorleucine (deH-HLNL).[101] With time, some of these immature crosslinks can interact with another allysine or hydroxyallysine, or another immature crosslink establishing trivalent pyridinium (pyridinoline (PYD) and deoxypyridinoline (DPD)) or pyrrole (pyrrololine (PYL) and deoxypyrrololine (DPL)) mature crosslinks respectively.[101]

Deoxypyridinoline and PYD are relatively specific for bone and measured to study bone disease.[101,115,39] Importantly, bone appears to have a significant pool of deH-DHLNL immature crosslinks and although its rate of conversion to mature crosslinks is a continuous process, it is not fixed and can increase when mechanical stress is applied to bone.[115,101] The increased conversion rate to mature crosslinks is an attempt to stabilize the collagen molecule.[101]

The total quantity of enzymatic crosslinks and the aggregation of collagen molecules into fibres is directly related to the expression and/or activity of LOX.[101] This enzyme is a copper (Cu) metalloenzyme and requires pyridoxine and lysine tyrosyl quinone as essential co-factors.[101] Activation and expression appear to be regulated by transforming growth factor-β, connective tissue growth factor, insulin-like growth factor 1, oestrogen, and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$).[101] Presence of basic fibroblast growth factor, high concentrations of prostaglandin E2, and TNF-α negatively affect the activity of LOX.[101]

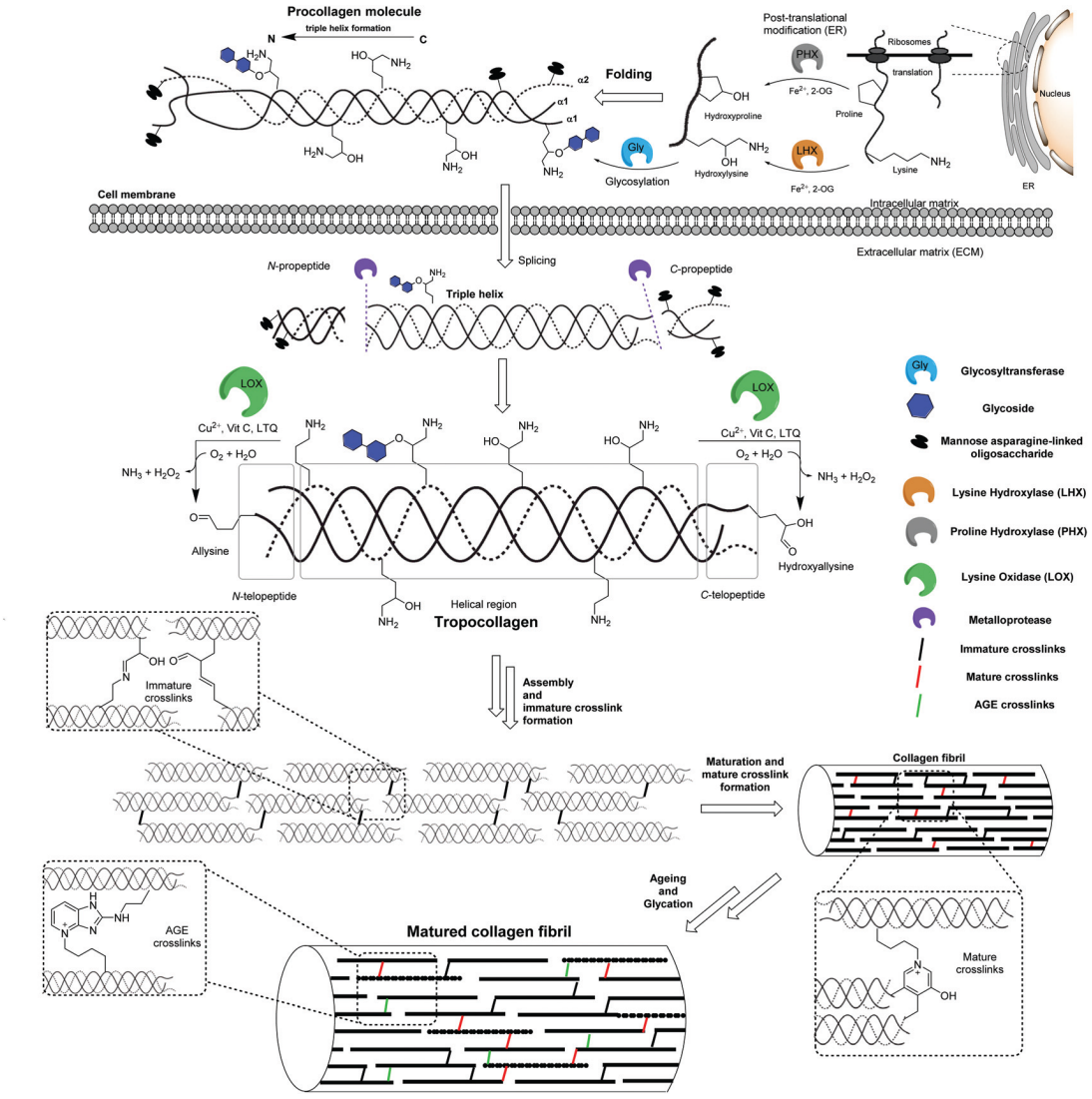


Figure 1.3 An overview of the steps involved in the synthesis of collagen and the formation of enzymatic and non-enzymatic crosslinks. ER, endoplasmic reticulum. Reprinted from *Gaar, Naffa & Brimble, 2020* with permission from Copyright Clearance Center, Inc.

Low LOX activity is associated with Cu and vitamin B6 deficiency as well as with ingestion of β-aminopropionitrile (a lathyrogens found in some legumes).[101] Other factors that affect the formation of collagen crosslinks include age, bone turnover activity (physiological or pathological changes), and bone location.[101] Formation of crosslinks also affects the way the collagen mineralises, and the way micro-damage is propagated.[8]

Finally, non-enzymatic or advanced glycation end product (AGE) crosslinks are formed by the Maillard reaction (where a sugar reacts with lysine, hydroxylysine, or arginine residue leading to the formation of a Schiff's base which undergoes rearrangement and formation of a Amadori product).[39,115,101] The glycosyl-lysine initially formed can then react with another lysine or arginine molecule in a close-by collagen molecule forming AGE crosslinks between collagen molecules.[101] In contrast to enzymatic crosslinks, AGEs are thought to weaken the mechanical and biological properties of collagen in bone and other tissues.[101,115]

1.4.1.3 Non-collagenous proteins

Approximately 25% of the non-collagenous proteins of the bone matrix are derived from serum (exogenous) and 75% are secreted by osteoblasts and/or other bone cells.[18] Exogenous non-collagenous proteins influence matrix mineralisation and bone cell proliferation and include mainly serum albumin and α2-Heremans-Schmid glycoprotein.[18] The functions of these proteins are numerous and new roles are constantly being described.

Osteocalcin, also known as bone γ-carboxyglutamic acid (Gla) protein, or bone Gla protein (B.G.P) is mainly secreted by osteoblasts.[18,25,126] Reported functions include inhibition of bone mineralisation, proliferation of β-cells in the pancreas, increased insulin secretion, stimulation of testosterone production, fatty acid oxidation, and thermoregulation.[125,126] Measurement of serum osteocalcin concentration is commonly used as a marker for bone turnover.[18] Alkaline phosphatase (ALP) is a glycosylated protein present in the osteoblast membrane that results in increased concentration of phosphate (promoting mineralisation)

and decreased concentration of extracellular pyrophosphate (mineralisation inhibitor).[18,43]

Other noncollagenous proteins found in bone include osteopontin and osteonectin. Osteopontin is found in the extracellular matrix and is involved in osteoclast differentiation and function.[25,3] Osteopontin also acts as a pro-inflammatory substance and interferes with glucose homeostasis in cells regulating energy metabolism.[125] Finally, osteopontin stimulates osteoblast differentiation and matrix mineralisation by promoting crystal growth.[18,3]

1.4.2 Bone mineral

The inorganic component of the bone matrix is hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] with smaller quantities of carbonate, Mg, Na, acid phosphate, and Zn.[18] Fluoride is also present in small amounts in the bone matrix.[25]

Mineralisation is associated with the activity of ALP, osteocalcin, osteonectin, and bone sialoproteins.[5,18] These proteins regulate the size and the amount of hydroxyapatite crystal deposition.[18] Osteoblasts secrete membrane-bound matrix vesicles that initiate the process of mineralisation.[70,25] These vesicles become entrapped in the newly synthesized extracellular collagen fibrils.[5]

Matrix vesicles contain molecules, such as phosphatidyl serine, calpactin (annexin II), anchorin CII (annexin V), and annexin VI with calbindin D9K, that bind and transport Ca into the matrix vesicles.[4] Phosphate concentration is increased around and inside the vesicles by the action of ALP, adenosine monophosphate phosphodiesterase, adenosine triphosphatase, ectonucleotide pyrophosphatase/phosphodiesterasel, and nucleoside triphosphate

pyrophosphohydrolase.[4,94] The continuous increase of Ca and phosphate within the matrix vesicles leads to precipitation and formation of $CaPO_4$ (a non-crystalline precursor of hydroxyapatite).[4,5] Matrix vesicle ATPases also contribute to $CaPO_4$ precipitation but in a lower amount than once believed.[5] This preformed mineral crystal perforates the membrane of the matrix vesicles (through the action of phospholipases and proteases) and is exposed to the extracellular space.[4,5] The second phase of mineralisation (termed mineral propagation) is then initiated which is characterised by the accumulation and growth of the crystal.[4,5] Collagen fibrils are arranged in a quarter-staggered array leaving gaps or "holes" at the end of the collagen triple helix bundles where crystals are deposited, known as intrafibrillar mineralisation.[5,93,81] Crystals can also be deposited between and on the surfaces of the collagen fibrils and this is known as interfibrillar mineralisation.[81]

1.5 Nutritional diseases of bone

Disturbances in bone development and growth, and abnormal bone metabolism can be due to a range of factors including nutritional deficiencies and/or disorders in the metabolism of proteins, lipids, carbohydrates, minerals, especially Ca and phosphorus, and vitamins, especially vitamin D.[78,42,34,72,25] Metabolic bone diseases or osteodystrophies in production animals include osteoporosis, osteomalacia/rickets, and fibrous osteodystrophy and are primarily caused by dietary deficiencies in Ca, phosphorus, vitamin D, copper, and/or protein.[22] Genetic defects (in enzymes or cellular receptors) can also cause osteodystrophies but these conditions are rare in production animals.[15,33]

The following sections will discuss the metabolism of Ca and phosphorus (principal causes of osteodystrophies) and the role they play in the emergence of osteodystrophies.

1.5.1 Calcium and phosphorus as elements associated with nutritional diseases of bone

1.5.1.1 Calcium

Almost 99% of body Ca is in bones with the remaining 1% in extracellular fluids and soft tissues.[100] In extracellular fluids, three distinct fractions of Ca are recognised, a protein-bound fraction (to albumin and other plasma proteins), a complexed to anions fraction (to citrate, phosphate or bicarbonate), and the physiologically active or the ionised fraction (Ca^{2+}).[99,25] In bone, Ca is present either as readily mobilizable extracellular Ca or forming part of hydroxyapatite crystals.[99]

In ruminants, Ca from the diet first becomes solubilized by acids in the abomasum and is predominantly absorbed in the small intestine.[22,40] Young growing animals absorb most of the Ca that is provided in the diet while adult cows only absorb the Ca that is needed to replace that lost due to renal or intestinal excretion.[99,22] However, this changes in periods of high Ca demand (pregnancy, lactation, and periods of Ca deficiency).[99,22] Calcium absorption is also dependent on the type of diet, the amount of Ca present, and the presence or not of substances that can interfere with Ca absorption, including phosphate, phytates, oxalate, and fatty acids.[99,40,22]

At the cellular level, intestinal absorption occurs either by a saturable, carrier-mediated, vitamin D-dependent transcellular transport mechanism or by a non-saturable intercellular or paracellular transport (relied on in animals with vitamin D deficiency).[123,40,99] Up to 98% of Ca excreted by the kidneys can be reabsorbed, with ~70% reabsorbed in the proximal convoluted tubules, ~20% in the thick ascending loop of Henle, and ~10% in the distal convoluted tubules.[99]

Primary Ca deficiency is due to an inadequate dietary supply and is uncommon.[22,40] Instead secondary deficiency is due to either hyperphosphatemia or vitamin D deficiency which reduces Ca intestinal absorption.[22,41]

1.5.1.2 Phosphorus

Between 80% – 85% of total phosphorus in the body is present in bones in the form of hydroxyapatite or as calcium phosphate.[61,88] The remainder of total phosphorus is present as inorganic phosphate or as part of phospholipids, phosphocreatine, adenosine molecules, and several carbohydrate metabolites.[42,88,61]

Phosphorus present in the diet and from biliary, pancreatic, and intestinal secretion is absorbed in the small intestine.[88] Factors that regulate absorption include the amount present in the diet, 1,25$(OH_2)D_3$, and PTH.[88,42] Other factors that affect phosphorus absorption include epidermal growth factor, glucocorticoids, oestrogens, metabolic acidosis, phosphatonins, and secreted frizzled related protein-4.[88] The phosphorus necessary for bone mineralisation is provided by ALP that cleaves phosphorus from the complexed form beta-glycerol

phosphate.[88] Renal phosphate reabsorption occurs principally in the proximal convoluted and straight tubules.[88]

Primary dietary phosphorus deficiency occurs predominantly in regions with low phosphorus content in the soil.[61] Generally, signs and symptoms of phosphorus deficiency manifest after months or years of deficiency.[22] However, in early-lactation cows an acute and transient phosphorus deficiency may occur two to four weeks after calving, is associated with lethargy, reduction in milk production, and may result in acute intravascular haemolysis (post-parturient haemoglobinuria).[107] Marginal phosphorus content in feed rations combined with reduced intake around calving and the increased mineral requirements for milk production are thought to be responsible.[22] Excessive Ca, Al, or Fe in soil reduces phosphorus availability to plants and can also lead to phosphorus deficiency.[22]

Serum inorganic phosphorus concentration can be used to assess short-term dietary phosphorus supply and intake, it is, however, not useful in the diagnosis of chronic phosphorus deficiency because of compensatory bone phosphorus mobilisation.[61] Additionally, serum phosphorus concentration fluctuates during the day and is affected by age, milk production, pregnancy, breed, and sample source.[61,22] A reliable method to estimate phosphorus status is estimating the dietary phosphorus content along with feed intake.[22]

Determination of total bone ash and total bone Ca and phosphorus concentration using a sample of the 12th rib, may provide useful information on the concentration of these elements in the animal.[22] Although there is conflicting information regarding threshold values of phosphorus in bone and the type of

sample used for analysis (rib bone vs tail bone).[61,22] Evaluation of Ca/ phosphorus concentrations must consider that changes in bone associated with Ca and phosphorus deficiencies occur slowly.[22]

In young animals, phosphorus deficiency causes slower growth rates and rickets.[34] In adults there is an initial subclinical stage, followed by reduced feed intake and finally osteomalacia.[22] In the first report of naturally occurring disease in cattle in the early 1900s an appetite for bones (osteophagia) was considered the main indicator of disease.[109] This behaviour is known as pica (which can lead to botulism) and animals can also eat wood and soil.[61,109] Ill thrift, anorexia, impaired growth, reduced fertility, and lameness are also reported with phosphorus deficiency in ruminants.[22] Lameness associated with phosphorus deficiency is referred to as styfsiekte in South Africa, creeps in Texas, and pegleg in Australia.[61]

1.5.1.3 Hormonal control of calcium and phosphorus metabolism

Calcium and phosphorus metabolism and regulation of plasma concentrations are controlled by PTH, $1,25(OH)_2D_3$, calcitonin, and the phosphatonin system, primarily fibroblast growth factor 23 (FGF23).

1.5.1.3.1 Parathyroid hormone

Parathyroid hormone is secreted when Ca-sensing receptors (predominantly on parathyroid gland chief cells and less on parathyroid oxyphil cells) sense low serum ionised Ca concentrations.[40] In the kidney, PTH stimulates Ca reabsorption in the distal convoluted tubules and phosphate excretion in the proximal tubules.[88,66,99] When the decrease in blood Ca concentration is minor,

renal Ca reabsorption might be all that is required to restore blood Ca concentration.[40] Parathyroid hormone-related protein due to its 70% sequence homology with PTH can also bind to PTH receptors in bone and renal tubular epithelial cells but does not stimulate the renal 1α hydroxylase enzyme (necessary for vitamin D formation).[99]

Parathyroid hormone promotes the conversion of 25-hydroxyvitamin D_3 to 1,25$(OH_2)D_3$ (active vitamin D) which stimulates intestinal Ca and phosphate absorption.[123,66] In bone PTH can have either a catabolic or an anabolic role.[66] The catabolic effect of PTH is through the stimulation of osteoblasts to secrete macrophage colony-stimulating factor and RANKL which activates osteoclasts to digest hydroxyapatite crystals for Ca and phosphorus release.[40,66] Osteocytes are also activated by PTH to pump Ca and phosphate from the bone extracellular space into the blood.[40] The net result of increased bone resorption is to release large amounts of Ca and phosphorus into the blood.[99,61,40] Conversely, when administered in intermittent low doses, for the treatment of osteoporosis in humans, PTH has an anabolic effect with increased bone formation due to increased osteoblast differentiation.[90]

Synthesis and release of PTH is inhibited by 1,25$(OH)_2D_3$ and increased serum ionised Ca concentration.[99,34] Fibroblast growth factor-23 can bind to a receptor in the parathyroid gland supressing PTH gene expression and serum PTH concentrations.[88] A decrease in PTH secretion in cows with hypomagnesemia is believed to be secondary to a reduction of adenylyl cyclase enzyme activity (necessary for the conversion of Mg-ATP to cyclic AMP).[40] Finally, diets low in Ca

during the prepartum period stimulate PTH secretion allowing an increase in the number of active osteoclasts, osteoclastogenesis, and enterocytes to become more efficient in the absorption of Ca thus reducing the possibility of the cow developing milk fever.[40]

1.5.1.3.2 Vitamin D

Vitamin D is available to animals from either or both the isomerisation of 7-dehydrocholesterol (7-DHC) in the skin to vitamin D_3 (cholecalciferol) during exposure to ultraviolet light and/or ingestion of vitamin D_2 (ergocalciferol) or D_3 in the diet.[22,40,34,54] The content of vitamin D_2 in forages is not consistent and vitamin D_2 is poorly metabolised in cattle compared to vitamin D_3 hence forages are not considered an important source of vitamin D in cattle.[80]

The production of vitamin D_3 by dairy cows is directly correlated with the amount of skin exposed to ultraviolet radiation, and blanketing or housing cows reduces vitamin D_3 production.[40,22] Other factors that negatively affect ultraviolet radiation include latitude (further from the equator, less ultraviolet radiation), altitude, sky conditions (cloudiness, overcast, presence of smoke), and season (winter).[34] In cows raised indoors and with poor sunlight exposure, vitamin D supplementation (feed additives or parentally) should be considered.[52] Primary hypovitaminosis D is most commonly due to a lack of ultraviolet solar irradiation of the skin, often coupled with a deficiency of preformed vitamin D in the diet.[22] Secondary hypovitaminosis D is due to excess carotenes present in lush green feed including cereal crops.[22]

Once produced in the skin vitamin D_3 plus the vitamin D_2 and D_3 ingested with the diet and absorbed by the small intestine are transported to the liver where 25-hydroxyvitamin D_3 and D_2 ($25(OH)D_3$ and $25(OH)D_2$) is produced by the action of hepatic vitamin D-25 hydroxylase.[34] The concentration of total $25(OH)D_3$ is used to assess vitamin D dietary intake and/or skin production.[40,34] Next, $25(OH)D_3$ is transported to the kidney, where at least two additional derivatives can be formed by the renal vitamin D-1α-hydroxylase enzyme.[88,123,34] The predominant active form is $1,25(OH)_2D_3$, produced due to phosphorus or Ca deficiency, and PTH secretion.[34] The other form is $24,25\text{-}(OH)_2D$ a biologically inert metabolite.[22]

Active vitamin D promotes Ca and phosphorus intestinal absorption and intracellular transport.[34,123,88] In the kidney, vitamin D promotes Ca absorption in the distal convoluted tubules and collecting ducts.[34] Increased renal and intestinal absorption of Ca and phosphorus, in turn, inhibits continual PTH secretion.[99,40,88,25] There is also negative feedback on the 1α-hydroxylase enzyme.[34]

In bone, $1,25(OH_2)D_3$ upregulates RANKL and inhibits OPG expression hence stimulating bone resorption.[34] Additionally, chondrocytes, osteoblasts, osteoclasts, and osteocytes can produce $1,25(OH)_2D_3$ locally thus increasing bone formation and mineralisation.[34]

1.5.1.3.3 Calcitonin

Calcitonin, is secreted by C-cells in the thyroid gland and acts contrary to vitamin D, decreasing plasma Ca concentrations.[25] Its receptor CTR is expressed on

osteoclasts and binding decreases bone resorption.[14] Calcitonin activity is diminished under metabolic acidosis.[97]

1.5.1.3.4 Fibroblast growth factor 23

Fibroblast growth factor 23 is produced by osteoblasts and osteocytes and secretion is increased with hyperphosphatemia or increased plasma $1,25(OH)_2D_3$ concentration.[48] The outcome of activation of FGF23 is a decrease in plasma phosphorus and $1,25(OH)_2D_3$ concentrations in blood.[48] In renal tubular cells FGF23 and its cofactor klotho, decrease phosphorus resorption.[48] Fibroblast growth factor-23 also decreases PTH secretion.[48] In the intestine, FGF23 can downregulate phosphorus absorption.[48]

1.5.2 Other compounds associated with osteodystrophy in ruminants

1.5.2.1 Copper

Copper (Cu) is a transition group metal essential for animals and plants.[112] It is a component of numerous enzymes including, cytochrome oxidase (necessary for cell respiration), ceruloplasmin (for Fe metabolism), tyrosinase (for melanin synthesis), superoxide dismutase (antioxidant), and LOX (an enzyme necessary for crosslinks in collagen and elastin).[24]

Since Cu deficiency is more common than toxicity in production animals in New Zealand, the literature review will be restricted to Cu deficiency.[112] Animals can obtain Cu from plants, feed additives (such as Cu sulphate, Cu chloride, and Cu oxide), chelated Cu in the diet, administration of Cu-containing boluses, or injections.[112,45] The principal site for Cu absorption is the small intestine although Cu absorption in ruminants is low (1.0–10.0%) compared to nonruminants.[19,105]

Factors that influence Cu absorption in ruminants and that can influence Cu availability in the body and the activity of Cu-dependent enzymes include:

- Age: absorption is greater in young animals.
- Season: absorption increases in autumn and diminishes during spring.
- Interaction with Mo and S: a dietary excess of these two elements will decrease Cu absorption and interfere with Cu hepatic metabolism by the formation of thiomolybdates.
- Interaction with Fe: high dietary intake of Fe reduces Cu absorption in cattle and sheep.
- Type of soil: deficiencies are described in cattle grazing on coastal sands, sandy soils, and peat soils.[78,45]

In the blood, Cu is transported to the liver by transcuprein and albumin.[45,112] Once in the liver, Cu can be either be stored in lysosomes, returned to the gastrointestinal tract via the bile, and/or combined with ceruloplasmin (for transport to cells in the body).[45,112] Finally, Cu readily crosses the placenta, very little is present in milk and only a small amount is excreted in the urine.[45]

As with other minerals, Cu deficiency can be primary (dietary deficiency) or secondary (associated with excessive Mo, S, sulphates, Fe, Ca, Zn, and Cd).[22] Signs of Cu deficiency in cattle include ill thrift, changes in coat colour and roughness, osteoporosis, significant tooth wear, delayed puberty, depressed fertility and impaired immune system, and sudden death (secondary to blood vessel fragility and rupture).[45,78] Bone-related clinical signs in ruminants grazing Cu-deficient pasture include poor growth and weight gain, lameness,

enlargement of joints, increased brittleness of bones, and increased incidence of spontaneous fractures.[44,50,78,108]

Assessment of Cu status in cattle can be complex and many different tests may be needed to achieve an accurate diagnosis.[64] The liver is the organ where Cu is stored (long-term storage pool) and liver biopsies for assessment of Cu status are considered a reliable test.[64,36] The liver concentration of most minerals represents what the diet has been over the last 30 days.[36] Plasma and/or serum concentration of Cu (transport pool) and ceruloplasmin (80% of Cu in serum is present in ceruloplasmin) may also provide information on Cu status in cattle but paired liver and blood Cu concentrations are probably of the greatest diagnostic value.[64,36,22] A lower-than-normal hepatic Cu concentration is the earliest sign of inadequate Cu consumption and inadequate storage in the liver.[36] When liver Cu stores are within a normal range, serum Cu concentrations remain stable.[36] Thiomolybdates can be absorbed into the bloodstream and have a Cu chelating effect, mobilizing Cu from tissue stores into a form that is not functional.[36] When there are significant concentrations of circulating thiomolybdates, serum Cu concentrations may be in the normal range, even though there is a Cu deficiency.[36]

1.5.2.2 Molybdenum

Molybdenum is an essential trace element and cofactor of redox enzymes.[31] Experimentally, deficiency and excess (toxicity) can inhibit early growth, and cause growth retardation and skeletal deformities.[31] Molybdenum in the diet is a Cu antagonist (and phosphorus antagonist), and as a result, the signs of toxicity

are those of Cu deficiency.[23,36] Molybdenum and S interactions can occur in the rumen with the formation of thiomolybdates (mono-, di-, tri-, and tetra thiomolybdates) that then form insoluble complexes with Cu.[105] The maximum tolerable dietary concentration of Mo is suggested to be 10mg/kg diet, although as little as 5mg Mo/kg diet can cause Cu deficiency in heifers.[23]

Molybdenum in the diet is readily absorbed and serum, whole blood, milk, liver, and kidney values reflect dietary intake.[36] The assessment of serum and hepatic Mo concentrations is useful as an indicator of excessive intake leading to secondary Cu deficiency.[36]

1.5.2.3 Zinc

Zinc is an important cofactor for many enzymes in the body (e.g. Cu/Zn superoxide dismutase, carbonic anhydrase, alcohol dehydrogenase, carboxypeptidase, ALP, RNA polymerase), it is necessary for prostaglandin synthesis and is also an antioxidant.[31,23] Zinc and Cu are antagonists, so an excess of Zn in the diet can cause Cu deficiency.[23]

Zinc deficiency is associated with bone growth retardation, skeletal deformities, and osteopenia, but determining the Zn status of an animal is difficult because there is no defined storage pool of Zn in the body.[31,36] Zinc concentrations can be measured in the liver, reproductive organs (especially the testes), pancreatic tissue, and bone and although liver Zn concentrations do not always correlate with Zn intake, they do decrease after short periods of dietary deficiency, as do serum Zn concentrations.[36]

1.5.2.4 Iron

Iron deficiency and excess are both associated with altered bone metabolism.[31] With deficiency there is decreased serum concentration of 1,25(OH_2)D_3, insulin-like growth factor 1, and osteocalcin.[31] Iron is a cofactor for prolyl and lysyl hydroxylases (for collagen crosslink formation), an essential cofactor in the enzyme 25-hydroxycholecalciferol hydroxylase necessary for vitamin D formation (affecting Ca absorption).[84] Excess concentrations are hypothesize to increased bone resorption, and increased risk of bone fractures.[31]

Ruminants are often exposed to high Fe intakes through ingestion of water, soil, or feedstuffs that are high in Fe.[105] Studies indicate that the addition of 250–1,200 mg of Fe (from ferrous carbonate)/kg of diet greatly reduces Cu status in cattle and sheep.[105]

Iron deficiency caused by a lack of Fe in the diet of production animals is uncommon.[36] Severe blood loss from a parasitic infection or blood loss from other causes may produce a secondary Fe deficiency in ruminants.[36] Determining liver and serum concentrations of Fe can be used to diagnose Fe deficiency and toxicosis.[36] Additional diagnostic methods used to determine the body Fe concentration include measuring a complete blood count, total Fe-binding capacity, and serum ferritin concentration.[36]

1.5.2.5 Cadmium

Toxic accumulation of Cd causes renal tubular dysfunction and can lead to osteoporosis, osteoarthritis, osteomalacia, and an increased risk of fractures in humans.[31,71] Bone disease is associated with a deficiency in the conversion of

$25(OH)_2D_3$ to $1,25(OH_2)D_3$.[31] Other effects of Cd toxicity include disturbance of Ca metabolism (increases calciuria) and the hormones that control Ca and phosphorus metabolism.[31] In humans, toxic levels of Cd have been found to decrease the liver concentration of Fe, Mg, and Se and increase Cu, Zn, and Mn liver concentrations.[31]

The environmental sources of Cd are related to industry and agriculture contamination.[71] In New Zealand, the main source of contamination is the use of phosphate fertilisers leading to Cd accumulation in topsoil, along with fluorine.[12,68] Other sources include air and sewage contamination from industry and from parent rock (mainly sedimentary).[68] The concentration of Cd present in fertilisers is dependent on the type of phosphate rock used to make the fertiliser.[68] In the late 1980's and early 1990's one in five cattle had kidney Cd concentration above the maximum permissible concentration.[68] The Cd concentration in New Zealand pasture herbage has been observed to be highest during autumn and lowest during spring.[68]

After absorption, ruminants eliminate most Cd (>90%) in the faeces.[71] Accumulation in the body is mainly found in the kidney, liver, small intestine and bone.[71,12] It is reported that in lactating dairy cows a limited increase in dietary Cd can be tolerated without causing significant issues for animal health.[12]

1.5.3 Metabolic bone diseases

1.5.3.1 Osteoporosis

Osteoporosis, one of the most common osteodystrophies in humans and animals, is defined by the World Health Organisation as a "systemic disease characterised

by low bone mass and microarchitectural deterioration of bone with a consequent increase in bone fragility and susceptibility to fracture".[77] It has been estimated that osteoporosis develops when for every 30 units of bone resorbed, only 29 are replaced.[6] This negative "bone balance" has three possible causes: increased osteoclastic activity without increased osteoblastic activity ("high turnover"); normal osteoclastic but decreased osteoblastic activity ("low turnover") and decreased osteoclastic and osteoblastic activity ("atrophic" or "adynamic" bone).[25]

1.5.3.1.1 Osteoporosis in ruminants

Osteoporosis in farm animals is uncommon.[22] An unusually high incidence of bone fractures without a history of trauma in a group of animals is very suggestive of osteoporosis and should prompt evaluation of animal nutrition and husbandry practices.[25]

The most important form of osteoporosis in animals is due to nutritional deficits of either a specific nutrient (Ca, phosphorus, Cu) or secondary to starvation when animals graze in areas prone to drought or when there is overstocking of paddocks.[25] Starvation is associated with reduced bone formation secondary to protein/calorie deficiencies in the diet leading to decreased insulin-like growth factor 1 and oestrogen, and increased peroxisome proliferator-activated receptor γ that induces adipocyte differentiation of osteoprogenitor cells instead of osteoblasts differentiation.[25]

Calcium deficiency alone only produces osteoporosis in sheep and cattle, in other animal species it results in fibrous osteodystrophy.[25] However, there is also

increased osteoclastic resorption due to PTH secretion in response to hypocalcaemia.[40] Lactational osteoporosis is observed in gilts fed rations deficient in Ca with normal or excess phosphorus.[25]

Severe gastrointestinal parasitism can produce osteoporosis in animals likely secondary to malabsorption of nutrients and inflammation.[2,55,69] In lambs, subclinical parasitism can lead to deficiency of phosphorus carrier proteins in the intestinal mucosa resulting in decreased serum phosphorus concentration and decreased availability of energy and protein.[25] Pro-inflammatory cytokines released with parasitism include TNF-α, IL-1, and IL-6 and these can induce RANKL production by osteoblasts and osteocytes thus increasing osteoclast differentiation.[82,25] Reduction in bone formation is secondary to cytokine-induced inhibition of runt-related transcription factor 2, increased dickkopf-1, and sclerostin.[82]

Chronic lead poisoning in lambs can cause osteoporosis as a result of deficient production of osteoid.[25] Chronic fluoride exposure in sheep leading to Ca deficiency was also found to be associated with osteoporosis and fragility fractures of long bones.[104] Other reported causes of osteoporosis include vitamin A toxicity, hyperthyroidism, chronic metabolic acidosis, chronic exposure to Cd, cyclosporin, and certain anticonvulsant drugs.[25]

Diagnosis of osteoporosis in veterinary medicine can be done through gross evaluation of the cut surface of bone at post-mortem examination, although is only accurate when there is advanced bone loss.[25,110] Suitable bones to evaluate for osteoporosis include vertebral bodies, scapula, ribs, and long bones although

bones with higher volumes of trabecular bone, such as vertebrae, are better to assess bone loss.[25,110] Bones may also be prone to breaking (fracture) when force is applied.[25] Other changes that can be observed grossly include thickened trabeculae extending through the medullary cavity (which may represent strengthening attempts in an area subjected to biomechanical stress and are called reinforcement lines or bone bars), enlargement of the medullary cavity, and thin cortices (due to cortical bone resorption).[25] In young animals there can be delayed tooth formation and eruption, teeth overcrowding with malocclusion, and malalignment.[25]

Microscopic evaluation of bone samples will provide information on bone quality and in some cases permit differentiation between osteoporosis due decreased bone formation (characterised by normal numbers of thin trabeculae) or due to increased bone resorption (characterised by a reduced number of trabeculae) as well as differentiation from other osteodystrophies.[25,32,124] In young animals, hypertrophic chondrocytes in the growth plate may be reduced in number, with the hypertrophic zone being narrow or absent.[25] Chondrocytes may appear smaller with increased intercellular cartilaginous matrix and the primary spongiosa may be completely absent.[25] Trabeculae may have microfractures, and growth arrest lines (indicates periods of malnutrition and/or starvation) may be present.[25,32] Serous atrophy of fat is a common feature of starvation-induced osteoporosis in animals.[25,110]

Histomorphometric evaluation of different histologic parameters including bone volume, trabecular thickness, osteoid volume, osteoblastic and osteoclastic

activity, and bone mineral apposition rate are not commonly used in veterinary medicine but if they were, could provide reliable information for the diagnosis of osteodystrophies allowing quantitative information on bone growth rate, bone modelling and remodelling.[37] Lastly, bone ash measurement may provide information on the matrix-mineral relationship.[25]

1.5.3.2 Rickets and osteomalacia

Rickets and osteomalacia are considered together due to similar causes and pathogenesis.[34] Rickets is secondary to vitamin D deficiency or phosphorus deficiency.[34] This results in abnormal Ca and phosphorus metabolism leading to abnormal endochondral ossification and defective bone formation, as such rickets occurs in young growing animals and children.[63,34] Osteomalacia occurs in adult humans and animals and is characterized by a failure of osteoid mineralisation leading to a reduction in bone strength.[22,25,63] Direct inhibitors of the mineralisation process such as metabolic acidosis, F, Fe, and Cd, are also considered causes of rickets and osteomalacia in humans.[63]

1.5.3.2.1 Rickets and osteomalacia in ruminants

Deficiencies of vitamin D and phosphorus predispose young animals to rickets due to the increased nutritional requirements for early growth and development.[22,113] On the other hand, adult animals have increased nutritional demands during periods of pregnancy, lactation, or both, which can predispose them to osteomalacia.[22,25]

Most herbivores rely on sunlight to produce vitamin D and if animals are kept indoors or graze at latitudes where solar irradiation is insufficient (parts of the

United Kingdom, South America, New Zealand, and southern Australia), especially during winter, minimal dermal photobiosynthesis of vitamin D occurs.[25,87,22] Most reported outbreaks of rickets or osteomalacia in cattle are associated with phosphorus deficiency and include, yearling steers wintered on *Brassica napus* (swede) for over 3 months during winter in New Zealand, after periods of drought in parts of Australia, and in calves kept indoors and fed oats, sugar beet pulp, barley and hay and raw potatoes in England.[111,98,106]

Hereditary forms of rickets confirmed by genetic testing have increasingly been recognised in animals.[113] Vitamin D–dependent rickets type I due to a defect in the renal 1α-hydroxylase enzyme (CYP27B1), has been described in Hannover pigs, Saint Bernard dogs, and cats.[15,59,25] Hereditary vitamin D–resistant rickets caused by a defect in the vitamin D receptor-effector system in the cells of target organs have been described in a Pomeranian dog and a cat with autosomal recessive mutations in the receptor.[34,113] Lastly, in New Zealand, autosomal recessive hypophosphatemic rickets type 1 caused by a nonsense mutation in dentin matrix acidic phosphoprotein 1 has been described in Corriedale sheep.[32]

Lameness and pathologic fractures are common clinical signs observed in some affected animals.[34,113] Pathologic fractures commonly occur in bones that show rapid growth (long bones), ribs, and vertebrae, and may be precipitated by sudden exercise, yarding, or handling of the animals.[22,113]

Gross lesions of rickets are prominent in the metaphyseal and epiphyseal regions of long bones, and costochondral junctions where the enlargement of these junctions is known in human medicine as the “rachitic rosary”.[34] Cut sections of

the ribs can show irregular often thickened growth plates with tongues and islands of unresorbed cartilage extending into the metaphysis admixed with haemorrhage and fibrous tissue.[34,113,110] In long bones, similar lesions are seen in physes with rapid growth such as the distal radius, proximal humerus, distal femur, and proximal tibia.[113,34] Different bones may show different severities of lesions within the same animal, depending on the rate of growth of that bone.[25,34,113] Physeal lesions are accompanied by signs of increased bone fragility including trabecular resorption, haemorrhages, infractions, and pathologic fractures.[113,34] Lesions in the epiphyseal bone or articular cartilage are usually milder due to the slower rate of endochondral ossification in that site, but there may be collapse of subchondral bone.[25] Rickets is also associated with delayed and irregular teeth eruption, poor teeth mineralisation, pitting, and pigmentation.[34,113] There is also malalignment, and irregular and rapid tooth wear.[25]

Histologically there is persistence of hypertrophic chondrocytes in the physis, forming irregular masses instead of columns.[113,34] In the primary spongiosa there are islands of chondrocytes with unmineralised and degenerate cartilage matrix, unmineralised osteoid and trabecular microfractures or infarctions are also common.[34] Bone trabeculae are thicker, irregular, and surrounded by unmineralised osteoid.[25] If the condition is associated with vitamin D deficiency, which causes both hypophosphatemia and hypocalcaemia, there is also increased numbers of osteoclasts along with prominent osteoblastic activity and proliferation of loose fibrous connective tissue (histological features of

hypocalcaemia leading to secondary hyperparathyroidism and fibrous osteodystrophy).[25,34,113]

The lesions of osteomalacia include increased unmineralised osteoid in locations where biomechanical stress is higher.[113] Trabeculae are reduced in size and number and are usually surrounded by unmineralised osteoid.[25,113,34] The cortex can be thin, soft and with advanced disease there may be pathological fractures.[25,34] Osteoporotic lesions are usually superimposed especially in animals with severe long-term phosphorus deficiencies, as severe deficiency results in anorexia leading to protein-calorie deficiency.[25]

A valuable diagnostic aid is the ratio of ash to organic matter in the bones.[22] Normally the ratio is 3 parts of ash to 2 of organic matter, but in rachitic bone, this may be depressed to 1: 2, or to 1: 3 in extreme cases.[22]

1.6 Dairy farming in New Zealand

New Zealand dairy systems are mainly pasture-based and characterised by seasonal calving.[17] Milking on most farms is done twice daily, although on some farms once-daily milking is preferred due to an associated reduction in farm costs, milk shed expenses, and improvement of mental health of farmers and staff.[17]

Dairy farms in New Zealand have grown in the past 20 years from an average herd size of 251 cows producing 310 kg of milk solids in 2000/01 to an average herd size of 440 cows producing an average of 385 kg of milk solids in the 2019/20 season.[28,27] This increase may have led to health issues not seen before. Despite most heifers achieving liveweight targets at 12 months of age, 44% of

heifers at mating and 65% of heifers at 22 months of age (pre-calving) are below target liveweights.[46] Handcock et al[47] reported that the growth trajectory in heifers is not linear with multiple factors affecting growth including breed, heterosis, feed supply, nutrition, and season (slower in winter months).[47]

Winter feeding is important because over 30% of the heifer's growth occurs during winter months (from 8-11 months and 20-23 months of age).[49] Although many improvements have been made, winter still represents the industry's most challenging feeding period with low pasture growth and corresponds with the lowest liveweight-gain targets.[49]

This has led to significant changes in feeding systems in New Zealand. Dairy systems have become more intensive and diverse (from relying entirely on pasture with little supplementation to being highly dependent on feed supplements).[51] Furthermore, in pasture-based systems, temperature and rainfall affect pasture quantity and quality.[47] Challenging winter conditions in the South Island of New Zealand have encouraged the use of forage crops (fodder beet (*Beta vulgaris*-FB) and brassicas (kale - *Brassica oleracea*) to make up for the low pasture growth during this season.[30] In particular FB has been used to meet body condition score targets at calving.[30] Compared to other winter crops such as kale, FB has higher growth performance, and cows have better reproductive performance and milk production.[30,38] However, when animals graze on FB they usually have the bulb of the plant available which is low in crude protein, low in neutral detergent fibre and low in phosphorus and Ca, which can lead to significant productive and health issues in the cow.[30] An increased incidence in

subacute ruminal acidosis in cows fed FB can lead to low milk yields response (acidosis reduces ruminal fermentation, feed intake, and milk production).[38] Other negative issues associated with FB feeding include high establishment costs, hypophosphataemia, hypocalcaemia, hypomagnesaemia, ketosis, and hepatic lipidosis.[38] It appears then that feeding kale and/or FB may prevent heifers from achieving target daily liveweight gains during the winter period in the South Island.[49]

1.6.1 The transition period and the metabolic predictors of peripartum diseases

The period from pregnancy to lactation (from three weeks prepartum to three weeks post-partum) or transition period is characterised by significant changes in energy and mineral requirements.[53] Increased energy utilisation, reduction in dry matter intake (DMI) (~30% lower), and mobilisation of Ca into colostrum and milk initiate adaptive mechanisms to meet the new requirements.[53,1,76]

For initiation and maintenance of lactation, the release of PTH is crucial.[53] The effects of the PTH and other hormones on the metabolism of Ca and phosphorus are described in section 1.5.1.3 of this chapter. If the homeostatic mechanism fails to deliver Ca, cows can develop periparturient hypocalcaemia (commonly known as milk fever).[53] The susceptibility of cows to mastitis (due to reduced ability of immune cells and/or deficient teat closure), retention of fetal membranes, displaced abomasum, dystocia, lameness, and ketosis increases with hypocalcaemia.[92,13]

The prevalence of hypocalcaemia increases with age and parity, grass silage, or maize silage feeding in the pre-calving period, and breed (higher in Jersey compared to Friesian).[95,13] The prevalence of clinical hypocalcaemia in pasture-based herds in New Zealand is around 2% and a prevalence of 52% is reported for subclinical hypocalcaemia in 3-year-old or older cows.[95] Correct mineral and anion-salt supplementation in the pre-calving period play a crucial role in reducing the prevalence of clinical hypocalcaemia.[95] Metabolic alkalosis reduces the affinity of PTH for its receptor (in bone and kidney) resulting in impaired bone resorption and production of $1,25(OH_2)D_3$ which results in hypocalcaemia.[40,53] Alternatively, with metabolic acidosis, H^+ ions in the blood are exchanged with Ca^{2+} cations in bone, increasing serum Ca concentrations and improving Ca homeostasis.[40]

Increased energy utilisation in the transition period and reduced dry matter intake can lead to negative energy balance in dairy cows.[1,76] Consequently, energy production is required to counteract the negative energy balance, and this is achieved mainly through lipolysis.[76,1] With lipolysis, non-esterified fatty acids (NEFA) are released into circulation to be used either as an energy source or for the formation of triacylglycerol.[76] When large amounts are present, NEFA are converted to ketone bodies (acetone, acetoacetic acid, and especially β-hydroxybutyrate (BHB)) which are then used as an energy source by various tissues in the body but especially muscle.[1,76] Excessive NEFA in the liver can overwhelm the organ leading to re-esterification of NEFA into triacyclglycerols that, in excess, can accumulate, leading to hepatic lipidosis.[76]

Cows that fail to adapt to the increases in energy demand are more prone to develop metabolic disease, have reduced milk production, low reproductive performance, and can be prematurely culled from the herd.[76] Excessive ketone bodies in the blood (hyperketonaemia) can lead to ketosis.[76] Hyperketonaemia without clinical signs is known as subclinical ketosis (SCK).[13]

Evaluation of negative energy balance in dairy cows can be done through testing of blood NEFA concentrations (more accurate) and/or blood BHB concentrations.[76] Serum NEFA concentration is more sensitive and specific in the determination of the risk of disease, as an indicator of reproductive performance and milk production compared with serum BHB concentrations.[76] Nonetheless, testing for BHB is more practical and less expensive when compared to NEFA.[76] The concentration of blood NEFA reflects the magnitude of fat mobilization, whereas the concentration of BHB reflects the completeness of oxidation of fat in the liver.[65] Cows with higher body condition score (>4) have higher reductions in DMI, leading to more profound negative energy balances and consequently higher concentrations of BHB and NEFA in blood.[89]

The blood BHB thresholds for SCK diagnosis in the literature range between 1.2 and 1.4 mmol/L.[13] New Zealand had the greatest prevalence of SCK (40.1%) in a report that examined prevalence around the word.[13] however, Compton et al[21] reported a cow-level prevalence for SCK of 16.8% at 7-12 days post-calving in cows from three regions of New Zealand.[21]

1.7 Tools for assessment of bone strength

Bone strength is determined by bone mass, geometry (macroscopic geometry and microscopic architecture), and quality (bone material composition and structure).[20,35] Analysis of bone quality requires tools that can assess bone turnover, microarchitecture, mineralisation, microdamage, and the composition of bone matrix and mineral.[20] Diagnosis of osteoporosis in humans tends to rely solely on the assessment of bone mineral density using dual-energy X-ray absorptiometry.[35] However, the need to improve the prediction of bone strength and fracture risk has led to the development of different methods to assess bone strength.[35]

Bone turnover can be evaluated using blood or urine biochemical markers and/or bone histomorphometry.[20] Assessment of blood/urine biochemical markers provides whole body information rather than about a specific bone.[20] Biochemical markers of bone formation commonly measured include osteocalcin, bone-specific ALP and procollagen type 1 N propeptides.[20] Markers for bone resorption include collagen type 1 telopeptides (Ctx, Ntx), deoxypyridinoline, and TRAP type 5b.[20]

Trabecular (namely the size and shape of trabeculae, their connectivity, and orientation (anisotropy)) and cortical bone microarchitecture (cortical width, porosity, and bone size) as determinants of bone strength can be assessed histologically, or with high-resolution magnetic resonance imaging (HR-MRI), high resolution peripheral quantitative computed tomography (HR-pQCT), micro-CT (μCT) and synchrotron radiation μCT which provide a three-

dimensional evaluation and quantification.[20] These imaging techniques also allow multiple measurements in the same individual over time.[35] Bone microdamage (microcracks and microfractures) can be evaluated using histology.[20]

Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), small angle X-ray scattering (SAXS), Raman spectroscopy, and biochemistry analysis are used for the evaluation of the bone material composition.[20] Nuclear magnetic resonance imaging (NMR) provides information on the structure of the mineral within the bone and the water content.[35] Fourier transform infrared and Raman spectroscopy use an incident light on the specimen that excites vibrations and energy release from molecules.[35] Infrared spectra arise from absorption of energy while Raman spectra arise from the scattering of visible or ultraviolet photons that have gained or lost part of energy.[86] Each molecule has its unique vibrational characteristics.[86] Both FTIR imaging and Raman imaging are complimentary to each other and are used to examine changes in tissue properties with developmental stage, tissue age, or disease within a bone specimen.[35,86] Both techniques were used in this thesis.

Quantitative determination of the total amount of collagen and collagen crosslinks can be analysed using fluorescence measurement and high-pressure liquid chromatography (HPLC) techniques.[35]

Bone mechanical properties can be assessed using whole-bone mechanical testing (a bone is loaded to failure in compression, bending, or torsion) providing information on structural stiffness, failure to load, and the energy absorbed to

failure.[35] Other tests include microbeam testing, microindentation, and nanoindentation.[35]

1.8 Humeral fractures

Bone fractures occur in animals either due to extreme forces applied to the bone or secondary to bone disease, termed pathological fracture.[25] In a study that examined 213 long bone fractures in cattle, the femur was the most commonly affected bone (32% of cases), with only 12 cases affecting the humerus (5.6%).[326] In young cattle the most frequently affected bones are the metacarpus and metatarsus, apparently due to the lack of soft tissue around these bones and in calves are often associated with the use of traction to resolve dystocia in the new-born.[26]

Humeral fractures are reported to be infrequent in ruminants due to the musculoskeletal configuration around the humerus and the need for very high forces to fracture it.[91] Situations associated with a humeral fracture in ruminants include fighting, falling, impact with objects, mating in young bulls, and calves that are stepped on.[91] Humeral fractures are described as the only fractures that can be caused by uneven compressive or rotational forces transferred particularly to the distal humerus from the ground.[79]

The most frequently described location for femoral and humeral fractures in cattle is the diaphysis.[26] Most humeral fractures in ruminants have an oblique or spiral configuration and are thought to happen when the animal falls on its side resulting in mediolateral bending of the bone.[26] Fighting and mating injuries

produce torsional loading of the bone and lead to comminuted spiral fractures.[26] Occasional physeal fractures are also reported.[91]

A recent biomechanical analysis of the bovine humerus demonstrated that the highest stress concentration was in the distal humeral diaphysis and as a result, this area was regarded as the weakest part of the bone.[9] The increased cortical thickness observed in this section is believed to be an adaptation to try and reduce maximum stress.[9]

Clinically, a humeral fracture is usually associated with dragging and flexion of the affected limb with a "dropped elbow" or "dropped shoulder" appearance.[91] Animals usually don't place any weight on the limb and there is extensive tissue swelling, pain, and crepitus of the tissue with palpation.[91] The prognosis is poor in most cases due to the size of the animal and the lack of effective treatment.[91]

While humeral fractures have always occurred in dairy cattle, the first reported large-scale outbreak of humeral fractures in New Zealand occurred in dairy heifers from a spring-calving dairy farm in the Manawatū-Whanganui region where 6/200 (3%) crossbred heifers suffered spontaneous humeral fractures in the first months of lactation.[121] Copper deficiency due to a missed Cu injection in early winter was thought to be responsible for the increase in bone fragility and spontaneous fracture.[121] Since then, many farmers and veterinarians have anecdotally reported cases of spontaneous fractures, although on some farms Cu deficiency was ruled out. Most cases are reported in first lactation heifers, with occasional cases seen prior to calving and in second lactation heifers.[122]

1.8.1 Fracture healing

The process of bone fracture repair is by regeneration thus returning the bone to its original shape and strength.[25] Regeneration begins with haematoma formation and ischaemic necrosis of bone prompting an acute inflammatory response.[25] Cytokines and growth factors are produced by neutrophils and macrophages at the site and stimulate the migration of multipotential mesenchymal cells.[25] Transforming growth factor-β, fibroblast growth factor-1 and bone morphogenetic proteins (BMPs) control fibroblast differentiation with production of fibrous connective tissue, and chondrocyte differentiation with cartilage production forming the soft callus that anchors the fractured bone ends. Invasion of endothelial cells is controlled by vascular endothelial growth factor, BMPs, and fibroblast growth factor-1.[25] Later, angiopoietin I and II control vessel formation.[25] Osteoprogenitor cells differentiate into osteoblasts and begin the formation of the new bone characteristic of the hard callus phase, this is controlled mainly by BMPs. The final phase or remodelling of the fracture site can take several months or even years.[25]

The remodelling that occurs after a bone is fractured is described as systemic rather than just in the fractured bone and is related to mechanical influences (disuse), acute and chronic inflammation, and dysregulation of hormones that control mineral homeostasis.[82] The systemic changes associated with bone fractures worsen if they occur in older individuals and if they are associated with mineral deficient diets.[82] A systemic acceleratory phenomenon may increase bone remodelling to make minerals available for bone repair hence animals with

an already deficient diet can experience greater systemic bone loss after a fracture. Bone mass may not return to pre-fracture levels in these cases.[82]

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UNIVERSITY OF NEW ZEALAND

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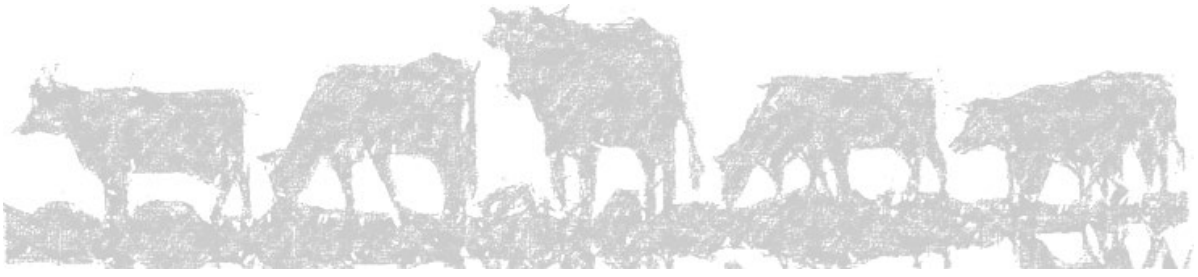
CHAPTER 2

SURVEY ANALYSIS OF FREQUENCY AND FACTORS ASSOCIATED WITH HUMERAL FRACTURES IN DAIRY HEIFERS



2.1 Introduction

Since 2008, when the first outbreak of humeral fracture in primiparous dairy heifers was described in New Zealand, anecdotally there has been a rise in the reported incidence and prevalence of this condition by veterinarians and farmers.[27] Furthermore, surveillance reports from the Ministry for Primary Industries (MPI) have raised concerns over the identification of potential causes or risk factors that may be associated with the condition.[6,1,5,2,4,8,23,25]

The increased incidence of cases has resulted in significant loss of income on affected farms, and the dairy industry in general, as well as substantial animal welfare costs. Results from a randomised national phone survey of 505 herds described most cases occurring in first-lactation heifers (11.7%), and a direct annual economic loss estimated to be over NZ$ 9 million (J Hunnam, unpublished data).

Although sporadic cases have been reported in Australia, only in New Zealand are outbreaks frequently reported.[24] Compared to other dairy systems around the world, the dairy system in New Zealand is unique, being seasonal-driven, extensive, and pastured-based.[11] The use of pasture as the main source of dry matter intake is a major difference between the New Zealand dairy industry and other dairy industries around the world.[9] These differences in dairy systems and husbandry practices have led to the hypothesis that the aetiology of humeral fractures might be a combination of nutritional factors and mineral deficiencies unique to New Zealand.[18,19] These factors potentially include inadequate protein-calorie nutrition in young heifers leading to deficient bone deposition, Cu

deficiency leading to poor bone collagen quality, and increased bone resorption secondary to the Ca demands of peak lactation.[18]

Although Cu deficiency has been described as an important contributory factor in the aetiology of fractured humeri, with low serum and/or liver Cu (LiCu) concentrations being a common finding in a large number of outbreaks, there have also been reports of farms with a high incidence of cases in where heifers had normal serum and/or LiCu concentration.[28,18,19,5,3,7,8] This failure of Cu deficiency, to consistently explain all outbreaks, has prompted the search for other causes that could be associated with or could predispose the appearance of cases on farms, including information regarding farm management, herd health, and nutrition.

The objectives of this chapter were to determine possible risk factors associated with humeral fractures in dairy heifers. To access information a survey was conducted collecting information on the management and nutrition of cows from birth to the first lactation using farms that have and have not had cases of humeral fractures in dairy heifers.

2.2 Materials and methods

This chapter reports a case-control study comparing farms that have had cases of humeral fractures in dairy heifers (case farms) with farms that have not had fractured humeri reported in dairy heifers (control farms). A questionnaire was designed and used to retrospectively compare how frequently the exposure to a risk factor was present in case farms and control farms and to determine the relationship between the risk factor and humeral fracture. The questionnaire was

divided into four sections that included: (a) contact information and farm location, (b) farm information, (c) herd health and nutrition, and (d) calf rearing practices.

Two delivery methods were used to collect information from farms across New Zealand: a one-page/printed questionnaire ("Humeral Fracture Questionnaire" - Appendix B) and an online survey (https://massey.au1.qualtrics.com/jfe/form/SV_eOKv5T2ffyqnsz3). The online survey was designed using commercially available software (Qualtrics®XM, Seattle, WAS, USA). A risk assessment through peer evaluation determined that the survey was low risk according to Massey University Human Ethics Committee, consequently, full ethical approval was not required.

The survey was advertised to veterinarians in the newsletter of the Dairy Cattle Veterinarians (HoofPrint), to veterinarians and farmers that contacted the members of the humeral fracture research group about heifer humeral fractures and/or that submitted samples from cases of spontaneous humeral fractures to Massey University, Palmerston North. Additionally, 100 printed versions with a stamped return envelope were sent to the Animal Care team at DairyNZ for distribution during their 2019/2020 farmer consults. A link for the survey was also posted on the Massey Heifer fracture Research group Facebook page (@masseyheiferfracture). Questionnaires were filled out by farmers and/or veterinarians between July 2019 and March 2020. Farmers and veterinarians decided whether they wanted to participate or not (self-selection).

2.2.1 Data Analysis

Before data analysis, partial responses (defined as respondents who did not complete all survey pages) and duplicates (defined as responses with the same contact's person name and/or farm address) were excluded. For questionnaires that were not excluded, responses were first divided into two groups (referred to as farm type): case farms and control farms. Case farms were those farms reporting a case or cases of humeral fracture and control farms were those farms that had no history or current cases of humeral fractures between July 2019 and March 2020 (2019/2020 dairy season). Data from those questions with multiple choice answers were grouped according to observed frequencies with percentages for each choice and according to farm type. The chi-square test of homogeneity was used to test whether a case farm and a control farm had the same frequency counts of a certain risk factor. Where the sample size was too small for the chi-square test, a Fisher's exact test was conducted. Answers with numerical data (questions 3 and 17) are presented as mean ± standard deviation (SD) and analysed using an independent sample-t-test. If the assumption of homogeneity of variance was violated a Welch t-test was run. Finally, open text answers were summarised.

2.3 Results

A total of 68 returned questionnaires were found suitable for analysis, with 35 responses from case farms and 33 responses from control farms. In the case of farm location, a similar distribution of case and control farms responses were observed from the North and South Island of New Zealand. There were twenty-six responses from the South Island (13 case farms and 13 control farms) and 38

responses from the North Island (20 case farms and 18 control farms). However, within each Island, the location distribution of case and control farms was not homogenous, Figure 2.1.

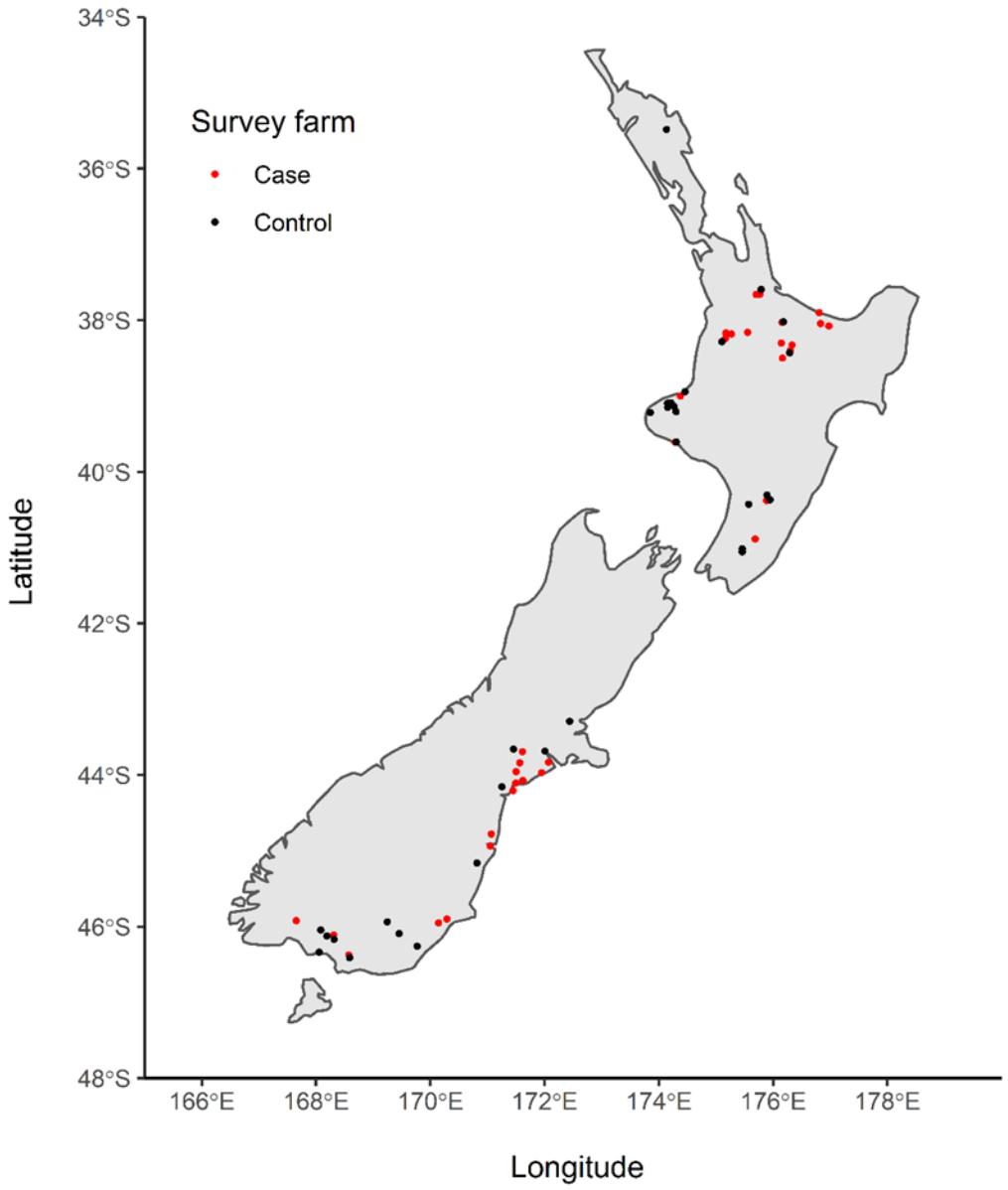


Figure 2.1 Map of New Zealand showing the geographical location of case (•) and control (•) farms from returned questionnaires, four farms (two case and two control) are not shown due to farmers withholding their addresses. Clusters of case farms (red dots) are observed in Waikato and Canterbury regions and clusters of control farms (black dots) are observed in Taranaki and lower South Island regions.

There was an excess of case farms from the Waikato and Canterbury regions and an excess of control farms from the Taranaki and lower South Island regions. For four returned questionnaires farm location was not given (two case and two control farms).

2.3.1 Farm information section

Questions regarding farm information can be found in Appendix B. Observed frequencies and percentages (in brackets) of the predominant breed of cows, calving pattern, and frequency of milking are presented in Table 2.1

Table 2.1 Distribution of response in case and control farms for the predominant breed, calving pattern, and frequency of milking.

	Farm information		
	Breed	**Calving pattern**	**Frequency of milking**
Case farm n=35	Friesian: 6 (17%)	Spring calving: 33 (94%)	All once a day: 2 (6%)
	Jersey: 1 (3%)	Autumn calving: 0 (0%)	All twice a day: 22 (63%)
	HFxJ: 27 (77%)	Split calving: 2 (6%)	Mix once/twice: 11 (31%)
	Other*: 1 (3%)		
Control farm n=33	Friesian: 17 (52%)	Spring calving: 31 (94%)	All once a day: 4 (12%)
	Jersey: 6 (18%)	Autumn calving: 0 (0%)	All twice a day: 21 (64%)
	HFxJ: 10 (30%)	Split calving: 2 (6%)	Mix once/twice: 8 (24%)
	Other: 0 (0%)		

* Not specified. HFxJ,
Holstein-Friesian Jersey crossbreed of unknown %.

For analysis of the predominant breed of cow on the farm, Fisher's exact test was conducted due to inadequate sample size for the chi-square test of homogeneity. There was a statistically significant difference in the multinomial probability distributions between case farms and control farms ($P<0.001$). Post hoc analysis

involved pairwise comparisons using multiple Fisher's exact tests (2 x 2) with a Bonferroni correction. Statistical significance was accepted at $P<0.0125$. There were statistically significant differences in the proportion of control farms in which it was reported that Holstein-Friesian was the predominant breed, as well as the proportion of case farms in which it was reported that Holstein-Friesian Jersey crossbreed (HFxJ) was the predominant breed ($P<0.0125$). Reducing breed to HFxJ or other (combined Holstein-Friesian, Jersey, and other), the odds of the predominant breed on a case farm being HFxJ were 7.4 times (95%CI 2.6 to 23.4) that compared to a control farm.

There was no evidence found for a significant difference in the type of calving pattern (Fisher's exact test $P=1$), and milking frequency (Fisher's exact test $P=0.61$).

The average herd production (in MS/cow) was normally distributed, as assessed by Shapiro-Wilk's test ($P>0.05$). The mean herd production for case farms was 410.4 milk solids (MS)/cow ± 51.6 and for control farms, was 441.3 MS/cow ± 83.4, a difference that was not significant ($P=0.09$).

The median number of years fractured humeri was reported on a case farm was two years, ranging from one to nine years. A total of 10/35 (29%) case farms had reported cases occurring for more than two years. In 16/35 (46%) case farms, the 2019/2020 season was the first-time cases of humeral fractures occurred.

2.3.2 Herd health and nutrition section

Observed frequencies and percentages (in brackets) of yes or no answers on whether Cu deficiency had been diagnosed in the herd, whether Cu

supplementation was used, whether Ca was supplemented through lime flour and whether fodder beet (FB) (*Beta vulgaris*) was offered to the herd are presented in Table 2.2.

Table 2. 2 Distribution of yes or no response in case and control farms regarding the previous diagnosis of Cu deficiency in the herd, use of Cu and lime four supplementation, and if cows were offered fodder beet for grazing.

Herd health and nutrition			
	Possible answer	**Case Farm n=35**	**Control Farm n=33**
Diagnosis of Cu deficiency	Yes	9/34 (26%)	5/33 (15%)
	No	25/34 (74%)	28/33 (85%)
Cu supplementation	Yes	29/35 (83%)	24/33 (73%)
	No	6/35 (17%)	9/33 (27%)
Lime flour supplementation	Yes	23/35 (66%)	23/33 (70%)
	No	12/35 (34%)	10/33 (30%)
Fodder beet offered	Yes	14/35 (40%)	10/33 (30%)
	No	21/35 (60%)	23/33 (70%)

For one case farm, there was no information regarding a previous diagnosis of Cu deficiency in the herd. There was no evidence for a difference in the proportion of the type of farms previously diagnosed with Cu deficiency (P=0.25). Similarly, there was no evidence for a significant difference in the proportions of type of farms using Cu supplementation (P=0.31). The distribution of type of Cu supplementation in the 29 case farms that used Cu supplementation was bolus (13/29, 45%), other (9/29, 31%), injection (9/29, 31%), and fertiliser (1/29, 3%). Three respondents selected more than one choice. On the 24 control farms that supplemented Cu, the type of supplementation used was: other (10/24, 42%),

bolus (7/24, 29%), injection (7/24, 29%), and fertiliser (2/24, 8%). The multinomial probability distributions were equal in the population (Fisher's exact test *P*=0.64).

The age groups that received Cu supplementation on case farms included: all ages (16/29, 55%), rising (R) ones (RIs) (9/29, 31%), and milkers (4/29, 14%). Similarly, for control farms, the distribution of age groups that received Cu supplementation included: all ages (13/24, 54%), RIs (4/24, 17%), milkers (6/24, 25%), and in 1/24 (4%) farm there was no information regarding what age group was supplemented. The multinomial probability distributions were equal in the population (Fisher's exact test *P*=0.38).

There was no statistically significant difference in proportions between the type of farms for Ca supplementation using lime flour (*P*=0.72). On case farms, different commercial formulations of Ca carbonate (80-100% limestone) were the most frequently used lime flour supplement. Supplement rates between 50 and 300 g/cow/day were reported for case farms and in 6 responses pasture dusting was described as the method of delivery. One respondent described mixing Ca with molasses, and another gave Ca as a drench. Similar supplementation rates (between 50 and 200 g/cow/day) were reported for control farms, with six respondents using pasture dusting and one administering it in molasses.

There was no evidence for a difference in proportions of case and control farms where FB was offered to cows (*P*=0.40). On six case farms, cows were grazed on FB during the winter and on four case farms, cows were grazed on FB during the autumn. The reported age groups of cows that grazed on FB on case farms

included: R1 on four farms, R2 on four farms, all cows on six farms, calves on one farm, and 3-years-olds on two farms. On control farms, cows grazed on FB during winter on nine farms, one farm reported FB being offered to milkers in autumn, one farm to milkers in summer, and one farm to R1 over autumn. Reported age groups on control farms that grazed on FB included: R1 on two farms, R2 on four farms, milkers on five farms, R3 on two farms, all ages on three farms, and calves on one farm.

Information regarding growth checks or health issues was available for 28 case farms and 30 control farms. Growth checks or health issues were reported in 8/28 (29%) case farms, compared to 2/30 (7%) control farms. There was a statistically significant difference in proportions (P=0.04, Fisher's exact test) for the presence of growth checks between case and control farms. Reported growth checks on case farms included: worm burden, slow growth, blood Se deficiency, Cu and Se liver deficiency, bovine viral diarrhoea positives in the herd, infectious bovine rhinotracheitis positives in the herd, unspecified numbers of calves that had coccidia and tested positive for *Cryptosporidium parvum* and rotavirus. For control farms, growth checks were reported at 10 weeks and 10 months (but the issue was not specified) and on one farm it was reported that calves tested positive for rotavirus.

2.3.3 Calf rearing section

Regarding feeding other than colostrum, calves from case farms were fed: whole milk (28/34 farms, 82%), milk powder (1/34 farms, 3%), and both (5/34 farms, 15%). On control farms, other than colostrum, whole milk was fed in 20/31 (65%)

farms, followed by milk powder in 2/31 (6%) and both in 9/31 (29%) farms. For one case farm and two control farms, this question was left unanswered. There was no evidence for a difference in feeding whole milk, milk powder, or both between case and control farms (P=0.34).

The mean milk fed per calf per day was 5.0 L/per calf/day ± 0.22 on case farms compared to 4.9 L/per calf/day ± 0.16 on control farms (P=0.77).

A meal supplement was fed pre- and post-weaning on 32/35 (91%) case farms and 28/30 (93%) control farms. The meal supplement was solely fed pre-weaning in 3/35 (9%) case farms and 1/30 (3%) control farm. The meal supplement was solely fed post-weaning in 1/30 (3%) control farm. In three questionnaires from case farms no response was selected. There was no evidence for a difference in meal feeding practices between case and control farms (P=0.47).

Finally, the mean age calves were allowed access to pasture on case farms was 3.4 weeks ± 1.9 compared to 4.5 weeks ± 2.2 on control farms, a difference that was significant (P=0.03).

2.4 Discussion

By surveying different aspects of farm management and husbandry practices on farms that have and have not had cases of spontaneous humeral fractures in heifers, three potential risk factors for humeral fractures in dairy heifers in New Zealand were identified. First, more cases were reported for farms where the predominant breed was HFxJ. Second, case farms reported more growth checks compared to control farms, and third, calves from case farms were on average

more than a week younger when allowed access to pasture compared to calves on control farms.

When analysing the New Zealand population of dairy cows by breed, a rapid growth in the population of HFxJ cows in the national herd over the last 30 years is observed.[20] From the 1998/1999 season to the 2008/2009 season (when the first outbreak of humeral fractures was reported in New Zealand), there was a 16% increase in HFxJ at the expense of Holstein-Friesian (14.2% less) and Jersey cows (2.2% less).[15,14] Over the next 10 years from the 2008/2009 season, a similar trend was observed with the number of HFxJ cows increasing by 14.7% reaching almost 50% of the population of dairy cows in New Zealand by 2021.[16,17] However, this breed data represents the total number of cows in New Zealand and not the numbers of farms with each breed.

In New Zealand, crossbreeding of dairy cattle is common, contrary to what happens worldwide.[10] The increased number of HFxJ cows in New Zealand is related to the better hybrid vigour of crossbred cows and their higher production worth index.[20,10] This survey suggests there may be a relationship between increased crossbreeding in New Zealand and the incidence of humeral fractures. This association may partly explain why fractured humeri seem unique to New Zealand.

Another significant finding in this survey was the tendency for more growth checks to be reported in heifers on case farms. Assessment and maintenance of animal health and/or welfare is crucial in dairy farms not only for productivity but also to follow international animal welfare standards.[9,26] This assessment is

done through visual evaluation of cows body condition score, lameness, injuries, and hygiene.[26] Although finding this tendency in case farms is quite exciting and potentially supportive of the hypothesis that imbalanced protein-calorie nutrition in young heifers is a risk factor associated with humeral fractures, it is also possible that the significant difference between case and control farms was due to recall bias. Recall bias is a well-reported systematic error that occurs when participants in a case-control study do not remember previous events or experiences accurately, for example, people with lung cancer are more likely to remember they smoked than people without lung cancer.[12] In this case it is possible that farmers with outbreaks of fractured humeri are more likely to remember growth checks than control farms.

The last risk factor that was significantly different between farms was that calves from case farms were turned out to pasture about a week earlier than calves from control farms. The time calves are put outside the shed to graze is dictated by the size of the shed, the number of calves it can house, and the geographical location (associated with weather conditions) of the farm.[9] Farmers usually turn calves out in fine weather or when the calf shed gets too full.[9] The non-homogenous distribution of case and control farms, with case farms on each Island tending to be further North, could mean that this association is spurious, however this finding needs more investigation. Certainly, a younger calf at turn out could be more likely to experience a growth check, which would also support the other significant finding that case farms had more growth checks.

Although not significant the higher average herd productions (in MS/cow) from control farms is likely due to the higher proportion of control farms with Holstein-Friesian cows compared with case farms. Holstein-Friesian cows tend to have higher milk yield compared to Jersey and HFxJ crossbred.[22] Other factors that can influence productivity include age, the efficiency of feed conversion, and, farm management (for example once a day versus twice a day milking frequency).[22,21] Considering case farms had lower average herd production compared to control farms, some of the factors formerly described might be influencing productivity.

The rates of lime flour supplementation reported here, when used, were similar to the industry-recommended lime flour supplementation rate (either through the dusting of pastures or by incorporating it into supplements being fed) that after calving cows receive between 100-300g of lime flour per cow/day.[13]

Finally, the response rate represents only 0.6% (68/11.179 dairy herds accounted for in the 2019-2020 season) of dairy farms responding.[16] Different surveying methods were developed in an attempt to collect a large number of completed questionaries, despite this, the response rate is very low and illustrates the difficulties in surveying farmers in New Zealand. A previous questionnaire, developed by the Massey Heifer fracture research group which also aimed to determine risk factors associated with humeral fractures only collected data from 9 case farms and 7 control farms (K Dittmer, unpublished data). The factors that affect survey response and completion need to be identified and addressed.

2.5 Conclusion

This questionnaire has identified the Holstein-Friesian Jersey crossbreed as a possible risk factor associated with the incidence of spontaneous humeral fractures in dairy heifers in New Zealand. The survey has also found possible associations between growth checks and age of turnout which also need further investigation.

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TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

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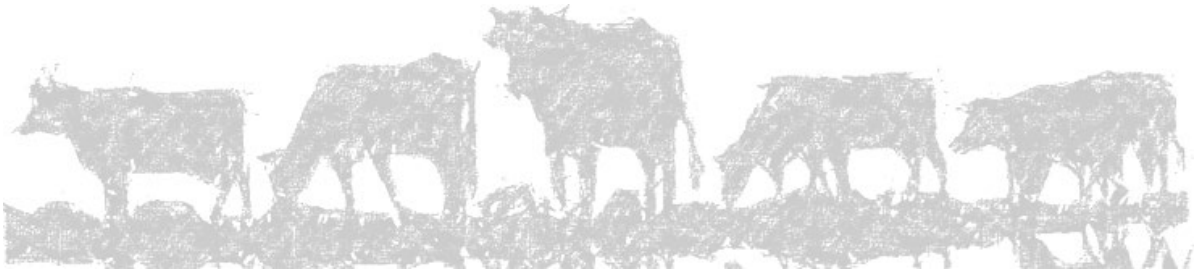
CHAPTER 3

BIOCHEMICAL PROFILE OF HEIFERS WITH SPONTANEOUS HUMERAL FRACTURES



3.1 Introduction

Spontaneous fracture of the humerus of dairy heifers was first reported in New Zealand in 2008, although, anecdotally, cases had been observed since the 1970s.[28] The incidence has increased significantly since, affecting animal welfare, impacting the mental health of farmers and veterinarians, and resulting in major economic losses to the dairy industry. A specific cause for the condition is not known and aside from sporadic cases in Australia, a high number of cases has only been reported in New Zealand.[12,9] The condition is described predominantly in first lactation heifers but occasionally prior to calving or in heifers in their second lactation.

When investigating an outbreak of pathological bone fractures in ruminants nutritional deficiencies, such as energy, protein, Ca, phosphorus, and vitamin D, should be considered as these can lead to abnormal bone growth or metabolic bone diseases such as osteoporosis and rickets/osteomalacia.[5,8] In the first reported outbreak, low liver and serum Cu concentrations were reported in many of the affected heifers and it was hypothesised that Cu deficiency in the months leading up to fracture had caused increased bone fragility resulting in fracture.[28] A recent investigation into humeral fractures in dairy heifers concluded that affected heifers had suffered from osteoporosis which was likely associated with periods of protein/calorie malnutrition, increased osteoclastic bone resorption to meet the Ca demands of lactation, and periods of Cu deficiency.[9] It was suggested that these factors decreased bone strength and led to the fracture of the humerus.[9]

To date, the energy and mineral status of dairy cows, which were euthanised due to a humeral fracture, has not been reported. It is well known that dairy cows go through significant changes in their metabolism to supply adequate energy and minerals to the developing fetus, for parturition and to initiate and maintain lactation.[15] Meeting the changing mineral and energy demands of the cow from pregnancy through to lactation are key for a successful transition through these periods and to avoid peri and post-parturient metabolic diseases.[15,17] It is possible that, as well as the more common presentations of metabolic diseases such as milk fever or acetonaemia, there can also be more gradual concurrent negative effects on bone growth and mineralisation when there is sub-optimal nutrition.

This report describes the serum concentrations of several biochemistry analytes associated with mineral and energy metabolism, serum Cu and liver Cu concentration, and their correlation in heifers that were euthanised due to spontaneous humeral fracture. We did not have access to a comparable set of samples from herd mates without fractures, hence it is unknown whether these biochemistry analytes would differ between affected and unaffected animals. Thus, concentrations of metabolites within the normal range in our dataset (animals with fractures) indicate there is unlikely to be an association between these metabolites and fractures. However, finding abnormal concentrations of metabolites in the fractured group does not necessarily imply associations as we could not define “normal” for these herds. In the absence of a control group, we have compared the concentrations in the fracture group to published reference ranges, but recognize that particularly for Cu, serum and liver concentrations

below the reference value are not synonymous with the development of clinical disease.

3.2 Material and Methods

3.1.1 Case History

This was an observational study using 35 liver and serum samples from spring calving heifers, on 17 different dairy farms, which fractured one or both humeri in the post-partum period from July to December 2019. The heifers were sampled after the humeral fracture was reported by the farmers to their veterinarian. Blood samples were collected as part of a diagnostic investigation into the cause of the fracture. A sample of the liver was collected from the affected heifers after euthanasia. The serum and liver samples were submitted to IDEXX Laboratories New Zealand Pty. Ltd., Palmerston North, NZ. The concentration of the following biochemistry analytes was measured on serum samples as part of the cow metabolic test profile: albumin, β-hydroxybutyrate (BHB), creatinine, Ca, Mg, phosphate, non-esterified fatty acids (NEFA), and serum Cu concentration. In liver samples, Cu concentration (LiCu) was measured.

Data on breed and predominant diet animals grazed in the winter months leading up to fracture occurrence was recorded by farmers and or veterinarians on the *pro forma* submission form for each case submitted (Appendix C). The feed category descriptors used within the submission form were pasture, FB, and other/mixed (described as mix of feeds including swedes (*Brassica napus*), maize (*Zea mays*) silage, oats (*Avena nativa*), and/or kale (*Brassica oleracea*), depending upon which one constituted the predominant feed on a dry matter intake basis.

3.1.2 Data Analysis

Control samples were not available for comparison; hence data were compared according to the reference interval provided by IDEXX Laboratories New Zealand Pty. Ltd., and the proportion of heifers with a humeral fracture with analyte concentrations outside of the reference range reported. Data were also summarised by mean and ± standard deviation (SD) for the biochemistry analytes. For analysis of Cu data, serum Cu concentrations were categorised as low (0-4.5 µmol/L), marginal (4.6-7.9 µmol/L), adequate (8.0-20.0 µmol/L) or high (20.0-50.0 µmol/L).[13] Liver Cu concentration was categorised as low (0.44 µmol/kg), marginal (45-94 µmol/kg), adequate (95-2000 µmol/kg) or high (2000-50000 µmol/kg).[13] A Pearson's correlation was run to assess the relationship between LiCu and serum Cu concentration. Statistical analysis was performed using SPSS statistics software (IBM® SPSS® Statistics version 27)

3.2 Results – Clinical findings

A total of 35 blood and liver samples were collected from cases of humeral fracture from 17 different farms. Serum samples from all 35 cases were analysed for albumin, creatinine, phosphate, Ca, Mg, and BHB and in 32/35 (91%) cases serum NEFA concentration was also measured.

Appendix D presents individual raw data and Table 3.1 shows the summary data, with the mean ± SD for each analyte and the proportion of cases with results below or above the reference range. Pertinent results included serum creatinine concentration below the reference range in 24/35 (69%) heifers, 20/35 heifers (57%) had high BHB serum concentrations and serum NEFA concentration was

increased in 3/32 (9%) heifers. The three heifers with high serum NEFA concentration also had high serum BHB concentration. Only one heifer had a serum phosphate concentration below the reference range. In two cases serum Ca and Mg concentrations were less than the lower detectable limit of the assay (analytical error) and these cases were not included, the remaining 33 heifers had serum Ca and Mg concentrations within the reference interval. All heifers with humeral fracture had serum albumin concentration within the reference range.

Case distribution according to the main breed of cows on the farm showed that 15/34 (44%) heifers were Holstein-Friesian Jersey crossbreed and 19/34 (56%) were Holstein-Friesian. In the case of farms, 9/17 (53%) farms had Holstein-Friesian Jersey crossbreed cows and 7/17 (41%) farms had Holstein-Friesian cows, with one submission not reporting breed data.

The main diet heifers grazed on in the months leading up to fracture occurrence (during winter) were described as predominantly pasture in 15/35 (43%) heifers, from nine different farms, predominantly FB in 14/35 (40%) heifers from three different farms, and mixed diet in 6/35 (17%) heifers from five different farms.

Table 3. 1 Mean (±SD), range, the proportion of case results with concentrations above or below reference range and the reference range used in this study for the concentrations of albumin, creatinine, PO4, Ca, Mg, non-esterified fatty acids, β-hydroxybutyrate, serum copper and liver Cu concentrations in 35 heifers with humeral fracture.

Analyte	n	Mean (± SD)	Range	Results	Reference range
Creatinine	35	48.7 ± 10.52	36-73	24/35 (69%) < 55 µmol/L 11/35 (31%) > 55 µmol/L	55-130
BHB	35	1.1 ± 0.31	0.4-2.1	20/35 (57%) ≥ 1.1 mmol/L 10/35 (43%) < 1.1 mmol/L	Adequate – 0.2 - 1.0 Increased – 1.1 - 10.0
NEFA	32	0.5 ± 0.36	0.2-1.4	3/32 (9%) ≥ 1.2 mmol/L 29/32 (91%) < 1.2 mmol/L	Adequate – 0.0 - 1.1 Increased – 1.2 - 4.0
Phosphate	35	2.1 ± 0.48	0.78-2.93	1/35 (3%) < 1.1 mmol/L 34/35 (97%) > 1.1 mmol/L	1.10-2.80
Ca	33	2.4 ± 0.14	2.12-2.74	33/33 (100%) > 2.00 mmol/L	2.00-2.60
Mg	33	0.9 ± 0.20	0.5-1.32	33/33 (100%) > 0.49 mmol/L	0.49-1.15
Albumin	35	32.4 ± 2.6	28-38	35/35 (100%) > 23 g/L	23-38
Analysis of Cu concentration					
SeCu	33	14.8 ± 7.34	3.2-31.4	2/33 (6%) < 4.6 µmol/L 5/33 (15%) < 8.0 µmol/L 18/33 (55%) < 20 µmol/L 8/33 (24%) ≥ 20 µmol/L	Low 0 – 4.5 Marginal 4.6 – 7.9 Adequate 8.0 – 20.0 High 20.0 – 50.0
LiCu	35	187 ± 342	15-1540	21/35 (60%) < 45 µmol/kg 3/35 (9%) < 95 µmol/kg 11/35 (31%) < 2000 µmol/kg 0/35 (0%) ≥ 2000 µmol/kg	Low – 0 – 44 Marginal – 45 – 94 Adequate 95 – 2000 High – 2000 - 50000

BHB, β-hydroxybutyrate; NEFA, non-esterified fatty acids; SeCu, serum Cu concentration; LiCu, liver Cu concentration.

Analysis of the analytes by predominant diet showed that creatinine concentration was below the reference range in 13/15 (87%) heifers grazing pasture, 7/14 (50%) heifers grazing FB, and in 4/6 (67%) heifers receiving a mixed diet. β-hydroxybutyrate was above the reference range in 5/15 (33%) heifers grazing pasture, 9/14 (64%) heifers grazing FB and 6/6 (100%) heifers receiving a mixed diet. There were no differences in the other biochemistry analytes when comparing diets prior to fracture.

Thirty-five heifers had liver samples analysed for LiCu concentration, and 33 heifers had paired serum and liver samples analysed. Many of the heifers (24/35, 69%) had low or marginal LiCu concentration (< 95 µmol/kg), but only 7/33

(21%) heifers had low or marginal serum Cu concentration (< 8 µmol/L) (Table 3.1). Of the heifers with low or marginal LiCu concentration, 11/15 (73%) heifers grazed pasture, 9/14 (64%) heifers grazed FB and 4/6 (67%) heifers received a mixed diet. For low or marginal serum Cu concentrations, 3/13 (23%) heifers grazed on pasture, 4/14 (29%) heifers grazed on FB, and 0/6 (0%) heifers received a mixed diet.

Paired liver and serum samples (n=33) showed that all 7 heifers with low or marginal serum Cu concentrations had low or marginal LiCu concentrations. The remaining heifers (16/33, 49%) had adequate or high serum Cu concentrations but low or marginal LiCu concentrations. Finally, 10/33 (30%) heifers had adequate serum Cu and LiCu concentrations. Pearson's correlation showed a statistically significant, moderate positive correlation between LiCu and serum Cu concentration, $r(31) = 0.43$, $P=0.014$.

3.3 Discussion

Analysis of the results indicated negative energy balance was common in heifers with a humeral fracture with 57% of heifers showing high BHB concentration and 69% of heifers having creatinine concentrations below the reference range. Furthermore, low/marginal LiCu concentration remains a significant finding in heifers with humeral fractures in New Zealand.

Although 57% of heifers had increased BHB, the increases were only slight, the mean for all heifers was within the adequate range, and the maximum value recorded was 2.1 mmol/L. These results alone would usually not be strongly indicative of a negative energy balance or sub-clinical ketosis (SCK) and this

observation is further supported by the finding that only 9% of heifers had concurrent increased NEFA concentration. There are several different published cut points when using serum BHB to diagnose SCK in New Zealand dairy cattle. Using a BHB blood concentration > 1.2 mmol/L, Brunner et al[3] found that 8.3 – 40.1% of New Zealand dairy cows were classified as having SCK, and using a cut point of ≥ 1.4 mmol/L, Compton et al[4] reported an SCK prevalence of 16.8% at 7-12 days post-calving in pasture-grazed dairy cows in New Zealand.[4,3] In this study 7/35 (20%) heifers had serum BHB concentrations > 1.2mmol/L and 3/35 (9%) heifers had serum BHB concentrations ≥ 1.4 mmol/L indicating SCK was present in some heifers at the time of humeral fracture. However, the prevalence of SCK reported in this study is similar to other studies and may indicate that our findings are normal for recently calved dairy heifers in New Zealand. If this is the case, then it is unlikely that SCK constitutes an important risk factor for spontaneous fracture of the humerus.

Discrepancies have been reported between the use of NEFA and/or BHB as indicators of energy status. Serum NEFA concentration appears to be a better indicator of negative energy balance, with higher sensitivity and specificity compared to BHB.[17] For accurate results on blood NEFA concentrations, samples should be collected in EDTA or red-top tubes (no anticoagulants), should be kept at 4°C after collection and the serum separated within 24 h of collection which can be difficult with field studies.[25] On the other hand, BHB is very stable, and can be measured in different fluids (urine, blood, milk), the cost is low and serum concentrations change less under different handling and storage conditions compared to NEFA.[25,17] In this study, information regarding sample collection,

handling, and/or time of collection was not reported by the submitters. However, most samples were sent overnight by courier, serum was not separated within 24 h and it is unlikely samples were collected onto ice. This means that some care should be taken when interpreting the serum NEFA concentration found in this study. Given the greater stability of BHB compared with NEFA, the BHB results in this study suggest a high proportion of heifers (57%) were in negative energy balance and mobilising adipose tissue to adapt to the transition period.

Heifers grazing on FB over the winter months, leading up to fracture occurrence, had a higher BHB blood concentration than those grazing on pasture. Fodder beet has increasingly been used as a winter feed in New Zealand, especially in the South Island due to its higher yield, compared to traditional winter crops, and the higher palatability associated with the high sugar content in the plant.[20] The higher energy content of FB often leads to higher body condition scores in those animals which winter on it which can lead to increased production of ketone bodies.[22,11] Fodder beet feeding may also increase the amount of butyric acid in the rumen which can be rapidly converted to BHB in the rumen with the consequent increase in blood BHB concentration.[2]

Serum creatinine concentration was below the reference range (< 55 μmol/L) in 69% of heifers with humeral fracture, with the mean value below 50 μmol/L. Serum creatinine concentration is used to evaluate kidney function but in normally hydrated animals with normal renal function, serum creatinine concentration can be used to evaluate muscle mass (it is weakly associated with muscle thickness) and undernutrition (correlates positively with body condition

score and low serum creatinine concentration appears to be secondary to mobilisation of muscle protein for energy production).[18,24,1] Additionally, a parity effect is described by Cozzi et al[7] with higher plasma creatinine concentration in primiparous (67 µmol/L) vs multiparous cows (64 µmol/L).[7] Considering the animals in this study were all heifers, serum creatinine concentration would be expected to be within or above the reference range.

The below reference range serum creatinine concentrations in a high proportion of heifers in this study may be indicative of periods of undernutrition (leading to decreased muscle mass) in heifers with humeral fracture and/or increased mobilisation of muscle protein for energy production, though this finding must be interpreted with caution considering the lack of a control group, information on the hydration status of cows, and body condition score. Additionally, serum creatinine concentration reference ranges reported by the diagnostic laboratory are for adult cows. Nevertheless, this potential lower muscle mass in affected heifers is an important factor not previously described. Indeed, humeral fractures are uncommon in ruminants, and this is thought to be because of the larger muscles around the humerus.[21] Hence, lower muscle mass (either due to decreased formation and/or increased mobilisation) could be a risk factor for bone fracture in these heifers. Moreover, muscle mass interacts with bone via the "muscle-bone unit" stimulating bone formation and strength, and lower body mass can reduce bone quality.[30]

Previous studies on humeral fractures reported that low LiCu and serum Cu concentrations in affected heifers had contributed to the appearance of humeral

fractures.[9,29] The link between Cu and bone is the enzyme lysyl oxidase, which is necessary for the formation of collagen and elastin crosslinks in bone providing bone strength.[6] The liver is the primary organ for Cu storage and serum Cu concentration is maintained through mobilisation of LiCu storage.[27] Additionally, the liver concentration of Cu and other minerals, represents what the diet has been for the last 30 days.[10] In this study, LiCu concentrations were low or marginal (< 95 umol/kg) in 69% of heifers indicating deficient Cu intake and liver Cu storage depletion of at least a month of duration, with 21% of these heifers having low or marginal serum Cu concentration. This indicates a more significant Cu depletion in these heifers that could lead to Cu deficiency. However, most heifers in the present study had adequate serum Cu concentration at the time of euthanasia, indicating that transport of Cu to tissues was likely sufficient for the functioning of Cu-dependent enzymes, such as lysyl oxidase. It can thus be suggested that although mostly depleted, the liver storages were still supplying enough Cu to maintain serum concentrations at the time of euthanasia for many of the heifers with a humeral fracture in this study.

Furthermore, previous reports on humeral fractures have not described clinical signs of Cu deficiency before heifers need to be euthanised due to the fractures. Clinical signs of Cu deficiency occur after long periods of deficiency; for example, when growing animals are fed low Cu diets, they first develop hypocupraemia (from ~100 days on the deficient diet), followed by coat colour changes, diarrhoea, and finally, alterations in gait and bowing of legs that takes at least 5 months to manifest.[19,26,14] Additionally, high variability in specific serum Cu and LiCu concentrations are described with clinical signs of Cu deficiency including

bone fractures.[16,19,26] The role of Cu deficiency in the pathogenesis of humeral fractures needs to be researched further, by investigating how the formation of collagen crosslinks in the bone is affected.

In the present study serum Ca, phosphate, and Mg concentrations in heifers with humeral fracture were within the reference intervals which could be explained in several different ways. Hypocalcaemia, hypomagnesaemia, and hypophosphataemia are uncommon in heifers compared to cows as the homeostatic mechanisms for maintenance of serum Ca, phosphorus, and Mg concentrations are meeting the extra demand.[23] Another reason why measured Ca concentrations were normal could be that the maintenance of serum Ca concentrations is at the expense of bone resorption, which if excessive can potentially affect bone strength.[15] However, serum total concentrations of Ca (rather than ionised), phosphorus, and Mg are considered unreliable methods to determine total body status and since the timing of fracture post-partum was not known, the interpretation of these results is limited.[24]

3.4 Conclusion

Despite its limitations, this study has shown that heifers with humeral fracture had β-hydroxybutyrate serum concentration above the reference range (although the results were like those measured in other studies on non-fractured heifers) and serum creatinine concentrations below the reference range. Together the β-hydroxybutyrate and creatinine results suggest protein/calorie undernutrition could be a component factor in heifers with humeral fractures, but the evidence is not strong and will again need further investigation. Although low liver Cu

concentration was a significant clinical finding in heifers with humeral fracture, further work needs to determine the true contribution of Cu to the pathogenesis of spontaneous humeral fractures in heifers and at what point in the animal's life and during which period of skeletal development this deficiency has the greatest effect.

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TE KUNENGA KI PŪREHUROA
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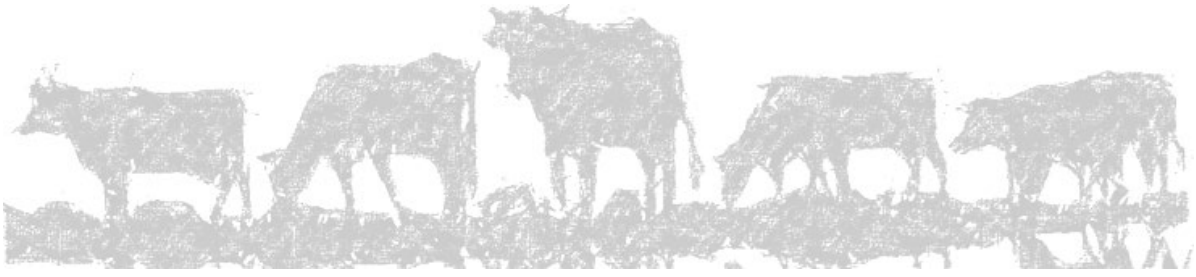
CHAPTER 4

OSTEOPOROSIS IS THE CAUSE OF SPONTANEOUS HUMERAL FRACTURE IN DAIRY COWS FROM NEW ZEALAND



4.1 Introduction

In 2008, an outbreak of spontaneous humeral fracture involving 6/200 first calving crossbreed dairy heifers was reported in New Zealand.[54] Investigations into this outbreak showed that several of the affected heifers had either low serum or low liver Cu (LiCu) concentrations, suggesting periods of Cu deficiency led to improper bone collagen crosslink formation and bone fragility, predisposing the heifers to spontaneous fracture.[55] Since then, the occurrence of spontaneous humeral fracture in dairy heifers has significantly increased (despite some farms implementing Cu supplementation to prevent deficiency) impacting animal welfare and mental health of farmers and resulting in economic losses to the dairy industry.[11]

Cases described so far have occurred mainly in first lactation cows post-calving, although occasional fractures have been seen prior to calving and in second lactation cows.[55] The clinical onset is sudden, and animals develop severe forelimb lameness (also referred to as dropped elbow or dropped shoulder).[54]

A small-scale study using computed tomography comparing the humeri of 10 heifers with humeral fractures and 10 unaffected age-matched controls found a significant decrease in the ratio of bone volume: total volume in affected animals.[11] In the same study, histological findings in the humerus of affected animals included the presence of short cartilage spicules, thin bone trabeculae with the presence of intratrabecular resorption, growth arrest lines, cortical osteoclastic resorption, and formation of additional woven bone between trabeculae.[11] Three affected animals had low LiCu concentration and one had low

serum Cu concentrations.[11] The authors concluded that the affected heifers had osteoporosis, with inadequate bone deposition during crucial growth periods (leading to lower peak bone mass) and/or increased bone resorption (associated with gestation and lactation) as the main mechanisms resulting in decreased bone density.[11] Limitations of the study included that it only examined 10 cases, the affected animals were from a specific region of New Zealand and there was no information regarding the animal's diet in the months prior to the occurrence of the fracture.

Diet is important because, anecdotally, humeral fractures are common in cows grazing on a diet that is predominantly fodder beet (FB) (*Beta vulgaris*) before parturition (during the winter months in New Zealand). This crop has increasingly been used as a winter crop in New Zealand, despite being phosphorus and protein deficient, and has been associated with rickets/osteomalacia in lambs born to ewes that grazed on FB during gestation.[12,21] However, humeral fractures also occur in cows that predominantly graze on pasture in the winter months, and it is hypothesized that these animals go through phases of protein-calorie malnutrition during important growth periods leading to osteoporosis and subsequent fracture.[11]

The aims of this study were 1) to determine the histological findings (qualitative) and to quantitatively evaluate histomorphometric changes in a large cohort of heifers affected by spontaneous fracture of the humerus; 2) to determine if histological and histomorphometric changes in bones from heifers grazing a diet of predominantly FB over winter are different to heifers that graze on a pasture-

based diet over the winter; 3) to describe and compare histological changes in heifers according to LiCu concentration, and 4) to describe the histological findings in the costochondral junction (rib).

4.2 Material and Methods

4.2.1 Study Design and Sample Collection

This was a case-control study using a convenience sample from fractured (affected) and non-fractured (control) heifers. The case definition for enrolling an animal in the affected group was a dairy cow of any breed, at least 2-year-old, which had suffered a spontaneous fracture of the humerus, without any history of trauma, within 6 months of calving. Samples were provided by farmers and veterinarians who after reporting a case of spontaneous fracture of the humerus were presented with a list of samples to be collected postmortem (Appendix C). These included the humerus (fractured and/or contralateral), ribs (specifically including the costochondral junction (CCJ)) and a piece of liver. A *pro forma* submission form (containing farm and animal data, main feed offered before parturition, and relevant clinical information) was developed and sent to the case submitter for completion (Appendix C).

A control was defined as a 2-year-old cow (a cow with an ear tag indicating they were born 2 years ago) of any breed that had calved recently (udder consistent with lactating) and had been culled for reasons unrelated to bone fracture of the humerus or any other bone. Control samples were obtained from an animal rendering plant (Wallace Corporation, Feilding, NZ) and Massey University School of Veterinary Science postmortem service. From each control case, a

sample of the humerus, the CCJ, and a piece of liver was collected postmortem. Owing to the method of sampling, no information regarding the cow diet and/or the reason for culling was available for the control animals.

4.2.2 Gross Evaluation

The humeri and CCJ were dissected away from surrounding soft tissues and examined grossly, and photos taken to record the gross appearance. Subsequently, several bone slabs (~3-5 mm thick) were obtained using a band saw. One slab from the humerus was then cleaned with water to remove the bone marrow and allow evaluation of the trabecular bone and cortex. Photos of the uncleaned and cleaned bone slabs were taken. For each animal, a subset of bone slabs from the humerus and the CCJ were placed in 10% neutral buffered formalin solution until further processing for microscopic evaluation.

Data recorded in the gross evaluation of the fractured humerus included the location and extension of the fracture, the appearance of the cortex, the appearance of the trabecular bone, and the presence/absence of growth arrest lines.

4.2.3 Histological Processing and Evaluation

Sections from the humerus and CCJ were processed for histological evaluation. Bone slabs were placed in glass jars with 10% hydrochloric acid (Decalcifier hydrochloric acid, Amber Scientific Ltd). Glass jars with samples were placed on a Thermo shaker incubator at 37°C and 180 rpm (Thermo shaker MB100-4A, Hangzhou Allsheng Instruments CO., Ltd). Every 24 h a manual evaluation of the decalcification process was performed and the decalcifier solution was changed.

When properly decalcified, bone samples were trimmed to fit a histology cassette. Samples were processed routinely for histology, embedded in paraffin, sectioned at 4-5 µm, and stained with haematoxylin and eosin (HE) for evaluation.

Appendix E shows the five locations in the humerus selected for histological evaluation and the histological features considered for evaluation. Measurements (growth plate and cortical thickness) were taken using imaging software (Olympus cellSens Standard 1.18, Olympus Corporation). Histological parameters were graded using a subjective dichotomous ordinal scale depending on whether the observed parameter was abnormal/present (1) or normal/absent (0).

In sections of the CCJ, histological findings were evaluated and recorded. Finally, the growth plate thickness was measured at 3 different sites (right, centre, and left side) and the mean was calculated.

4.2.4 Histomorphometry Processing and Evaluation

One 1 cm x 1 cm formalin-fixed sample of the primary spongiosa of the humerus was used for histomorphometric evaluation from a subset of 20 affected heifers (10 were randomly selected from heifers that grazed on FB during the winter months and ten randomly selected from heifers that grazed on pasture during the winter months) and 10 control heifers.

The undecalcified bone sections were dehydrated in graded alcohol, cleared with xylene, and placed first in resin infiltration solution I, then solution II for 24 h in each (90% Methyl methacrylate (MMA) and 10% Dibutyl phthalate (BPO), Sigma-Aldrich). Next, samples were placed in a resin embedding solution (95%

MMA/5%BPO) for 24 h. Benzoyl peroxide (Luperox® A75, Sigma-Aldrich) was used as the polymerization agent. Samples were then cut at 6 µm using a super-microtome (Mikrotom 2050 supercut, Reichert Jung). Sections were stained with Goldner's modified trichome stain. For each case bone area (B.Ar) in μm^2, total area (T.Ar) in μm^2, B.Ar/T.Ar, mean perimeter in µm, mean trabecular width (mean Tb.Wi) in µm, mean osteoid area (mean O.Ar) in μm^2, and mean osteoid perimeter (mean O.Pm) in µm were measured. For B.Ar, T.Ar, and B.Ar/T.Ar, standard BoneJ plugins (Domander, R., Felder, A. A., & Doube, M. (2021). BoneJ2) for ImageJ (version 1.53c, National Institute of Health) were used. Evaluation of mean trabecular perimeter, mean Tb.Wi, mean O.Ar, and mean O.Pm were measured using ImageJ software (version 1.53c, National Institute of Health). Results were compared between affected and control cases, and according to the main diet over winter (FB vs pasture).

4.2.5 Determination of Liver Copper Concentration

Liver samples from the affected and control groups were submitted to a commercial diagnostic laboratory (IDEXX Laboratories New Zealand Ltd.) for determination of LiCu concentration using inductively coupled mass spectrometry (NexION 2000B ICP Mass Spectrometer, PerkinElmer). Liver Cu concentration between 0-94 µmol/kg was considered low/marginal concentration and values >94 µmol/kg were considered adequate LiCu concentration.

4.2.6 Statistical analysis

An independent-samples-t-test was used to determine if there were any significant differences in the values of growth plate thickness, cortical thickness,

and quantity of resorption in the distal humerus between affected and control heifers, between heifers that grazed either FB or pasture as their main winter feed and between heifers with low/marginal and adequate LiCu concentration. Values from these parameters were first log transformed to achieve a normal distribution. If the assumption of homogeneity of variances was violated, a Welch t-test was run to determine differences between groups.

For all the dichotomous parameters, a chi-square test for homogeneity was done to compare differences in the distribution of proportions between heifers in the affected and control group, between heifers in the affected group that grazed either FB or pasture as their main winter feed, and between heifers with low/marginal or adequate LiCu concentration. If the minimum sample size for each expected frequency was not met (greater than or equal to 5), then results for Fisher's exact test were presented instead. In all tests, a *P* value of < 0.05 was considered significant.

For the comparison between heifers in the affected and control group, the parameters were put into a multivariable logistic regression model to predict the probability of heifer suffering from humeral fracture. For a variable to be included as a parameter in the model it had to have an observed count (distribution) of more than 1 and the *P* value obtained from the t-test or chi-square had to be significant ($P<0.25$).[8] The model was constructed using forward selection, with variables retained at $P<0.05$, and the model fit was assessed using the Hosmer-Lemeshow (HL) test and the Nagelkerke R^2.

Finally, an independent-samples t-test was conducted to compare differences in the histomorphometric data between the affected and control group and between heifers that grazed on FB compared to heifers that grazed on pasture. Differences were considered significant if $P<0.05$. All statistical analysis was done in SPSS statistics (IBM® SPSS® Statistics version 27).

4.3 Results

4.3.1 Study Population

A total of 80 humeri were collected from farms throughout New Zealand from heifers that fitted the case definition. Out of the 80 samples of humerus, 47/80 (59%) cases also included one or several sections of CCJ and in 66/80 (83%) cases a piece of liver was submitted. Forty-three of the 80 (54%) affected heifers were Kiwi cross heifers (Holstein-Friesian x Jersey, of unknown proportions), 28/80 (35%) Holstein-Friesian, 4/80 (5%) Jersey, and 5/80 (6%) had no breed information.

Case distribution according to the predominant diet offered in the winter months showed 33/80 (41%) cases grazed on FB, 28/80 (35%) cases grazed on pasture, 14/80 (17.5%) cases were fed another type of feed, and in 5/80 (6.5%) cases information regarding main winter feed was not provided. Diets in cases classified as "other" contained a mix of feeds including swedes (*Brassica napus*), maize (*Zea mays*) silage, oats (*Avena nativa*), and/or kale (*Brassica oleracea*).

The only reported clinical sign was non-weight-bearing lameness of the affected leg. One case reported that the heifer was lame after turning around in the cow shed, another case reported bumping into an object, and in four cases the heifer

was reported to be in oestrus, artificially inseminated, and/or bulling the day prior to the fracture.

A total of 22 humeri were sourced from control animals, 17/22 (77%) controls also included one or several sections of CCJ and 22/22 (100%) included a piece of liver.

4.3.2 Gross Findings

In 51/80 (64%) cases, the affected humerus was included, whereas for 29/80 (36%) the non-affected (contralateral) humerus was submitted. The gross changes described below are for the 51 humeri with fractures. Fractures in 49/51 cases (96%) were complete, nonarticular, simple, spiral fractures extending from the lateral side of the humeral head, just beneath the greater tubercle, spiralling along the diaphysis distally, to end just above one of the condyles (Figure 4.1a). In 2/51 cases (4%), the fracture was complete, comminuted, and transverse through the diaphysis.

Evaluation of bone slabs from affected cases revealed that 39/51 (76%) had a mild reduction (~30% less) in the amount of trabecular bone associated with an expansion of the bone marrow cavity towards the primary spongiosa when compared to controls (Figure 4.2a and c). The distal humerus in 20/51 (39%) cases had evidence of increased cortical resorption identified as numerous red

streak marks (Figure 4.2d). No cases had macroscopically visible growth arrest lines.

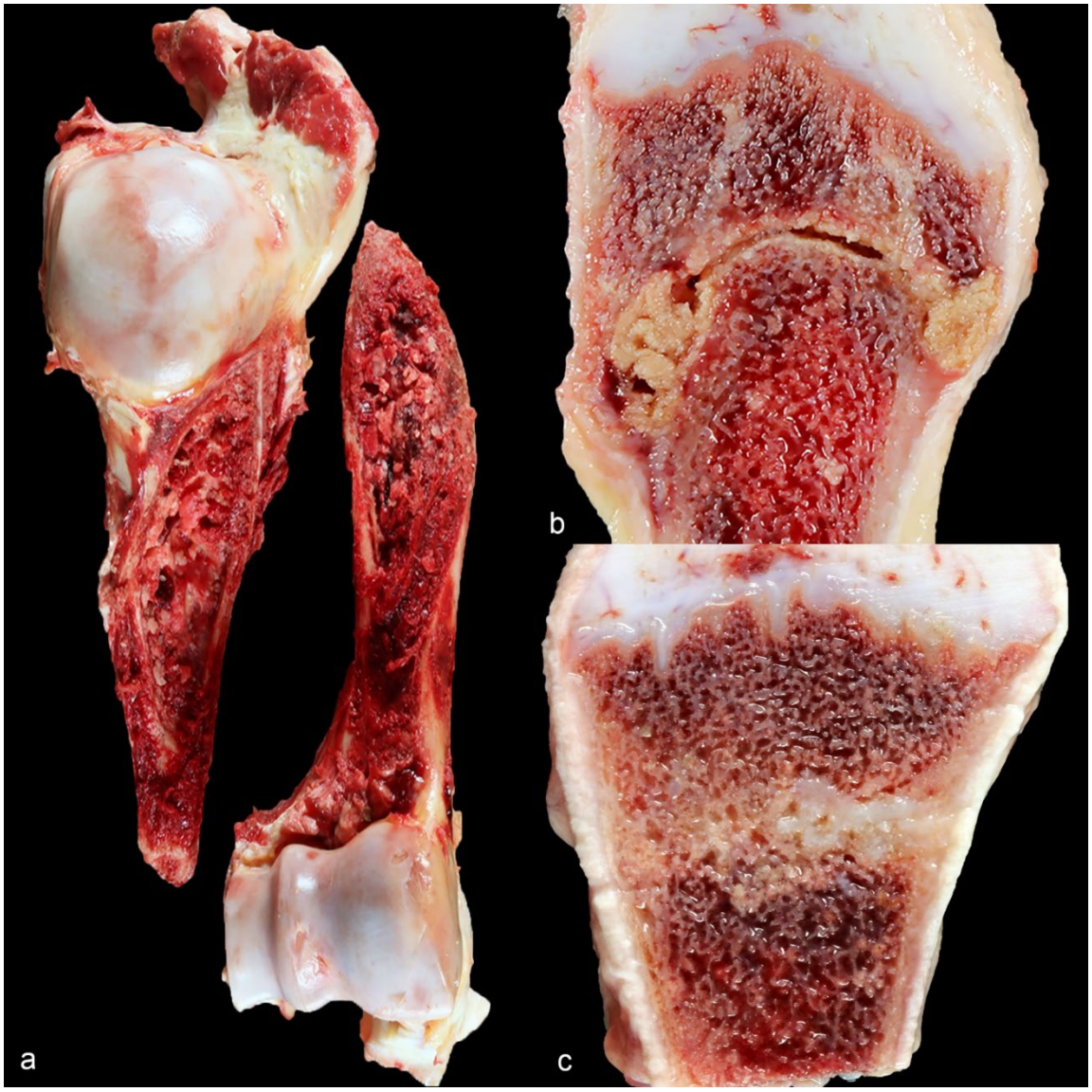


Figure 4.1 Humerus and ribs from affected heifers. (a) Spiral fracture, humerus, heifer. The fracture line extends from the lateral side of the humeral head (just beneath the greater tubercle) and extends distally to end just above one of the condyles. (b) Costochondral junction (CCJ), heifer. Cut surface of the rib and CCJ from an affected heifer with a bulging, irregular cortex and a fracture line extending cortex to cortex associated with the presence of necrotic bone and haemorrhage. (c) Costochondral junction (CCJ), heifer. Rib and CCJ from an affected heifer show an older fracture line with the presence of cartilaginous tissue.

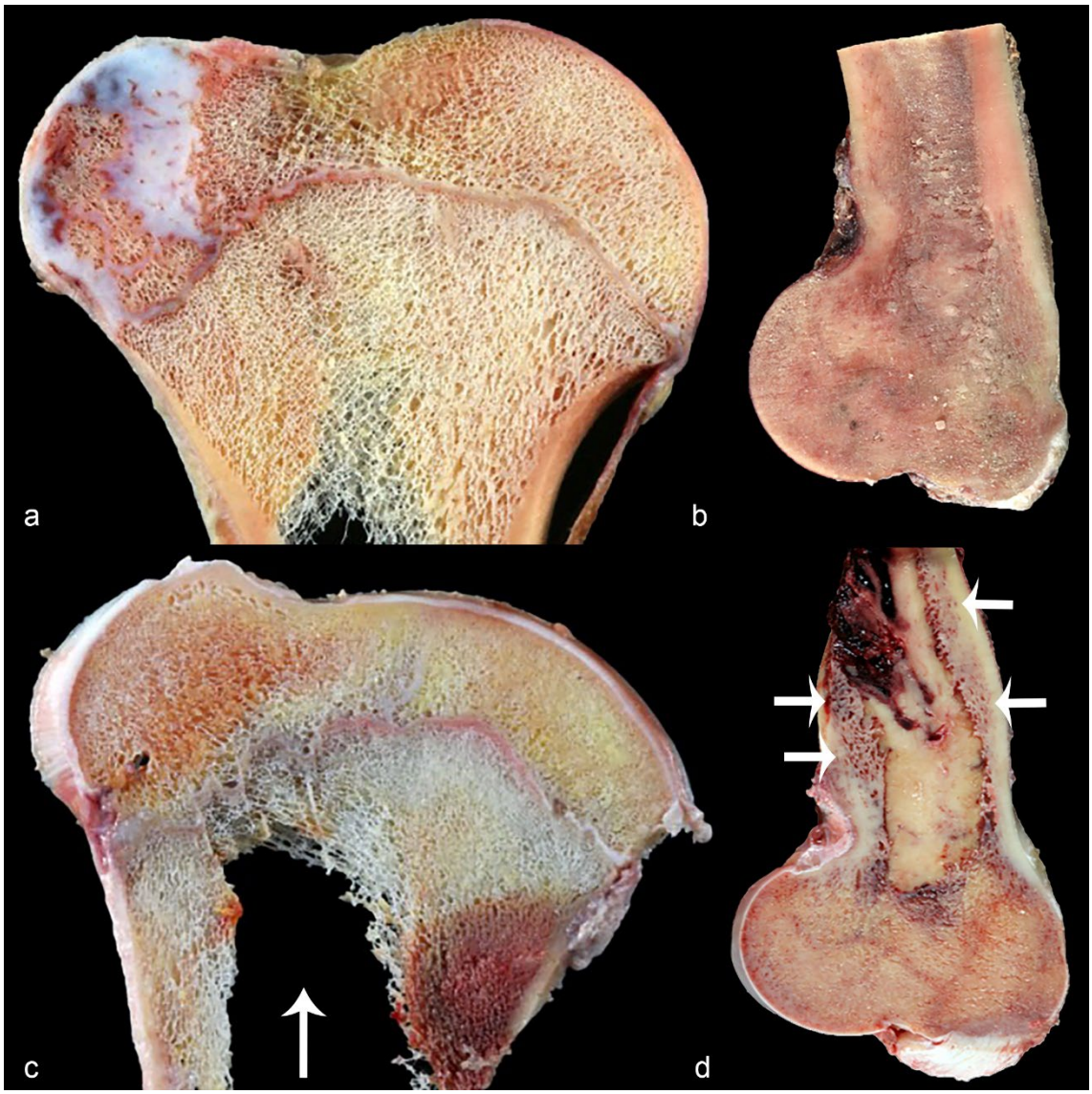


Figure 4.2 Humeral bone slabs, control and affected heifer. (a) Control heifer, proximal humerus. Normal appearance of the growth plate and abundant trabecular bone. (b) Control heifer, distal humerus. Normal appearance of the distal humerus with thick cortex and absence of resorption cavities. (c) Affected heifer, proximal humerus. There is an expansion of the marrow cavity (arrowhead). (d) Affected heifer, distal humerus. Variable thickness and numerous red marks in the cortex (arrows) are associated with resorption cavities.

Evaluation of 32/47 (68%) ribs revealed moderate to marked enlargement of the CCJ (up to a 3-fold increase compared to controls). The cut surface in these cases revealed the presence of a fracture in the metaphysis, extending in most cases from cortex to cortex and characterized by a bulging, rounded, irregular cortex mixed with areas of necrotic bone, haemorrhage, and proliferation of tissue that appeared either oedematous or fibrous and/or cartilaginous (Figure 4.1b). In 9/47 (19%) the CCJ did not appear enlarged, but the cut surface revealed the presence of lesions in the metaphysis consistent with remnants of previous fractures or fractures in an advanced stage of healing, characterised by proliferation of fibrous connective tissue and/or bony cartilaginous tissue (Figure 4.1c). In one (2%) case the growth plate was thickened, irregular, and had small tongue-like extensions of cartilage into the primary spongiosa. In 5/47 (11%) cases the cut surface of the rib and CCJ appeared grossly normal.

Finally, 4/17 (24%) heifers from the control group, had lesions like affected heifers, consistent with a rib fracture and formation of callus tissue. In the remaining 13/17 (76%) controls, the ribs were grossly normal.

4.3.3 Histology Findings

4.3.3.1 Humerus

Affected vs control: the proximal humeral growth plate was significantly thicker (a difference of 35.3 µm, 95% CI, 18.2 to 52.4) in affected heifers compared with control heifers, while the humeral cortex was thicker (a difference of 153.8 µm, 95% CI, 97.5 to 210.1) in control heifers compared to affected heifers. Heifers with fracture had a higher number of resorption cavities (12 more, 95% CI, 6.7 to 16.7)

in the distal humerus compared to heifers in the control group. Mean ± standard deviation (SD) and *P* values are presented in Table 4.1.

In the case of the dichotomous parameters, most heifers in the affected group had decreased trabecular density, abnormal trabecular architecture, abnormal growth plate appearance, additional woven bone formation in the primary spongiosa and proximal metaphysis, and 17/80 (21%) heifers in the affected group had histological evidence of growth arrest lines (Figure 4.3). A greater number of heifers in the affected group showed evidence of periosteal reactive bone formation and abnormal cortical resorption compared to heifers in the control group (Figure 4.3h). Finally, 24/80 (30%) had evidence of additional woven bone formation in the cut-back zone (Figure 4.3i). Abnormal cortical resorption was characterised by numerous coalescing resorption cavities extending transversely through the cortex (Figure 4.4a).

In contrast, most heifers in the control group had normal trabecular density, trabecular architecture, and growth plate appearance; absence of growth arrest lines, periosteal reactive bone formation; and no additional bone formation in the primary spongiosa, proximal metaphysis, or distal humerus (Figure 4.3). Finally, cortical resorption was limited to the periosteal and/or endosteal side of the cortex with sparse intracortical resorption cavities (Figure 4.4b). Statistical significance for each of the dichotomous bone parameters evaluated is presented in Table 4.2 and case distributions are presented in Appendix F. All parameters evaluated were significantly different ($P<0.05$) when comparing heifer in the affected and control groups.

Table 4.1 Mean ± SD of the proximal growth plate thickness of the humerus and costochondral junction, humeral cortical thickness, and the number of resorption cavities in the distal humerus comparing cases in the affected and control group, fodder beet vs pasture group, and low/marginal vs adequate liver Cu concentration group.

	Humerus		
	Growth plate thickness, µm	**Cortical thickness, µm**	**Resorption[a]**
Affected n=80	126 ± 38.89	220.9 ± 106.17	19 ± 11.68
Control n=22	91.1 ± 20.49	374.8 ± 154.41	7 ± 2.69
P value	0.0005*	0.0005*	0.0005*
Fodder beet n=33	128.5 ± 36.22	214.1 ± 113.35	20 ± 11.93
Pasture n=28	106.3 ± 19.90	251.5 ± 113.80	16 ± 8.03
P value	0.016*	0.01*	0.03[b]*
Low/marginal LiCu n=45	117.5 ± 32.81	237.8 ± 122.25	20.7 ± 12.87
Adequate LiCu n= 43	114.6 ± 34.66	293.6 ± 149.27	12.0 ± 9.19
P value	0.556	0.050*	0.005*
	Costochondral junction		
	Growth plate thickness, µm		
Affected n=47	147.5 ± 38.0		
Control n=16	116.3 ± 38.2		
P value	0.002		
Fodder beet n=21	162.6 ± 42.1		
Pasture n=17	136.2 ± 31.5		
P value	0.032		
Low/marginal LiCu n=27	131.7 ± 31.7		
Adequate LiCu n= 33	146.9 ± 46.7		
P value	0.306		

[a]Quantity of resorption cavities in the distal humerus.
[b]Welch t-test.
*P value <0.05.
LiCu, liver Cu concentration.

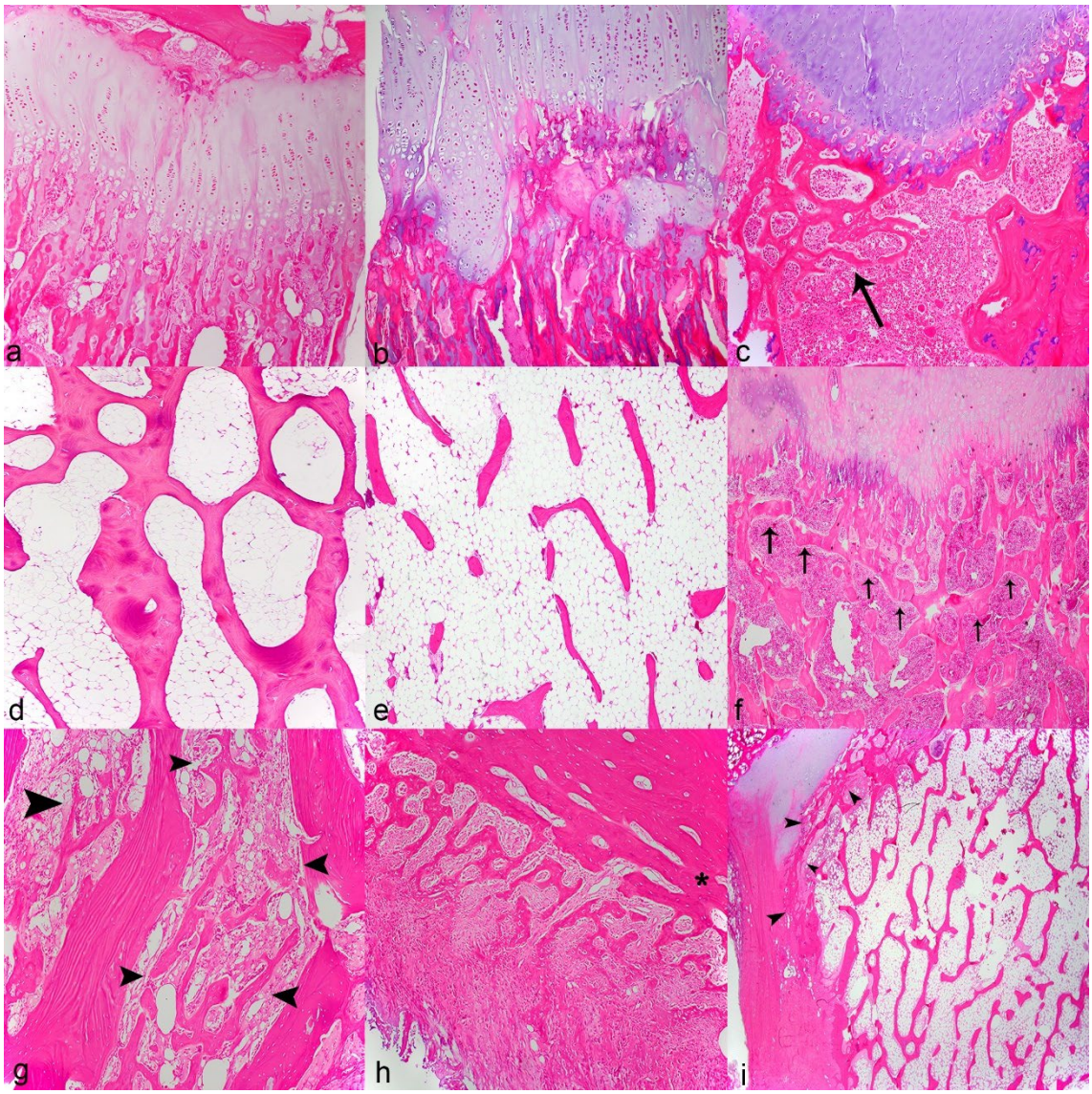


Figure 4.3 Histological appearance of humeral sections evaluated in affected and control heifers. Haematoxylin and eosin (HE). (a) Growth plate and primary spongiosa, control heifer. Normal growth plate architecture with thin growth plate, organised chondrocyte columns, and normal appearance of the primary spongiosa. (b) Growth plate and primary spongiosa, affected heifer grazed on fodder beet. Abnormal growth plate architecture with thicker, irregular growth plate, disorganised chondrocyte columns, and extension of cartilage into the primary spongiosa. (c) Growth plate and primary spongiosa, affected heifer grazed on pasture. Normal growth plate architecture with thin growth plate, organised chondrocyte columns but with the formation of additional woven bone in the primary spongiosa (arrow). (d) Trabecular bone, control heifer. Normal trabecular architecture with abundant, thick, and interconnected bone trabeculae. (e) Trabecular bone affected heifer. Abnormal trabecular architecture with fewer, thin, long, unconnected bone trabeculae. (f) Growth plate and primary spongiosa, affected heifer. Transverse bone trabeculae below the growth plate (growth arrest line) (arrows). (g) Proximal metaphysis affected heifer. Formation of woven bone (arrowheads) between trabeculae. (h) Cortical bone affected heifer. Periosteal reactive woven bone mixed with fibrous tissue extending from the cortex (*). (i) Cut-back zone, affected heifer. Additional woven bone formation in the cut-back zone (arrowheads).

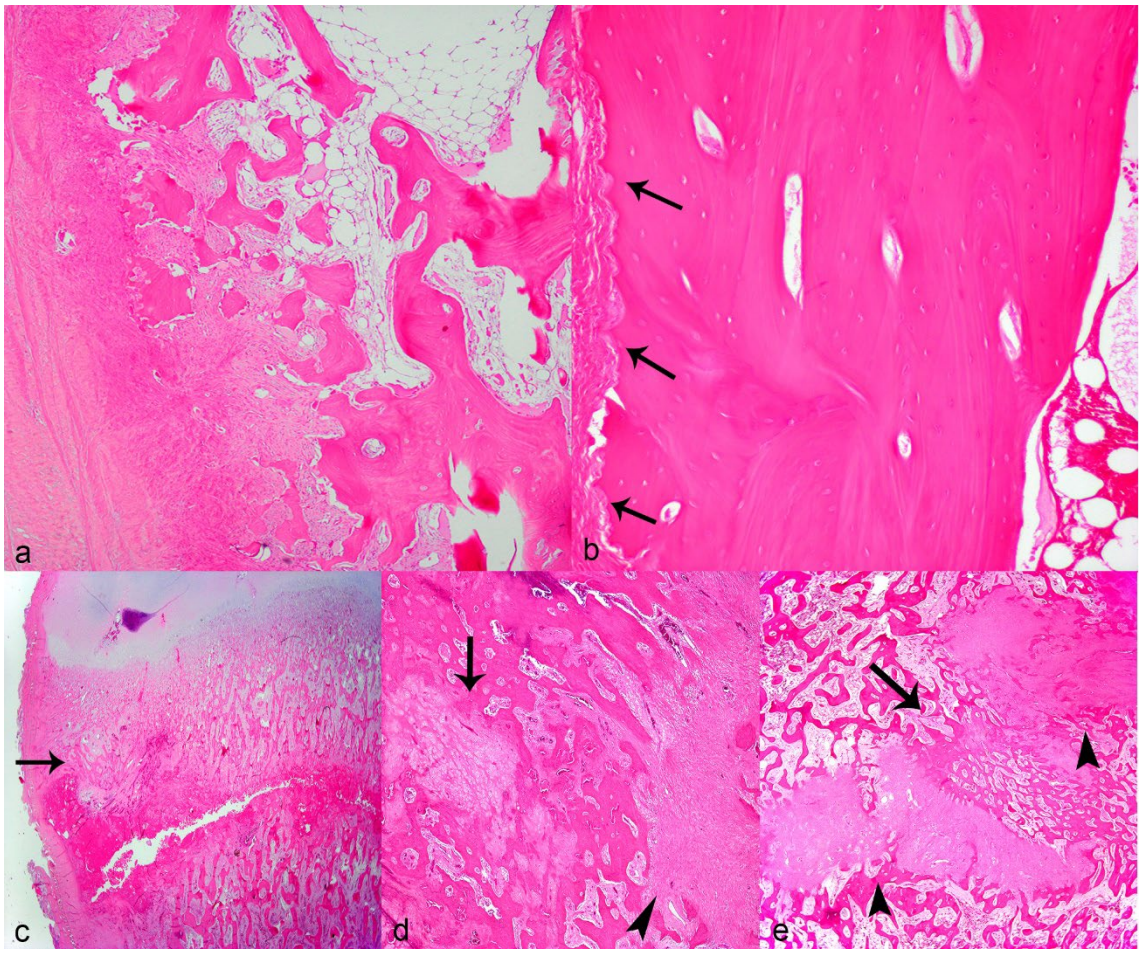


Figure 4.4 Histological appearance of humeral cortical bone, costochondral junction, and rib from affected and control heifers. Haematoxylin and eosin (HE). (a) Metaphysis, humeral cortical bone, affected heifer. Abnormal cortical thickness and appearance of the cortical bone from an affected heifer with osteoclasts extending transversally through the cortex, leaving a cortex with a moth-eaten appearance. (b) Metaphysis, humeral cortical bone, control heifer. Thick cortex, only the periosteal surface shows cortical scalloping due to resorption lacunae (arrow). (c) Costochondral junction and metaphysis, rib, affected heifer. Fracture line extending from the cortex into trabecular bone associated with haemorrhage and disorganised trabecular bone (arrow). (d) Trabecular bone, rib, affected heifer. Older fracture line with proliferation of fibrous connective tissue (arrowhead) and formation of soft callus tissue (arrow). (e) Trabecular bone, rib, affected heifer. Irregular replacement of fracture line by a proliferation of fibrous connective tissue (arrowheads) and additional woven bone formation (arrow).

Table 4.2 Statistical significance of each of the bone parameters analysed comparing cases in the affected and control group, fodder beet vs pasture group, and low/marginal vs adequate liver copper concentration group.

	Groups compared		
Bone parameter analysed	**Affected vs Control**	**Fodder beet vs Pasture**	**Low/marginal Cu vs adequate[a]**
Trabecular architecture	<0.0005*	0.945	0.08
Trabecular density	<0.0005*	0.330	0.02*
Growth plate appearance	<0.0005*	<0.0005*	0.83
Growth arrest lines	0.020[c]*	0.107	0.45
Additional bone[b] in the PS	0.011*	0.629	0.06
Additional bone[b] in the CB	0.003*	0.921	0.28
Cortical resorption	<0.0005*	0.483	0.008
Periosteal reactive bone formation	<0.0005*	0.036*	0.03*
Additional bone[b] in the PM	<0.0005*	0.029*	0.76
Additional bone[b] in the DH	0.011[c]*	0.583	0.14

[a]Refers to liver Cu concentration.
[b]Additional woven bone formation.
[c]Fisher's exact test.
* *P* value <0.05.
PS, primary spongiosa; CB, cut-back zone; PM, proximal metaphysis; DH, distal humerus.

Fodder beet vs pasture: The growth plate was thicker (a difference of 22.3 µm, 95% CI, 6.91 to 37.63) and growth plate appearance was significantly abnormal in heifers grazing FB compared to heifers grazing pasture (Figure 4.3b and c). Cortical thickness and quantity of cortical resorption in the distal humerus were not significantly different between groups. Mean ± SD and *P* values are presented in Table 4.1. Heifers that grazed on pasture had significantly more woven bone formation in the primary spongiosa and greater formation of periosteal reactive bone compared to heifers that grazed on FB. Statistical significance for each of the dichotomous bone parameters evaluated are presented in Table 4.2 and case distribution is presented in Appendix F.

Low/marginal vs adequate liver Cu concentration: A significantly greater proportion of affected heifers 41/66 (62%) had low/marginal LiCu concentrations compared to control heifers 4/22 (18%). When analysing the number of resorption cavities in the distal humerus, regardless of the fracture status, the mean number of resorption cavities was greater (9 more, 95% CI, 3.8 to 13.5) in heifers with low/marginal LiCu concentration compared with heifers with adequate LiCu concentrations. Also, cortical bone was thicker (a difference of 55.73 µm, 95% CI, 2 to 113.4) in heifers with adequate LiCu concentration compared to heifers with low/marginal LiCu concentration. There were no significant differences in growth plate thickness between groups. Mean ± SD and *P* values are presented in Table 4.1.

For the dichotomous parameters, significantly more heifers with low/marginal LiCu concentration had decreased trabecular density 34/45 (76%), abnormal

cortical resorption (36/45, 80%), and formation of periosteal reactive bone (31/45, 69%) compared to heifer with adequate LiCu concentration. There were no significant associations between all other dichotomous bone parameters analysed. Statistical significance for each of the dichotomous bone parameter evaluated are presented in Table 4.2 and case distribution is presented in Appendix F.

4.3.3.2 Ribs

The main microscopic changes seen in the rib sections from affected heifers were an abnormal growth plate appearance characterised by an irregular growth plate with the extension of hypertrophic chondrocytes into the primary spongiosa forming tongues and/or islands of cells. Furthermore, all sections of the rib evaluated had incomplete or complete fracture lines extending cortex-to-cortex. Fractures had a variety of changes ranging from the presence of necrotic bone mixed with congestion, haemorrhage, and oedema to callus tissue formation (Figure 4.4c). In a few cases, attempts at callus tissue formation were observed, characterised by a proliferation of cartilaginous tissue (soft callus) and/or woven bone (hard callus) (Figure 4.4d). In most cases, fracture lines were filled with a proliferation of fibrous connective tissue associated with an irregular proliferation of woven bone at the periphery of the fracture line (Figure 4.4e). Nonetheless, in most cases where callus tissue was identified, there was also a proliferation of fibrous connective tissue interspersed with cartilage and woven bone indicative of an abnormal repair process. Other findings included the presence of numerous microfractures, thickening of the periosteum by a

proliferation of fibrous connective tissue, identification of granulation tissue, marked trabecular resorption, and an irregular bulging cortex.

Lesions in the ribs from control heifers showed 5/22 (23%) cases had small (2-5 cells thick) nests of chondrocytes in the primary spongiosa. In four cases where grossly a fracture line was observed, these were histologically characterised by proliferation of fibrous connective tissue and multifocal areas with soft callus and hard callus proliferation. Lesions in control animals also had areas of haemorrhage, formation of additional bone between trabeculae, and thickened periosteum.

The rib growth plate was thicker (a difference of 31.1 µm, 95% CI, 9.1 to 53.1) in heifers in the affected group compared with heifers in the control group. Finally, the growth plate of the rib was thicker (a difference of 26.4 µm, 95% CI, 1.4 to 51.4) in heifers that grazed FB in winter compared to heifers that grazed pasture. Mean ± SD and *P* values are presented in Table 4.1.

4.3.4 Histomorphometry Findings

Comparison between the affected and control group showed heifers in the control group had a greater bone area ($P<0.005$), higher bone area/total area ($P<0.005$), longer mean trabecular perimeter ($P=0.027$), and mean trabecular width ($P=0.03$) values. Osteoid area ($P=0.24$) and osteoid perimeter ($P=0.18$) were not significantly different between groups. There were no statistically significant differences in any of the histomorphometric parameters evaluated when comparing FB and pasture as the main winter feed. Mean ± SD and *P* values are presented in Appendix G.

4.3.5 Logistic Regression Analysis

The following nine bone parameters were tested in the logistic regression model: trabecular architecture, trabecular density, growth plate appearance, formation of additional bone in the primary spongiosa and proximal metaphysis, type of cortical resorption, growth plate thickness, cortical thickness, and amount of resorption in the distal humerus. Four were retained in the final model: decreased trabecular density, abnormal cortical resorption, presence of additional woven bone formation in the proximal metaphysis, and the number of resorption cavities in the distal humerus. The logistic regression model was statistically significant, $\chi 2(4) = 85.152$, $P<0.0005$. The Hosmer and Lemeshow test was not statistically significant ($P=0.95$) indicating the model is a good fit. The model explained 88.6% (Nagelkerke R2) of the variance in humeral fracture and correctly classified 94.9% of cases. Sensitivity was 96.1% and specificity was 80.9%. The positive predictive value was 97.3% and the negative predictive value was 86.9%. The odds of an animal with decreased trabecular density having a fracture were 249.4 times higher than the odds of an animal with normal trabecular density having a fracture. Abnormal cortical resorption was also associated with an increased likelihood of humeral fracture (odds ratio 54.2) as was the presence of additional bone in the proximal metaphysis of the proximal humerus (odds ratio 37.2).

4.4 Discussion

Humeral fractures are infrequent in ruminants due to the musculoskeletal configuration around the humerus and the need for very high forces to fracture the humerus.[41] The fact that the fractures in affected heifers in this study are

spontaneous and numerous heifers can be affected at the same time within a single herd implies a significant reduction in bone strength/quality as the main mechanism leading to the appearance of fracture. Furthermore, the gross and histological changes present in sections of ribs from a large proportion of heifers with humeral fractures, showing fractures of different ages and stages of healing, indicate that the issues with bone strength and quality are systemic and have been compromised for some time.

The histological and histomorphometric results from this study reveal that heifers with humeral fracture have osteoporosis characterised by reduced trabecular density, abnormal trabecular architecture, reduced cortical thickness with increased abnormal cortical resorption, and the presence of growth arrest lines. We believe that these characteristics are the main factors that contributed to decreased bone strength resulting in humeral fracture.

Osteoporosis is characterised by a reduction in bone mass, bone mineral content, and bone matrix, which severely compromises bone quality and strength and increases the risk of bone fracture.[35,40,45] Histological changes of osteoporosis are characterised by decreased trabecular number, trabecular thickness, cortical thickness, and increased trabecular separation and cortical porosity, which are remarkably similar to the findings presented in this study of heifers with humeral fracture.[50,18]

Mechanisms leading to osteoporosis include failure to achieve peak bone mass (failure of bone formation), high bone turnover (excessive bone resorption), and/or low bone turnover (normal osteoclastic activity with reduced osteoblastic

activity).[9,40] Considering the histological and histomorphometric findings in this study, both failure of bone formation and excessive bone resorption have contributed to the osteoporosis observed in affected heifers.

When considering bone formation, the impact of nutrition on bone formation/growth (achievement of peak bone mass) and strength, especially during the early years of life, is important. Failure to achieve peak bone mass, and low protein/caloric intake during adolescence leads to osteoporosis and increased fracture risk in humans.[4,5] Similarly, prepubertal nutrition has an important effect not only on mature body weights in dairy heifers but also on milk yield and bone growth.[22,23,52,20] In heifers, live weight is important because it determines the bone size and is a major determinant of the strain stress index (a proxy for bone strength) in the humerus of dairy heifers.[22,23] Furthermore, although calves born from dams with high milk production tend to be lighter, if heifers reach pre-mating target live weights (before 12-14 months of age), there are no significant differences in skeletal (frame) size in later months, further emphasising the importance of nutrition in early life.[25,48]

Analysis of growth patterns in New Zealand dairy heifers shows there are normally two periods of reduced growth rate, the first is ~9 months of age (coinciding with the period of lowest pasture quantity and quality in winter), and the second between 22-24 months of age, corresponding to the second winter.[26] This is significant because when the growth of the metacarpus is compared with the humerus in growing dairy cows, the humerus keeps growing during the second year of life making it susceptible to additional growth checks.[23]

Several microscopic changes in the bone can provide information regarding nutrition and bone growth. Firstly, the presence of growth arrest lines is important. Growth arrest lines are transverse bone trabeculae that appear histologically when the activity of the growth plate stops for some time and is suggestive of starvation and/or malnutrition for a period of time.[38,9] The presence of these growth arrest lines in cases of humeral fracture previously described led to the suggestion that protein/calorie undernutrition was one of the factors contributing to reduced bone strength and osteoporosis.[11] While only 21% of heifers with a humeral fracture in the present study had growth arrest lines, remodelling can cause resorption and disappearance of growth arrest lines.[31] Increased remodelling was observed in many sections of humeral fractures in the current study and could have masked the identification of greater numbers of growth arrest lines.

Secondly, the histological appearance of the growth plate can also provide valuable information on patterns of growth in long bones.[49,9] The significant difference in growth plate thickness (both in the humerus and CCJ) and growth plate appearance in affected heifers compared to control heifers in this study may indicate a mismatch between the actual bone mass and the expected bone mass. Catch-up growth (referred to as compensatory growth) is a phenomenon that occurs after a period of nutrient restriction or another inhibitory factor whereby there is inhibition of chondrocyte senescence leading to longer chondrocyte columns and a thicker growth plate compared to age-matched controls, such as was seen in physes from heifers with humeral fractures.[17,19]

The other mechanism that can lead to the appearance of osteoporosis and that needs to be considered is increased bone resorption. In dairy cows, increased osteoclastic bone resorption is observed physiologically during gestation and this increases up to three-fold during lactation with the majority of this resorption occurring in trabecular bone.[28,42] Furthermore, primiparous cows and cows with higher milk yield have more active bone resorption than multiparous cows and cows with lower milk yield, as assessed by plasma and urine bone biochemical markers.[33,32] Excessive bone resorption can lead to fewer and thinner trabeculae (observed in 81% of affected cases), with loss of trabecular connectivity (observed in 78% of affected cases), compromising bone architecture and strength leading to fracture.[44] These frequently observed trabecular changes, plus the higher probability of breaking a bone with decreased trabecular density suggest increased resorption is an important factor impacting bone strength in these heifers. Decreased trabecular density, trabecular connectivity, and thickness affect the biomechanical strength of bone interfering with the capacity of trabecular bone to withstand a load.[37]

As a result of the reduction in the quantity and quality of trabecular and cortical bone, additional bone may be formed between trabeculae, particularly in areas with marked stress and strain.[16,51] In affected heifers additional woven bone formation was observed in the primary spongiosa (66% of cases), cut-back zone (30% of cases), proximal metaphysis (75% of cases), and the distal humerus (24% of cases). This formation of "extra" bone in affected heifers indicates that numerous areas of the humerus from affected heifers are under mechanical stress.[43] Additional woven bone between trabeculae appears histologically around

7 days after trauma in small animals and around 2 weeks in humans.[43] However, this new bone is not readily available for resorption and this could mean that osteoclastic activity increases along the already small and poorly formed pre-existing trabeculae to supply Ca for lactation.[43] Finally, the formation of periosteal reactive bone is also a mechanism to increase bone strength at sites of maximum stress-strain, and for fracture repair, as was observed in 75% of samples from heifers in the affected group.[3,13,51]

In the humeral cortex of affected heifers, there was a significant reduction in cortical thickness and a significant increase in resorption activity that was abnormal. Cortical bone thickness is determined by the rate of endosteal bone resorption (not by a lag in bone formation) and is key for bone strength.[2,44] Contrary to trabecular bone, only minimal resorption is observed in cortical bone before and after gestation.[42] Clumping and enlargement of resorption canals in the cortex causes a reduction in cortical thickness and focal areas of weaker bone leading to fracture, as seen in humans with nontraumatic intracapsular femoral neck fractures.[30] Abnormal cortical microstructure affects maximum stress distribution facilitating propagation of microcracks that can lead to bone fracture.[1] A similar pathophysiological process is likely occurring in the humeral cortex of affected heifers with enlargement of resorption cavities severely compromising cortical bone thickness and strength.

Additionally, increased cortical resorption was observed in the distal humeral sections in affected heifers and this is an important observation considering the distal humerus has been described as the biomechanically weaker area of the

bovine humerus.[6] Bouza-Rodriguez and Miramontes-Sequeiros[6] performed a biomechanical analysis on a three-dimensional model of the bovine humerus and found that when basic stimulations (compression, bending, and torsion) were applied to the model, the maximum stress was reached in the distal metaphysis.[6] Additionally, this study found that the lower diameter of bone in the distal humerus may also contribute to making this zone weaker and that the increased cortical thickness in this area is a type of bone adaptation, trying to decrease the maximum stress.[6] The increased resorption observed in the distal humerus in affected heifers combined with the observation that this is a potential weak point suggests the distal humeral metaphysis is the origin of the fracture line, contradicting a previous hypothesis that identified the cut-back zone as a weak area and the zone where the fracture most likely started.[11]

Finally, the binomial logistic regression model used in this study showed that decreased trabecular density, abnormal cortical resorption, formation of additional bone in the metaphysis, and the number of resorption cavities in the distal humerus strongly predicted the presence of humeral fracture in heifers, further supporting these parameters as important in the determination of bone strength and risk factors for fracture.

Fodder beet is an alternative crop that has been increasingly fed to pregnant, non-lactating dairy cows in parts of New Zealand.[36] Recently, health and welfare issues in animals grazing this crop have been reported, including poor live weights gains and nutritional congenital rickets in sheep.[21,53,12,14] Health issues are associated with the high sugar content of FB, which is also relatively low in

protein, fibre, and minerals (phosphorus, Ca, and Mg).[21,14] Heifers grazing FB in this study had a significantly thicker and abnormal growth plate appearance, compared to heifers grazing pasture, and therefore may indicate the presence of rickets in animals that had FB as their main winter feed, accelerating the onset of fractures. However, there was a lack of a significant difference in osteoid area and perimeter as evaluated by undecalcified bone sections. This suggests there is not an apparent lag in the mineralisation of bones between the groups evaluated (affected vs control and FB vs pasture), which does not support the diagnosis of rickets in animals on FB.

The presence of significantly more additional woven bone formation and periosteal reactive bone in pasture-grazed heifers imply bone from these heifers may be under greater mechanical stress compared with heifers that grazed FB.[10,7]

Another important factor that was studied in this group of heifers was the relationship between LiCu concentration and humeral fractures. Copper deficiency results in inadequate formation of crosslinks in collagen and elastin molecules leading to decreased mechanical strength and potential bone fracture.[27] Decreased lysyl oxidase activity (due to Cu deficiency) leads not only to inadequate collagen crosslink formation but also a reduction in bone formation by osteoblasts.[15]

Experimentally, Cu deficient calves and lambs have thickened and disorganised growth plates, decreased osteoblasts, and increased osteoclasts, along with a decreased number of bone trabeculae and loss of trabecular connectivity.[46,47,29] Other bone-related clinical signs in ruminants grazing Cu-deficient pasture

include poor growth and weight gain, lameness, enlargement of joints, increased brittleness of bones, and increased incidence of spontaneous fractures.[24,27,34,46] Additionally, several studies have described a relationship between Cu and osteoclast differentiation and activity. One study found significant inhibition of resorption in bone cultures when treated with copper sulphate (associated with activation of the enzyme prostaglandin endoperoxide reductase).[56] Induction of a hypoxic microenvironment that may inhibit bone resorption is also described in the presence of Cu ions.[39]

In the heifers in this study, the cortex was significantly thinner and there was significantly more resorption in the distal humerus of heifers with low/marginal LiCu concentrations compared to heifers with adequate LiCu concentrations, suggesting Cu deficiency could have resulted in increased osteoclastic activity. Low/marginal LiCu concentrations were also associated with decreased trabecular density, formation of additional bone, abnormal cortical resorption, and presence of periosteal reactive bone formation. These findings imply a likely correlation between decreased bone strength and increased fracture risk in heifers with low/marginal LiCu concentration. The activity of lysyl oxidase associated with the formation of collagen crosslinks and the relationship between the activity of osteoblasts and osteoclast with Cu concentration in tissues requires investigation.

4.5 Conclusion

Qualitative (histology) and quantitative (histomorphometry) analysis of bone sections support a diagnosis of osteoporosis as the main disease occurring in

these heifers. Periods of inadequate feed quality and perhaps Cu deficiency have led to a lag in bone formation with heifers failing to achieve peak bone mass at a critical point in their production cycle (i.e., the start of lactation), resulting in a reduction in trabecular number, width, and connectivity. In addition, there is increased cortical bone resorption leading to a marked reduction in cortical thickness and bone strength. Spontaneous fractures of the humerus are a direct consequence of these changes.

Moreover, the influence of grazing on FB during important growth periods and depletion of Cu concentration on bone quality, structure, and the occurrence of bone fractures in animals needs to be further investigated

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In which chapter is the manuscript/published work?	5
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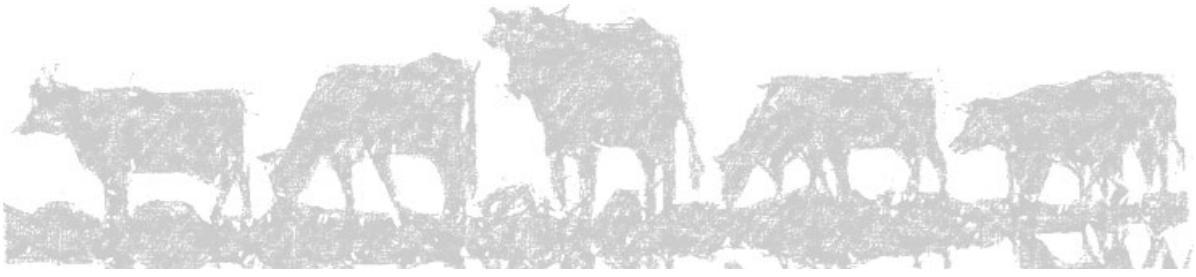
CHAPTER 5

RAMAN AND FOURIER TRANSFORM INFRARED SPECTROSCOPY ON HUMERI FROM DAIRY HEIFERS



5.1 Introduction

The increased incidence of spontaneous humeral fractures since 2008 in dairy heifers in New Zealand has prompted the study and analysis of normal and fractured bone material using a variety of techniques.[10,22,13] For example, histological analysis of a small set of bone samples from heifers with humeral fractures determined that heifers with humeral fractures developed osteoporosis secondary to periods of poor bone formation (probably due to protein-calorie malnutrition), increased bone resorption associated with lactation, and periods of Cu deficiency.[10] Peripheral quantitative computed tomography of the mid-diaphysis of the humerus showed that fractured animals have reduced cortical bone mineral density which reduced the stress-strain index.[12] These factors were thought to contribute to reduced bone strength/quality and the incidence of spontaneous humeral fractures in dairy heifers. Results from chapter 4, confirmed the diagnosis of osteoporosis on a larger sample of affected animals, and suggested periods of inadequate feed quantity/quality as the likely cause of this.

Bone strength is determined by multiple factors including bone architecture, bone quality, and bone mass.[8,20] For example, bone mass (determined by the amount of the bone organic matrix and mineral crystal hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)) influences bone physicochemical properties.[1,2] Similarly, bone quality is directly influenced by the rate of bone turnover (the process of bone resorption followed by new bone formation) which in turn can affect bone microarchitecture, mineralisation, microdamage repair, and the relative amount of bone organic matrix and mineral crystals.[8,1] Furthermore, the ability of bone to

resist fractures (or bone toughness) is dependent on the quality and quantity of the matrix and mineral component of bone.[20] As such, bone quality can be used to estimate/predict bone strength and potential bone fracture risk.[1,20,17]

Because variations in the chemical composition of bone can influence the structural quality of bone, assessment of bone chemical composition (matrix and mineral components) achieved using Raman and Fourier transform infrared spectroscopy (FTIR) is important.[17,8,20,4,7] These techniques rely on the unique vibrational characteristic of each molecule that is forming bone and are reliable methods to evaluate the relative chemical composition of bone.[20,17]

The objective of this chapter were (1) to evaluate and compare the bone matrix and mineral composition/quality from humeral cortical and trabecular bone samples from heifers with humeral fractures and age-matched heifers without humeral fractures using band intensity ratios measured by using Raman spectroscopy and attenuated total reflectance (ATR)-FTIR spectroscopy and (2) to compare the total concentration of Ca and phosphorus (as components of the bone hydroxyapatite crystal) in humeral bone samples from heifers with and without humeral fractures.

5.2 Materials and methods

5.2.1 Study design and sample collection

This was a case-control study using a convenience sample of fractured (affected) and non-fractured (control) animals. The case definition for enrolling an animal in the affected group was a dairy heifer of any breed, at least 2-year-old, which had suffered a spontaneous fracture of the humerus, without any history of

trauma, within 6 months of calving. The humeral bone samples were provided by farmers and veterinarians who after reporting a dairy heifer who fitted the case definition were asked to collect the bone sample post-mortem. The collection of samples occurred between July and December 2019.

Control samples were obtained from an animal rendering plant (Wallace Corporation, Feilding, NZ) and Massey University School of Veterinary Science postmortem service. Samples were taken from dairy cows of any breed, with an ear tag indicating they were at least 2 years old, who had calved recently (udder consistent with lactating) and had been culled for reasons unrelated to bone fracture of the humerus or any other bone. From each control animal, a sample of the humerus was collected post-mortem.

For both groups (affected and control), no information regarding sample handling and/or time between collection and reception was reported and/or recorded in this study. Most samples from affected heifers were sent overnight by courier.

5.2.2 Preparation of bone samples

A total of 81 bone samples from 67 affected cases and 14 control cases were received. Next, several bone slabs (~3-5 mm thick) from the proximal epiphysis and metaphysis of the affected and control humeri were obtained using an industrial-grade band saw. For Raman and ATR-FTIR analysis, one slab from the humerus was selected and then cleaned with high-pressure cold water to remove the bone marrow. From each slab, three locations were selected for analyses: the cortex, the primary spongiosa, and the metaphysis (Figure 5.1). Each location was

ground separately for 4 min using a cryogenic grinder filled with liquid nitrogen (6875 Freezer/Mill®, SPEX® SamplePrep, Metuchen, NJ, USA). The grind protocol included two cycles each with a pre-cool step, a run step of 2 min, a cool step, and a second run time of 2 min. The impactor rate was set at 5 cycles per second. Once the cycle was finalised, powdered material was stored in Eppendorf tubes covered with aluminium foil at -80°C until further processing.

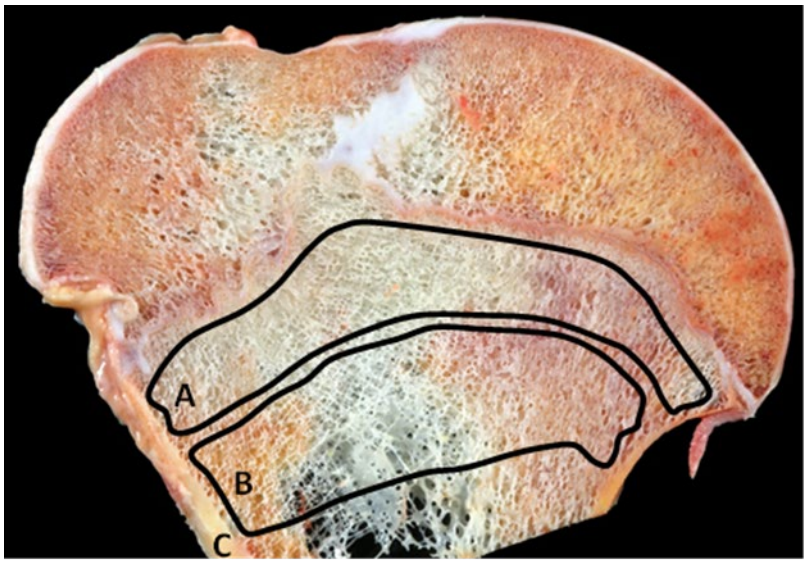


Figure 5.1 Humeral bone slab, affected heifer, proximal humerus. The three locations selected for spectroscopic analysis are shown. A, primary spongiosa; B, metaphysis; C, cortex.

5.2.3 Acquisition of Raman spectra

A total of 81 heifer powdered bone samples from 67 affected cases and 14 control cases were processed. For each heifer bone sample, three locations were analysed: cortex, primary spongiosa, and metaphysis, and for each location, three smaller (triplicates) portions of the powdered bone were mounted onto a glass slide and covered with a cover slip (22 x 22 mm) to form three thin films of randomly oriented powdered specimen. Thus, giving nine samples per heifer.

Raman spectra were collected using a custom-made Raman microscope system with a 532 nm monochromatic laser (Laser Quantum Torus 532). An Olympus IX-70 inverted fluorescence microscope body fitted a custom-manufactured 532 nm band-edge filter with a 12° incident angle (Iridian Technologies, Ottawa, Ontario, Canada) directed the laser into a 10x magnification objective (NA 0.25, Edmund Optics, Singapore). Raman scattering was collimated by the same objective. Rayleigh scattering was partially rejected by the incident band-edge filter and a second 532 nm Raman edge-filter (150cm^{-1} cut-off) placed immediately before the spectrograph provided complete Rayleigh rejection. The collimated Raman scattering was focused onto the entrance slit (50 µm) of a FERGIE spectrograph from Princeton/Teledyne Instruments equipped with a 1024 x 256 pixel charged coupled device controlled Lightfield software (version 6.0.4.1611, Princeton Instruments, Trenton, New Jersey).

Raman scattered light was collected over a spectral range of 200 to 4000 cm^{-1}, the resolution was 4 cm^{-1}, with laser power set to 5-10 mW. One hundred and twenty individual frames were acquired for each replicate spectrum with an exposure time set to 1 second per frame. Acquisition of individual frames allowed for a quick and straightforward assessment of sample damage (i.e., compare the first and last frame for each replicate). No sample damage was observed using this method.

5.2.4 Acquisition of ATR-FTIR spectra

A total of 81 heifer powdered bone samples from 67 affected cases and 14 control cases were processed. From each bone location (cortex, primary spongiosa, and

metaphysis), a small portion of powdered bone was placed onto the diamond crystal until fully covered. The pressure control arm was then positioned on top of the diamond crystal and maximum pressure was applied. Background spectra were collected before each experiment, with no sample present on the diamond crystal.

FTIR spectra of the powdered bone were collected using an FTIR spectrometer (Nicolet™ iS5™ FTIR Spectrometer, Thermo Scientific, MA, USA) with an iD7 diamond ATR attachment. OMNIC series software (v9.0) was used for data collection. Spectra were acquired in reflectance mode with a frequency region from 4000 – 400 cm^{-1} with 32 scans per spectrum and a spectral resolution of 2 cm^{-1}. Spectra were sampled to give 14936 data points for the spectrum. No online corrections were applied to the collected data.

5.2.5 Determination of the concentration of calcium and phosphorus

To determine the absolute concentration of Ca and phosphorus from affected and control animals, a total of 40 heifer bone samples from 26 affected cases and 14 control cases were submitted to a commercial diagnostic laboratory (Gribbles Scientific – Mosgiel, NZ) for determination of the percentage of bone Ca and phosphorus (in-house method modified from Bosnak C, Ewa S and Shelton CT[5]) using inductively coupled plasma with mass spectrometry (NexION 2000B ICP Mass Spectrometer, PerkinElmer, USA). Budgeting constraints limited the number of samples tested. For each heifer, a pooled sample (~ 300 mg) of the three bone locations (cortex, primary spongiosa, and metaphysis) was submitted.

The affected and control cases used for this analysis were also used for Raman and FTIR spectroscopy.

5.2.6 Spectral data analysis

For Raman spectra, a total of 729 spectra were collected that represented three spectra ("replicates") for each bone location (cortex, primary spongiosa, and metaphysis) in an individual heifer (i.e., nine spectra for 81 animals). In the case of FTIR spectra, a total of 240 spectra were collected: one spectrum for each bone location (cortex, primary spongiosa, and metaphysis) in an individual heifer (i.e., three spectra each for 80 animals). Before analysis, cases were categorised as affected or control and further categorised by bone location (cortex, primary spongiosa, and metaphysis).

5.2.6.1 Pre-processing of spectral data

Spectral data (Raman and FTIR) was analysed by bone location (cortex, primary spongiosa, and metaphysis) using Python™ 3.9.x code (www.python.org) written in Jupyter notebooks (https://jupyter.org), or as scripts from the command line. Before peak fitting, individual spectra were normalised and a baseline correction (using a modified asymmetric least squares baseline algorithm) was applied. For the Raman data, an average spectrum over the three replicates was calculated and used for further analysis. Visual inspection of the baseline functions was conducted to identify any potential artefacts introduced by baseline subtraction (Appendix H). If necessary, the baseline parameters were adjusted to limit the introduction of any artefacts.

5.2.6.2 Peak-fitting of spectra data

A plot was created of one case. After performing a visual inspection of the peaks, a working spectral range was selected. The working spectra range included at least one of the peaks selected for the study. Lorentzian or pseudo-Voigt functions were used as the component functions in the fit. A component function was added for each selected peak in the working spectral range and was fitted for position, amplitude, and width manually to begin with. Due to the complexity of the spectral signal, components were only added for the most significant spectral features (Appendix I). In some cases, the unfitted components were accounted for by the tails of the added components which likely results in an over-estimation of the spectral width and amplitude (however, analysis of the peak parameter ratios limits any impact of these effects). Once a reasonable fit had been obtained manually, a nonlinear least squares optimisation using the SciPy.Optimise.CurveFit package (https://docs.scipy.org/doc/scipy/reference/generated/scipy.optimize.curve_fit.html) was used to find the optimal parameter values.

For each location, peak parameters (including peak location, amplitude, and width) were obtained, and in the case of Raman spectra averaged. The integrated intensities of characteristic bands were measured; integration was performed for each fitted component in the peak fitting function. The value used was the integrated area of the band as a direct proportion of the concentration of the specific chemical component. The integrated intensities of four characteristic bands were measured: v_2 phosphate (422-454 cm^{-1}), carbonate type B (1046-1110

cm^{-1}), v_1 phosphate (903-991 cm^{-1}), and amide III (1243 - 1320 cm^{-1}).[16,7] Ratios of these bands areas resulted in the following parameters:

- Mineral/matrix ratio: v_2 phosphate / amide III.
- Carbonate/phosphate ratio: carbonate/ v_1 phosphate.
- Crystallinity: 1/width of v_1 phosphate.

Similarly, for FTIR the band assignments selected for analysis were: v_1v_3 phosphate (1180-911cm^{-1}), CO_3^{2-} (890-856 cm^{-1}), and amide I (1709-1606 cm^{-1}).[7,20] Ratios of these band areas resulted in the following parameters:

- Mineral/matrix ratio: v_1v_3 phosphate / amide I.
- Carbonate/phosphate ratio: CO_3^{2-}/ v_1v_3 phosphate

Vibrational spectroscopy analysis is based on the principle that the integrated area of a band is directly proportional to the concentration of the specific molecular moiety giving rise to the specific band.[20] Absolute measurements of Raman band intensities are difficult, especially in turbid media such as bone. Therefore, band intensity ratios are used when analysing bone chemical composition.[16] Bone matrix band assignments are mostly those of collagen type I.[16]

5.2.6.3 Data Statistical analysis

As the independent variables were not distributed normally, nonparametric tests of significance were used. An independent-samples Mann-Whitney U test was used to determine if there were any significant differences in the values of the ratios according to each bone location (cortex, primary spongiosa, and primary metaphysis) between affected and control heifers. Results are presented as

median, the *U* statistic, and *P* value. A *P* value of <0.05 was considered significant.

An independent-samples t-test was used to determine if there were any significant differences in humeral bone Ca and phosphorus percentage concentration between affected and control heifers. Results are presented as mean ± standard deviation (SD), unless otherwise stated. All statistical analysis was done in SPSS statistics (IBM® SPSS® Statistics version 27).

5.3 Results

5.3.1 Spectral analysis

Raman spectra were obtained from 67 affected cases and 14 control cases, and FTIR spectra were obtained from 66 affected cases (acquisition of spectra for one case was irregular hence that case was removed from this study) and 14 control cases. A representative spectrum of combined cases by fracture status and bone location are presented in Figure 5.2 for Raman spectroscopy and Figure 5.3 for FTIR spectroscopy. Results of the calculated ratios are represented in Tables 5.1 and 5.2.

5.1.1.1 Cortex

Bones from affected cases showed a significantly lower mineral/matrix ratio as measured by Raman and FTIR spectroscopy ($P<0.005$ and $P=0.019$ respectively). The carbonate/phosphate ratio was significantly lower in the cortex of affected cases compared to control cases when measured by Raman spectroscopy ($P<0.005$). In contrast, FTIR results showed a lower carbonate/phosphate ratio in control cases compared to affected cases ($P=0.018$). Finally, crystallinity

determined using Raman spectroscopy was significantly higher in control cases compared to affected cases (P<0.005).

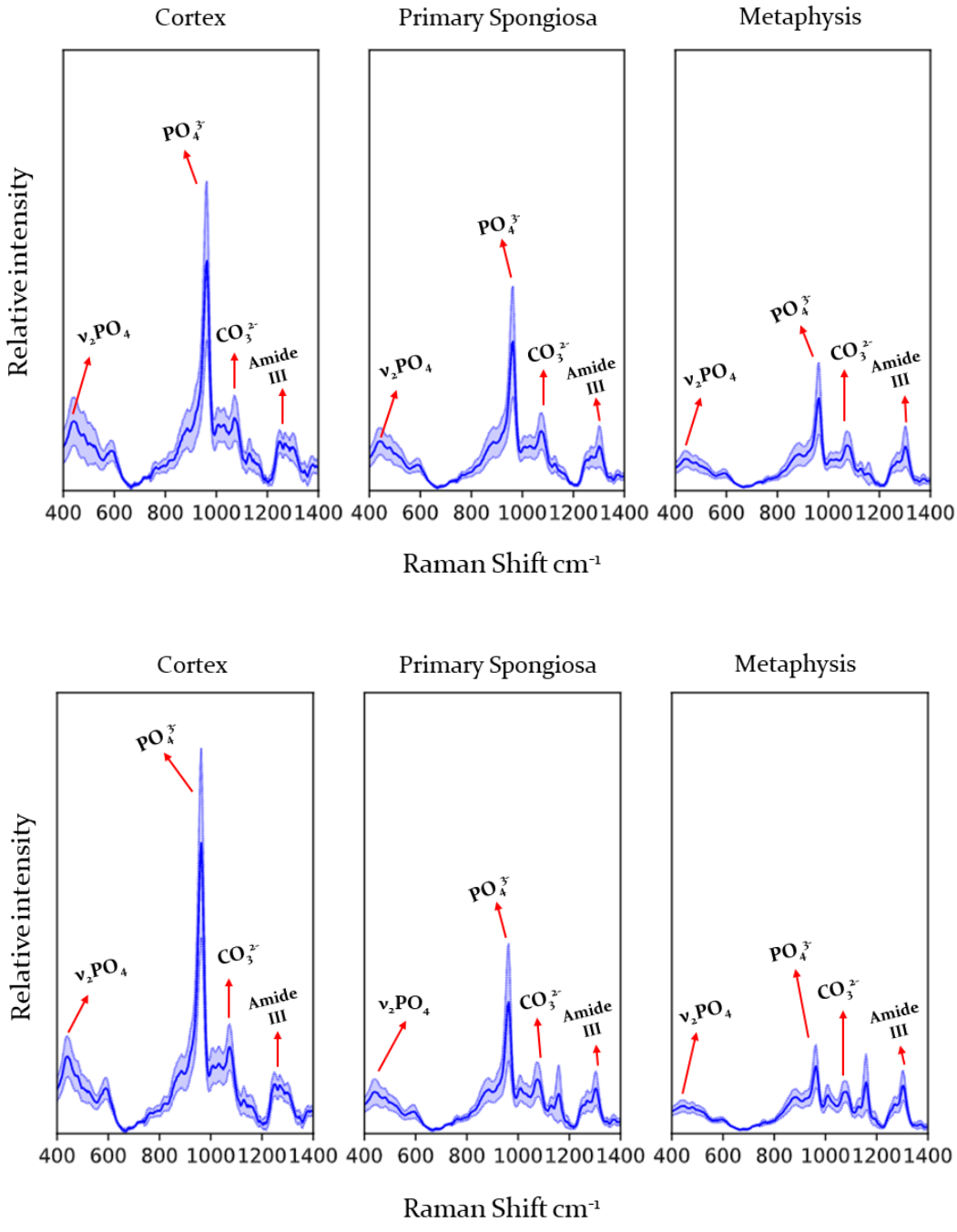


Figure 5.2 Raman spectra obtained from the humerus from heifers with humeral fracture (upper graph) and control heifers (no fractures) (lower graph) by location (cortex, primary spongiosa, and metaphysis). Labels indicate peaks used for the calculation of mineral/matrix ratio, carbonate/phosphate ratio, and crystallinity. The dark line indicates the mean, pale blue line 95% confidence interval. PO_4^{3-}, phosphate; CO_3^{2}, carbonate.

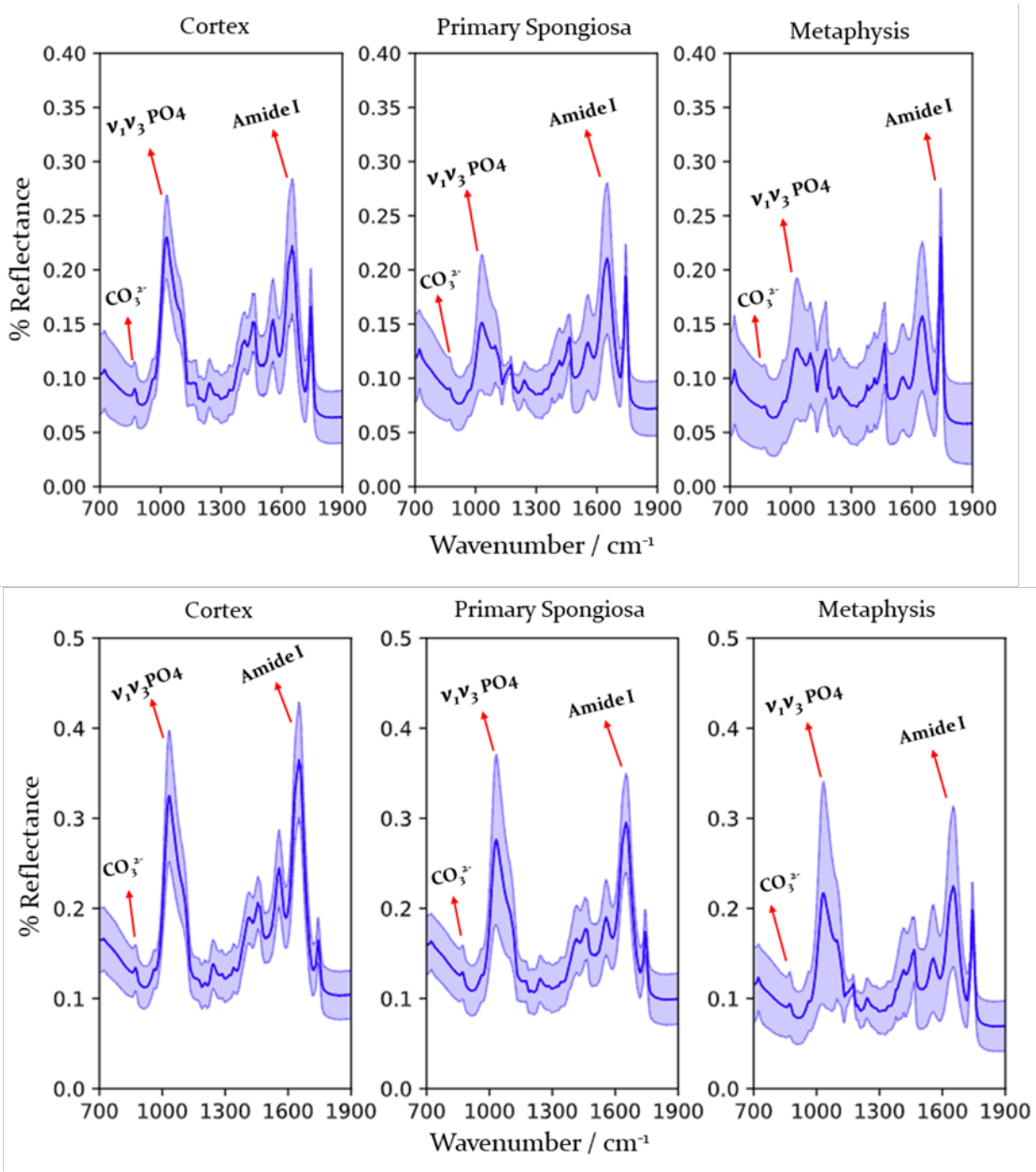


Figure 5.3 FTIR spectra obtained from the humerus from heifers with humeral fracture (upper graph) and control heifers (no fractures) (lower graph) by location(cortex, primary spongiosa, and metaphysis). Labels indicate peaks used for the calculation of mineral/matrix and carbonate/phosphate ratio. The dark line indicates the mean, pale blue line 95% confidence interval. PO_4^{3-}, phosphate; CO_3^{2}, carbonate.

5.1.1.2 Primary spongiosa

Bones from affected cases had significantly lower carbonate/phosphate ratio when measured by both Raman and FTIR spectra analysis ($P<0.005$ and $P<0.005$ respectively). The use of Raman spectra showed no evidence for a difference in the mineral/matrix ratio ($P=0.318$), contrary to when FTIR spectra was used, which showed a lower mineral/matrix ratio in control cases ($P=0.001$). Finally,

crystallinity was determined using Raman spectra and was significantly higher in control cases compared to affected cases (P<0.005).

5.1.1.3 Metaphysis

Bone from affected cases had a significantly lower carbonate/phosphate ratio when measured by Raman and FTIR spectra analysis (P=0.003 and P=<0.005 respectively). The mineral/matrix ratio was significantly lower in bones from control cases when measured by both Raman (P<0.005) and FTIR (P=0.001) spectroscopy. Crystallinity was significantly higher in control cases compared to affected cases (P<0.005).

Table 5.1 Mann-Whitney U test results of Raman spectra ratios and crystallinity by location (cortex, primary spongiosa, and metaphysis) for affected and control heifers.

	Case	Median	Mean Rank	*U*	*P* value
CORTEX					
Mineral/matrix	Affected (n=67)	0.75	35.63	829.000	<0.005*
	Control (n=14)	1.14	66.71		
Carbonate/phosphate	Affected (n=67)	0.12	34.07	933.000	<0.005*
	Control (n=14)	0.21	74.14		
Crystallinity	Affected (n=67)	0.06	35.21	857.000	<0.005*
	Control (n=14)	0.07	68.71		
PRIMARY SPONGIOSA					
Mineral/matrix	Affected (n=67)	0.99	39.81	549.000	0.318
	Control (n=14)	1.08	46.71		
Carbonate/phosphate	Affected (n=67)	0.24	36.69	758.000	<0.005*
	Control (n=14)	0.35	61.64		
Crystallinity	Affected (n=67)	0.06	34.36	9140.000	<0.005*
	Control (n=14)	0.07	72.79		
METAPHYSIS					
Mineral/matrix	Affected (n=67)	0.67	46.70	84.000	<0.005*
	Control (n=14)	0.34	13.71		
Carbonate/phosphate	Affected (n=67)	0.32	37.49	704.000	0.003*
	Control (n=14)	0.51	57.79		
Crystallinity	Affected (n=67)	0.07	34.51	904.000	<0.005*
	Control (n=14)	0.09	72.07		

* *P* value <0.05.

Table 5.2 Mann-Whitney U test results of FTIR spectra ratios by location (cortex, primary spongiosa, and metaphysis) for affected and control heifers.

Calculated ratio	Case	Median	Mean Rank	U	*P* value
CORTEX					
Mineral/matrix	Affected (n=66)	0.96	37.70	277.000	0.019*
	Control (n=14)	1.23	53.71		
Carbonate/phosphate	Affected (n=66)	0.12	43.33	275.000	0.018*
	Control (n=14)	0.09	27.14		
PRIMARY SPONGIOSA					
Mineral/matrix primary	Affected (n=66)	0.88	44.36	207.000	0.001*
	Control (n=14)	0.58	22.29		
Carbonate/phosphate	Affected (n=66)	0.25	36.15	175.000	<0.005*
	Control (n=14)	0.53	61.00		
METAPHYSIS					
Mineral/matrix	Affected (n=66)	0.84	44.30	211.000	0.001*
	Control (n=14)	0.46	22.57		
Carbonate/phosphate	Affected (n=66)	0.09	33.50	0.000	<0.005*
	Control (n=14)	0.49	73.50		

* *P* value <0.05.

Phosphorus and Calcium percentage concentration

A total of 26 pooled bone powder from affected cases and 14 samples from control cases were submitted. Results of the statistical analysis are presented in Table 5.3. Percentages of bone Ca and bone phosphorus concentration were normally distributed, as assessed by Shapiro-Wilk's test ($P>0.05$), and there was homogeneity of variances, as assessed by Levene's test for equality of variances ($P=0.057$ for Ca and $P=0.0156$ for phosphorus). There were no significant differences in the percentage of Ca and phosphorus in bone from heifers with humeral fracture compared with control humeri ($P=0.5$ and $P=0.6$ respectively).

Table 5.3 Mean ± SD of the Ca and phosphorus percentage concentration in humeral samples from affected and control heifers.

	Case	Mean ± Std. Deviation	*P* value	*d**
Bone Ca (%)	Affected (n=26)	19.38 ± 2.41	0.5	0.22
	Control (n=14)	18.91 ± 1.56		
Bone Phosphorus (%)	Affected (n=26)	8.96 ± 1.10	0.6	0.15
	Control (n=14)	8.81 ± 0.78		

*d** Cohen's d

5.4 Discussion

Results of this study demonstrate that there are significant differences in the relative chemical composition (bone quality changes) of cortical and trabecular bone from affected and control heifers when measured by Raman and FTIR spectroscopy. More importantly, significant changes in the chemical composition of the cortical bone suggest bone quality in the cortex is substantially reduced, thus compromising bone strength.

5.4.1 Mineral/matrix ratio

The mineral/matrix ratio has been used extensively to study bone quality and strength and is an alternative method for reporting bone density. [17,20,11,19] It directly quantifies the amount of bone organic matrix and is known to be reduced in humans with osteoporosis.[20,4] Compared to controls, heifers with humeral fractures had a significantly lower mineral/matrix ratio in cortical bone when measured by both Raman and FTIR spectrometry. These findings suggest there is a decreased mineralisation in the cortical bone of heifers with humeral fracture, consistent with these animals having osteoporosis.

Furthermore, the mineral/matrix ratio can provide information on the mechanisms of bone loss that lead to osteoporosis.[3] A lower mineral/matrix ratio is reported in iliac crest bone biopsies from women and men with high-turnover osteoporosis (defined as the increased number of resorption surfaces and higher than normal number of osteoclasts) compared to bone biopsies samples from women and men with low-turnover osteoporosis and normal bone activity.[3] In a more recent study, McCreadie et al[17] correlated the mineral-matrix ratio with

both the bone remodelling activity and the amount of mineralisation.[17] The study showed that a reduction in the mineral/matrix ratio in damaged bone from individuals with non-traumatic femoral subcapital fracture was associated with increased remodelling activity occurring at the site before bone fracture.[17] Increased remodelling activity yields bone tissue that is younger and hence less mineralised.[17]

Cortical bone from affected heifers in this study also had a lower mineral/matrix ratio which is similar to McCreadie et al[17] and suggests in bones from affected heifers there is a reduction in the mineral content and increased remodelling activity associated with an increased osteoclastic activity. These results corroborate the findings in a previous analysis done with this set of bone samples (Chapter 4). Histological and bone histomorphometry evaluation on bone samples prior to spectroscopic analysis showed that bones from heifers with humeral fractures had thinner cortices with increased abnormal cortical resorption. These results are supported by peripheral quantitative computed tomography data that showed a reduction in cortical bone mineral density in the mid-diaphysis of the humerus of fractured heifers.[12] Less mineralised bone has less resistance to fracture.[17]

Moreover, a strong interdependence is described between reduced mineral/matrix ratio, bone bending stiffness (a measure of the bone resistance to deformation under an applied load), and failure moment in cortical bone of rats with reduced BMD.[11,6] Findings in the current study indicate that in affected heifers cortical bone strength and quality are significantly compromised with less

organic matrix and lower mineralisation due to active bone remodelling, likely resulting in decreased bending stiffness and decreased resistance to fracture.

A decreased mineral/matrix ratio was found in the metaphysis (when measured by Raman and FTIR) and primary spongiosa (measured by FTIR) of control heifers compared with affected heifers. These results suggest that in control heifers the primary spongiosa and metaphysis are the sites of active bone remodelling activity. This is consistent with the knowledge that, although resorption occurs normally in both cortical and trabecular bone, the rate of remodelling is much greater in trabecular bone (20%) compared to cortical bone (5%).[20,18] It appears that in affected heifers, trabecular bone is not readily available for remodelling and for Ca supply, meaning animals are relying on the cortical bone for these processes. Histological findings from bone samples with humeral fractures, described in chapter 4 of this thesis, support this hypothesis. Heifers with humeral fractures have significantly reduced trabecular bone density and abnormal trabecular bone architecture which may not be readily available for remodelling.

There is an important effect of tissue age on the mineral/matrix ratio with the ratio increasing with tissue age when measured on the bone trabecular surface of healthy females.[19,20] Using this ratio to determine tissue age, older tissue was found in the cortex and younger bone in the metaphysis of control heifers. In contrast, this relationship was reversed in affected cases with the oldest tissue being found in metaphyseal bone followed by the cortex. This is another finding

that supports the switch in remodelling activity from the trabecular bone to cortical bone in affected heifers.

It is important to remember that bone tissue is a very heterogenous material, different bones in the same individual/animal and different locations within one bone may be undergoing active bone remodelling or be completely inactive.[20,11] Because tissue homogenates were used in this study variability in tissue microstructure (temporal and spatial organisation) was not considered when interpreting and comparing values. Lastly, although both spectroscopic techniques are complementary to each other, they use different techniques for obtaining bands and intensities which could lead to some of the variability in the ratios reported in this study.[16]

5.4.2 Carbonate/phosphate ratio

The carbonate/phosphate ratio measures the amount of carbonate substitution in bone.[1] Pure apatite crystals found in nature consist of Ca, phosphate, and hydroxyl ions.[20] In contrast, apatite crystals found in bone are highly substituted, are poorly crystalline, and vary according to the location in bone (cortical vs trabecular) and with the bone metabolic state.[20,15] Carbonate can substitute for either hydroxyl ions (type A substitution) or phosphate (type B substitution).[20,7] Type B substitution was measured in this study.

The carbonate/phosphate ratio impacts bone mechanical properties and in very old subjects can be associated with deterioration in the structural and mechanical material properties of bone.[1,4,21] For example, carbonate substitution (carbonate/phosphate ratio) was significantly higher in skeletally mature, old

murine bones compared to skeletally mature, young murine bones, and the carbonate/phosphate ratio positively correlated with bone plasticity (deformation) index.[21] McCreadie et al., found a greater carbonate/phosphate ratio in the iliac crest cortical bone from women (> 50 years old) with osteoporosis when measured by Raman spectrometry and concluded that the carbonate/phosphate ratio was an important determinant in the occurrence of osteoporotic fracture.[17]

In the present study, significant differences were found in all three locations examined (cortex, primary spongiosa, and metaphysis) and with both spectroscopic methods used. Higher carbonate substitution was found in the metaphysis and primary spongiosa of control heifers compared with affected heifers. It appears that in control heifers, increased carbonate substitution (increased carbonate/phosphate ratio) in trabecular bone is contributing to bone quality by increasing bone mechanical strength and that less/poor carbonate substitution in affected heifers may be an important contributory factor in the appearance of spontaneous humeral fractures.

Changes in the chemical composition of bones are irregular (nonlinear), and complex, and there is a wide range of other mechanisms that can influence tissue chemical composition that need to be considered.[17] These include genetically/environmentally determined differences in the chemistry of bones and bone damage.[17] Damage itself may result in changes from location to location within the same bone thus influencing bone remodelling and repair which may greatly influence chemical composition within the same bone.[17] Several of these

mechanisms may have confounded the results in this study. For example, these spectra were measured from dairy heifers that have recently calved and have a body machinery dedicated to milk production that could have affected the chemical composition of these bones. Since control cases were the same age and lactating it is believed we controlled for any effect of age or lactation. However, milk production, milking frequency, and body condition score, all of which also influence the chemical composition of bone, were variables that we were unable to control for and could have caused some bias in the results.

5.4.3 Crystallinity.

The term crystallinity refers to the size and shape of bone apatite crystals.[20,23] Carbonate substitutions and crystallinity are closely correlated, whereby the type and extent of substitutions influence crystal solubility, size, and shape.[20] Crystal size also changes with stage of mineralisation (less mature mineral, less crystallite size), with crystallinity increasing with age, suggesting a more ordered crystal lattice.[7,1]

Results from the current study showed crystallinity was significantly reduced in the cortex, the primary spongiosa, and the metaphysis of bones from affected heifers compared to control heifers. In cortical bone increasing crystallinity is positively correlated with tissue-level strength and stiffness and negatively correlated with ductility (the ability of a material to be plastically deformed without fracture).[23,1]

Therefore, reduced crystallinity in bone tissue from affected heifers may contribute to decreased bone strength in all locations (cortex, primary spongiosa,

and metaphysis) due to mineral crystals that are of reduced size, less mature, less mineralised, and less ordered, likely resulting in weak bone in affected heifers, predisposing them to humeral fractures.

5.4.4 Bone Calcium and Phosphorus content

The phosphorus and Ca percentages were remarkably similar in bone from affected and control heifers, with no significant differences between groups. In animals with osteoporosis, there is a reduction in the quantity of bone with a proportionate reduction in the chemical composition of bone, as such changes in proportion are not likely to be present.[9] Furthermore, Holst et al[14] used rib bone to assess bone mineralisation in range cattle and found that ash density (mg/mL), not phosphorus or Ca percentage, was better for identifying cattle with impaired bone mineralisation.[14] However, in the same study, cows between 2 and 3 years old had mean phosphorus of 12.2% and a mean Ca of 27.5%.[14] These values are much higher than those found in the affected and control heifers in the current study, which may suggest that the heifers were deficient in bone Ca and bone phosphorus, although the Holst et al[14] measurements were made on beef cattle and not dairy cows.

5.5 Conclusion

Raman and ATR-FTIR spectra analysis of bone from heifers with humeral fractures compared to age-matched control heifers indicates that in heifers with humeral fractures there is a significant difference in the relative bone chemical composition and quality. Affected bone showed a significantly reduced amount of bone matrix and mineral component, increased bone remodelling, lower

mineralisation and reduced tissue age, lower carbonate substitution, and reduced crystallinity.

All these changes in the bone chemical composition of affected heifers (specially in cortical bone) significantly impact bone quality, reduce bone strength, and likely contribute to the increased incidence of spontaneous fractures in first calving dairy heifers in New Zealand.

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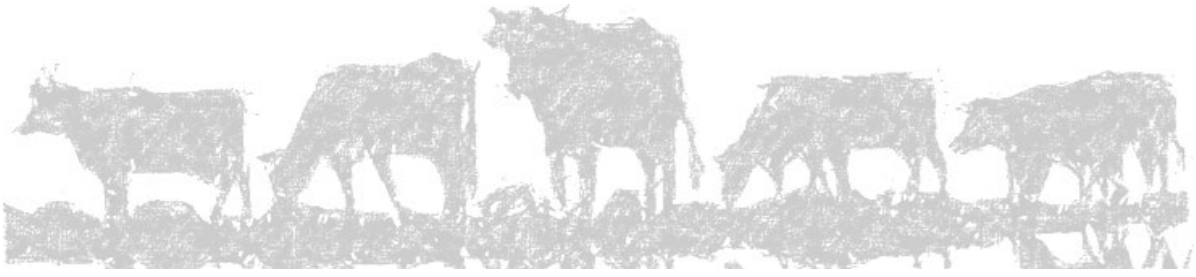
CHAPTER 6

COLLAGEN AND ITS CROSSLINK CONTENT IN BONES FROM HEIFERS WITH OSTEOPOROSIS FROM NEW ZEALAND



6.1 Introduction

Since the first reported case of humeral fracture in dairy heifers in New Zealand, several putative risk factors have been hypothesised as associated with an increased incidence of fractures.[37,11,15] The presence of growth arrest lines, identified in the largest histological study completed on humeral fractures to date, suggested that periods of protein-calorie inadequate nutrition, leading to inadequate bone deposition during the first months of the heifer's life, could contribute to the development of osteoporosis in affected animals (chapter 4).[11] A more recent study, using peripheral quantitative computed tomography, has shown a consistent trend for reduced bone length, reduced cortical bone mineral density, and a tendency for reduced cortical content and total bone content in heifers with humeral fractures compared with control heifers.[15] The findings from both these studies support the hypothesis that periods of inadequate nutrition act as a contributory factor in the occurrence of humeral fractures in heifers and furthermore, suggest these changes are recent.[15,11]

Another relatively common finding from previous studies is that many heifers affected with humeral fractures have either low liver and/or serum Cu concentrations.[36,11,15] For example, in the very first reported outbreak of humeral fractures in dairy heifers, Cu deficiency was diagnosed and associated with the outbreak.[36] A follow-up larger study also reported low serum and/or liver Cu (LiCu) concentration in some of the affected animals and proposed Cu deficiency as a contributing factor, which weakened bone, and contributed to osteoporosis and subsequent humeral fracture.[11] The same study also described increased

osteoclastic bone resorption secondary to lactation, as a third factor contributing to bone weakening.[11]

Copper deficiency is one of the most common trace mineral deficiencies in cattle in New Zealand.[18] Secondary Cu deficiency is more common than primary deficiency and is associated with excessive concentrations of Cu antagonists (including Mo, S, Fe, Zn, and Mn) in the diet and/or soil.[18,9] Molybdenum and S bind Cu in the rumen reducing its bioavailability.[12] Similarly, Fe and Zn interfere with Cu absorption and can interfere with cellular use of Cu.[12]

The relationship between Cu and bone strength is related to the activity of lysyl oxidase (LOX), a pivotal Cu-dependent enzyme in the formation of collagen crosslinks.[34,14] Collagen crosslink formation contributes to the mechanical properties of bone and lysyl oxidase oversees the most important step in the formation of collagen crosslinks, the conversion of specific ε-amino groups and the stabilisation of the triple helical collagen molecule.[30,35] Collagen crosslinks can be classified as either immature or mature crosslinks (among other types).[31] Immature collagen crosslinks convert spontaneously to mature collagen crosslinks, and normal human healthy bone is characterised as having 2-4 times the content of immature compared to mature collagen crosslinks.[31] As a result of Cu deficiency (primary or secondary), changes in the amount and type of collagen crosslinking can occur.[31,26,29] This can impact bone mechanical properties and strength, and as a result, Cu deficiency has been associated with increased bone fragility and an increased incidence of spontaneous bone fracture, among other clinical signs.[34,16,33]

The objectives of this chapter were 1) to quantify and describe any significant alterations in collagen and collagen crosslink content in humeri from heifers with humeral fractures due to osteoporosis compared with age-matched heifers without humeral fracture; 2) to assess the relationship between liver and bone Cu concentration and collagen and collagen crosslinking in bone; and 3) to compare the liver concentration of Cd, Fe, Mo, and Zn (Cu antagonists) between heifers with and without humeral fracture, to determine if Cu antagonists are causing secondary Cu deficiency.

6.2 Materials and methods

6.2.1 Study design and sample collection

This was a case-control study using a convenience sample of cortical bone from heifers with osteoporosis that fractured (affected group) one or both humeri and non-fractured (control group) animals. The case definition for enrolling an animal in the affected group was a dairy heifer of any breed, at least 2-year-old, which had suffered a spontaneous fracture of the humerus, without any history of trauma, within 6 months of calving. The case samples were provided by farmers and veterinarians who after reporting a case of spontaneous fracture of the humerus were presented with a list of samples, to be collected postmortem. These included the humerus (affected and/or contralateral) and a piece of liver. Blood samples were also collected by the referring veterinarian as part of a diagnostic investigation into the cause of the fracture. The collection of samples occurred between July and December 2019.

Control samples were obtained from a cow rendering plant (Wallace Corporation, Feilding, NZ) and Massey University School of Veterinary Science postmortem service. Samples for control cases were taken from dairy cows of any breed, with an ear tag indicating they were at least 2 years old, who had calved recently (udder consistent with lactating) and had been culled for reasons unrelated to bone fracture of the humerus or any other bone. From each control animal, a sample of the humerus and a piece of liver was collected postmortem, antemortem blood samples were however not available owing to the method of sampling.

For both groups (affected and control), no information regarding sample handling and/or time between collection and reception was reported and/or recorded in this study. Most samples from affected heifers were sent overnight by courier.

6.2.2 Preparation of bone samples

A total of 26 affected and 14 control humeri were received. Bone slabs (~3-5 mm thick) from the proximal epiphysis and metaphysis of the affected and control humeri were obtained using an industrial-grade band saw. For collagen and collagen crosslink quantification one slab from the humerus was selected and then cleaned with high-pressure cold water to remove the bone marrow. From each slab, cortical bone was dissected out and ground using a cryogenic grinder filled with liquid nitrogen (6875 Freezer/Mill®, SPEX® SamplePrep, Metuchen, NJ, USA). The grind protocol included two cycles each with a pre-cool step, a run step of 2 min, a cool step, and a second run time of 2 min. The impactor rate was

set at 5 cycles per second. Once the cycle was finished, powdered material was stored in Eppendorf tubes covered with aluminium foil at -80°C until further processing.

6.2.3 Collagen and collagen crosslink analysis of cow bone

6.2.1.1 Materials

The following chemicals, used in this study, were purchased from Sigma-Aldrich (St. Louis, MO, USA), and included sodium hydroxide, sodium borohydride 98%, chloramine T trihydrate, and 4-(Dimethylamino) benzaldehyde. Mass spectrometry grade water, acetonitrile (MeCN), formic acid, methanol, acetic acid glacial, hydrochloric acid S.G 1.18 (~37%), and sodium hydroxide were sourced from Fisher Scientific (Fair Lawn, NJ, USA). Perchloric acid 70% and propan-1-ol were purchased from BDH Chemicals Limited (Poole, England). Deionized water was obtained from a Milli-Q Ultra-pure water system (Dubuque, IA, USA). Dihydroxylysinonorleucine standard (DHLNL) was purchased from Santa Cruz Biotechnology (CA, USA). Pyridinoline (PYD) (95%) and deoxypyridinoline (DPD) (98%) standards were purchased from BOC Science (NY, USA). Hydroxylysinonorleucine (HLNL), histidinohydroxylysinonorleucine (HHL), histidinohydroxymerodesmosine (HHMD) standards, were isolated and purified by Dr R. Naffa in the Protein Structure and Function Laboratory, Massey University's School of Natural Sciences.[24]

6.2.1.2 Bone powder preparation

Around 100-150 mg of powdered bone sample from each case was freeze-dried for 24h. For this, Eppendorf tubes with powdered material were placed in a

stainless-steel stockpot, with their lids open but covered with parafilm® sealing film with multiple holes in it. The stockpot was closed-sealed and connected to the freeze dryer (SP VirTis Freezemobile 25XL Freeze Dryer (SP equipment, PA, USA). Bone samples were then reduced using sodium borohydride ($NaBH_4$) as previously described.[23] Briefly, 10 mg of $NaBH_4$ was dissolved in 1 mL of 1 mM cold sodium hydroxide to give a 1:10 ratio of sodium borohydride to bone powder, which was subsequently added to the bone samples. The mixture was incubated for 24 h at 37°C. The reduction was stopped using glacial acetic acid, added until the pH dropped to 3.0. Samples were then centrifuged at 5000g for 5 min at 19°C and the supernatant was discarded. The pellets were then washed with 5 mL of distilled water, to remove excess acetic acid and salts, centrifuged again (5000g for 5 min at 19°C), supernatant discarded, and freeze-dried overnight.

The following day samples were first weighed and then acid hydrolysed using 1.5 mL of 6 M HCl at 105°C for 24 h. After that, to neutralise the HCl in the resulting hydrolysate, 2.5 mL of 6 M NaOH was added to the sample, mixed, and filtered through glass-wool plugged plastic syringes. Two filtrations were carried out using 2.5 mL of distilled water, samples were then frozen in liquid nitrogen, connected to the freeze dryer, and freeze-dried for 24-48 h. Samples were then stored at -80°C until further testing.

6.2.1.3 Crosslink separation and quantitation

Before crosslink separation and quantification, samples of the prepared bone powder were rehydrated in 1000 µL of water and 200 µL of this was inserted into a 1.5 mL short thread vial (Thermo Scientific, Waltham, MA, USA). Separation of

collagen crosslinks was performed, as previously reported, by liquid chromatography (LC) using a LC system (Dionex UltiMate™ LPG-3400RS Rapid Separation Quaternary Pump, ThermoFisher Scientific, USA) with a Cogent Diamond Hydride™ HPLC column (PM Separations, Capalaba, Queensland, Australia).[23] The LC system was coupled to a Q Exactive™ focus mass spectrometer equipped with a high-energy collision-induced dissociation collision cell, an Orbitrap mass analyser, and a HESI-II ion source (Thermo Fisher Scientific, USA) for crosslink quantification. Parallel reaction monitoring using tandem mass spectrometry acquisition with an inclusion list of ions was used to detect and quantitate the relevant ions for each crosslink. To quantify the different collagen crosslinks in bone powder samples from affected and control heifers, three biological replicates were used, with each biological replicate being tested for extraction efficiency using three technical replicates. Details for the chromatographic and mass spectrometry settings are listed in Appendix J.

6.2.1.4 Determination of total collagen content in cow cortical bone

The amount of collagen in the prepared bone powder was determined using a hydroxyproline (HYP) assay with an aliquot of the acid hydrolysate as described by Reddy and Enwemeka.[28] In summary, aliquots of the standard HYP (2-20 µL) prepared from stock solution (1 mg/mL of HYP in water) and the bone powder hydrolysate (at different volumes; 2.5 to 10 µL) were mixed with water in a total volume of 50 µL. Next, 450 µL of chloramine-T was added, mixed gently, and incubated for 25 min at room temperature. Then 500 µL of Ehrlich's aldehyde reagent was added, and the sample mixed and incubated at 65°C for 20 min for the chromophore reaction to develop. Finally, absorbance was read at 550 nm

using a microplate spectrophotometer (BioTek PowerWave XS, VT, USA). Collagen content was calculated on the dry weight of the bone assuming 14% HYP in type I collagen.[25]

6.2.4 Determination of bone copper concentration

The same case and control samples that were used for the determination of bone Cu concentration were also used for collagen and collagen crosslink quantification. From the 26 affected cases and 14 control cases, ~ 300 mg of bone powder (cortical and trabecular bone) was submitted to a commercial diagnostic laboratory (Gribbles Scientific – Mosgiel, NZ) for determination of bone Cu concentration (in mg/kg) using inductively coupled plasma with mass spectrometry (NexION 2000B ICP Mass Spectrometer, PerkinElmer, USA).

6.2.5 Determination of serum copper concentration

Twenty-six blood samples from affected heifers were submitted to IDEXX Laboratories New Zealand Pty. Ltd. (Palmerston North, NZ). Serum Cu concentration was measured using inductively coupled mass spectrometry (NexION 2000B ICP Mass Spectrometer, PerkinElmer, USA).

6.2.6 Determination of liver copper, cadmium, iron, molybdenum, and zinc concentration

Liver samples from 26 affected and 14 control heifers were submitted to a commercial diagnostic laboratory (Gribbles Scientific – Mosgiel, NZ) for determination of liver Cu, Cd, Fe, Mo, and Zn concentration using inductively coupled mass spectrometry (NexION 2000B ICP Mass Spectrometer, PerkinElmer). A LiCu concentration between 0-94 µmol/kg was considered low/marginal and values >94 µmol/kg were considered adequate according to the

reference interval provided by IDEXX Laboratories New Zealand Pty. Ltd and published references.[17]

6.2.7 Data Analysis

Mass spectrometry data were processed using Xcalibur™ Software v.4.1.31.9 (Thermo Scientific™, USA), and the results were exported into Excel for quantification. The FreeStyle™ Software v.1.3.115.19 (Thermo ScientificTM, USA) was used for data visualisation and the TraceFinder™ 4.1 version 4.1.265.0 software was used to extract and quantify the ion peaks.

Statistical analysis was done on the total collagen content, DHNLN, PYD, and DPD crosslinks. Before statistical analysis, all values for the crosslinks were normalised to the HYP concentration in bone (total crosslink/total HYP in sample). Each bone crosslink result, for fractured and control bones, was the mean of three biological replicates, each the mean of their respective three extraction technical replicates.

An independent-samples t-test was used to determine if there were any significant differences between Cu concentration in the liver, serum, and bone, liver Cd, Fe, Mo, and Zn concentration, total crosslink content (DHLNL + PYD + DPD), total collagen content, DHLNL, PYD, and DPD content and DHLNL/(PYD+DPD) ratio in bone from affected and control heifers. Values were first natural log transformed to achieve a normal distribution. Results are back transformed and presented as mean ± standard deviation (SD) unless otherwise stated. A P value of < 0.05 was considered significant. If the assumption of

homogeneity of variances was violated a Welch t-test was run to determine differences between groups.

A Spearman's rank-order correlation test (results displayed as r_s) was used to determine the strength and direction of the relationship between liver and bone Cu concentration, HYP, DHLNL, PYD, and DPD content in bone. A *P* value of <0.05 was considered significant. A point-biserial correlation test (results displayed as r_{pb}) was used to determine the strength and direction of the relationship between liver and bone Cu concentration (as the continuous variable) and fracture status (affected vs control) as a dichotomous variable.

Next, a simple linear regression test was built to determine the relationship between liver and bone Cu concentration (as the predictor variable) based on the value of DHLNL, PYD, and DPD collagen crosslinks and collagen content (HYP) in bone (outcome variables).

Finally, two binomial logistic regression models were built. The first, to determine the effect of liver and bone Cu concentration on the odds of a heifer having a humeral fracture and the second, to determine the effect of the quantity of collagen crosslinks on the odds of a heifer having a humeral fracture. For a variable to be included as a parameter in the logistic model it had to have an observed count (distribution) of more than 1, and the *P* value obtained from the t-test a *P* value <0.25.[8] The model was constructed using forward selection, with variables retained at *P*<0.05, and the model fit was assessed using the Hosmer-Lemeshow (HL) test and the Nagelkerke R2. All statistical analyses were performed using SPSS statistics (IBM® SPSS® Statistics version 27).

6.3 Results

A total of 40 bone cortices from 26 affected heifers and 14 control heifers were used in this study. Individual results for Cu concentration (in liver, serum, and bone) and liver concentration of Cd, Fe, Mo, and Zn are presented in Appendix K.

Changes in copper concentration

Table 6.1 provides an overview of the Cu data. The mean LiCu concentration was adequate (>94 µmol/kg) in affected and control heifers. However, the mean LiCu concentration was significantly higher in control heifers compared with affected heifers, *P*<0.001. Serum Cu concentration was only available in affected heifers, with a mean of 15.8 ± 1.42 µmol/L, which is considered adequate. Bone Cu concentration was significantly higher in affected heifers compared with control heifers (*P*<0.001).

Table 6.1 Mean ± SD of liver and bone Cu concentrations in heifers comparing fracture status (affected vs control) and mean ± SD bone Cu concentration comparing low vs adequate liver copper concentration.

	Case	Mean ± Std. Deviation	*P* value	*d**
Liver copper (µmol/kg[a])	Affected (n=26)	237.6 ± 385.3	< 0.001**	1.28
	Control (n=14)	468.0 ± 285.3		
Bone copper (mg/kg[b])	Affected (n=26)	0.69 ± 0.27	< 0.001	1.24
	Control (n=14)	0.41 ± 0.12		
	Low LiCu (n=16)	0.68 ± 0.28	0.07	.52
	Adequate LiCu (n=24)	0.54 ± 0.24		

[a]micromoles per kilogram
[b]milligram per kilogram
*d** Cohen's d
** Welch t-test

Spearman's rank-order correlation showed no statistically significant correlation between liver and bone Cu concentration, $r_s(38) = -0.12$, *P*=0.46. Point-biserial correlation showed a statistically significant correlation between liver ($r_{pb}(38) = 0.504$, *P*=<0.001) and bone Cu concentration with fracture status ($r_{pb}(38) = -$

0.582, *P*=<0.001), with affected heifers having the lowest LiCu concentration and the highest bone Cu concentration.

Data on serum Cu concentration was only available for affected heifers' so correlation testing was solely conducted within this group. The relationship between serum and LiCu concentration was a statistically significant, positive correlation $r_s(38) = 0.39$, *P*=0.05. Finally, the relationship between serum and bone Cu concentration in affected heifers was not significant, $r_s(24) = 0.13$, *P*=0.51.

The following two parameters were tested in the logistic regression model: LiCu and bone Cu concentration. Both were retained in the final model. The logistic regression model was statistically significant, $\chi2(4) = 21.56$, *P*<0.001. The Hosmer and Lemeshow test was not statistically significant (*P*=0.30) indicating the model was a good fit. The model explained 58.1% (Nagelkerke R2) of the variance in humeral fracture and correctly classified 85% of cases. Sensitivity was 69.2% and specificity was 92.6%. Increased bone Cu concentration was associated with increased odds (56.1 times) of having a humeral fracture while for decreased LiCu concentration, the odds of having a humeral fracture increased by a factor of 3.2 (Table 6.2).

Table 6.2 Results of the logistic regression test for predicting humeral fracture in heifers based on LiCu and bone Cu concentration.

	B	SE	Wald	*df*	*P*	Odds Ratio	95% CI for Odds Ratio	
							Lower	Upper
Bone Cu	0.40	1.55	6.75	1	0.01	56.1	2.7	1168.9
LiCu	1.17	0.46	6.33	1	0.01	3.2	1.3	8.1
Constant	9.79	3.30	8.78	1	0.003			

B, B coefficient for the constant; S.E, standard error; Wald, Wald chi-square test; df, degrees of freedom; CI, confidence interval; Cu, copper; LiCu, liver copper concentration

Collagen content

The total collagen content was significantly higher in control heifers compared with affected heifers (*P*=0.004 Table 6.3).

Table 6.3 Mean ± SD of total collagen content in heifers comparing fracture status (affected vs control) and liver copper concentration status (low vs adequate).

	Case	Mean ± Std. Deviation	*P* value	*d**
Total collagen (mg[a])	Affected (n=26)	1.71 ± 0.43	0.004	1.02
	Control (n=14)	2.18 ± 0.46		
	Low LiCu (n=16)	1.79 ± 0.44	0.463	0.24
	Adequate LiCu (n=24)	1.93 ± 0.53		

[a] milligram
*d** Cohen's d
HYP, hydroxyproline; LiCu, liver copper concentration

There was no significant correlation between LiCu concentration and collagen content in bone, $r_s(38) = 0.05$, *P*=0.74. Instead, a significant negative correlation was found between bone Cu concentration and total collagen content in bone, $r_s(38) = -0.45$, *P*=0.003.

Collagen crosslinks

Bone samples did not contain HHMD crosslinks and the quantity of HLNL was either not found or extremely low. All bone samples analysed contained DHLNL, DPD, and PYD collagen crosslinks.

Table 6.4 provides the summary statistics for collagen crosslinks. The total crosslink content (DHLNL+PYD+DPD) was significantly higher in affected heifers and heifers with low LiCu concentration compared with control heifers ($P<0.001$) and heifers with adequate LiCu concentration ($P=0.007$).

Bone samples from affected heifers contained significantly more DHLNL and PYD crosslinks compared with control heifers ($P=0.009$ and $P=0.002$, respectively). Finally, there was no significant difference in the immature/mature crosslink ratio between affected and control heifers ($P=0.46$).

Bone samples from heifers with low LiCu concentration had significantly higher DHLNL and PYD compared with heifers that had adequate LiCu concentration, $P=0.045$ and $P=0.030$, respectively.

There was no statistically significant correlation between LiCu concentration and DHLNL, DPD, PYD, and immature/mature assessed by a Spearman's rank-order correlation test. There was a significant correlation between bone Cu concentration and the quantity of DHLNL in bone, $r_s(38) = -0.38$, $P=0.017$. There was no significant correlation between bone copper concentration and DPD, PYD, and immature/mature crosslinks.

Table 6.4 Mean ± SD of total collagen crosslink content, DHLNL, PYD, DPD, and DHLNL/(PYD+DPD) comparing fracture status (affected vs control) and liver Cu concentration status (low vs adequate). The crosslinks were normalised to the total HYP content (total crosslink in sample/total HYP in sample).

	Case	Mean ± Std. Deviation	*P* value	*d**
Total crosslink[a]	Affected (n=26)	3979.07 ± 797.72	<0.001	1.27
	Control (n=14)	3058.71 ± 548.70		
	Low LiCu (n=16)	4082.32 ± 859.21	0.007	0.92
	Adequate LiCu (n=24)	3373.36 ± 711.16		
Immature crosslink				
DHLNL[a]	Affected (n=26)	2847.23 ± 717.27	0.009	0.91
	Control (n=14)	2257.38 ± 489.29		
	Low LiCu (n=16)	2943.09 ± 835.07	0.04	0.67
	Adequate LiCu (n=24)	2439.25 ± 519.41		
Mature crosslinks				
PYD[a]	Affected (n=26)	1043.89 ± 403.18	0.002	1.13
	Control (n=14)	724.98 ± 159.41		
	Low LiCu (n=16)	1049.00 ± 334.29	0.03	0.73
	Adequate LiCu (n=24)	854.45 ± 377.64		
DPD[a]	Affected (n=26)	87.95 ± 25.75	0.16	0.47
	Control (n=14)	76.34 ± 23.13		
	Low LiCu (n=16)	90.23 ± 26.07	0.19	0.43
	Adequate LiCu (n=24)	79.65 ± 24.22		
Immature/mature crosslinks				
DHLNL/(PYD+DPD)[a]	Affected (n=26)	2.77 ± 1.02	0.46	0.25
	Control (n=14)	2.92 ± 0.67		
	Low LiCu (n=16)	2.81 ± 1.12	0.77	0.09
	Adequate LiCu (n=24)	2.83 ± 0.76		

*d** Cohen's d
[a]mg/mol
LiCu, liver copper concentration; DHLNL, dihydroxylysinonorleucine; PYD, pyridinoline; DPD, deoxypyridinoline

Linear regression analysis showed LiCu concentration explained very little of the variation in crosslink concentration. In contrast, linear regression showed bone Cu concentration could statistically predict DHLNL and total collagen content concentration ($F(1, 38) = 4.21$, $P=0.047$ and $F(1, 38) = 7.8$, $P=0.008$, respectively. Bone Cu concentration accounted for 10% of the variation in DHLNL concentration (with adjusted R^2= 7.6%) and 17% of the variation in total collagen concentration in bone (adjusted R^2= 15%). Table 6.5 shows the summary statistics for linear regression.

Table 6.5 Linear regression summary statistics testing the relationship between liver and bone Cu concentration with the different collagen crosslinks in bone and the total collagen content.

Crosslink	Tissue	R^2	Adjusted R^2	*p* value
DHLNL	Liver	0.056	0.031	0.140
	Bone	0.100	0.076	0.047
PYD	Liver	0.043	0.018	0.199
	Bone	0.047	0.022	0.181
DPD	Liver	0.092	0.068	0.057
	Bone	0.001	-0.025	0.831
Total Collagen	Liver	0.001	-0.025	0.830
	Bone	0.170	0.15	0.008

DHLNL, dihydroxylysinonorleucine; PYD, pyridinoline; DPD, deoxypyridinoline

The following three parameters were tested in the logistic regression model: total collagen, DHLNL, and PYD. Dihydroxylysinonorleucine and PYD were retained in the final model. The logistic regression model was statistically significant, $\chi2(4) = 16.81$, $P<0.001$. The Hosmer and Lemeshow test was not statistically significant ($P=0.63$) indicating the model is a good fit. The model explained 47.9% (Nagelkerke R2) of the variance in humeral fracture and correctly classified 77.5% of cases. Sensitivity was 85.2% and specificity was 61.4%. Table 6.6.

Table 6.6 Results of the logistic regression test for predicting humeral fracture in heifers based on pyridinoline (PYD) and dihydroxylysinonorleucine (DHLNL) content in bone.

	B	SE	Wald	*df*	*P*	Odds Ratio	95% CI for Odds Ratio	
							Lower	Upper
PYD content	0.002	0.001	4.259	1	0.04	1.002	1.000	1.003
DHLNL content	0.006	0.003	4.385	1	0.04	1.006	1.000	1.012
Constant	-8.314	3.134	7.038	1	0.008			

B, B coefficient for the constant; S.E, standard error; Wald, Wald chi-square test; df, degrees of freedom; CI, confidence interval; Cu, copper; LiCu, liver copper concentration

Molybdenum, zinc, iron, and cadmium liver concentration

There were no significant differences in the mean liver concentration of Mo, Zn, Fe, and Cd between affected and control heifers and between heifers with low or adequate LiCu concentration. Table 6.7 shows the summary statistics.

Table 6.7 Mean ± SD of molybdenum, zinc, iron, and cadmium liver concentration comparing fracture status (affected vs control) and liver copper concentration (low vs adequate).

	Case	Mean ± Std. Deviation	*P* value	*d**
Molybdenum (mg/kg)	Affected (n=26)	1.02 ± 0.25	0.95**	0.02
	Control (n=14)	1.06 ± 0.38		
	Low LiCu (n=16)	0.98 ± 0.22	0.48	0.23
	Adequate LiCu (n=24)	1.08 ± 0.34		
Zinc (mg/kg)	Affected (n=26)	61.58 ± 18.86	0.63	0.16
	Control (n=14)	68.04 ± 34.09		
	Low LiCu (n=16)	60.05 ± 15.67	0.65	0.15
	Adequate LiCu (n=24)	66.37 ± 29.72		
Iron (mg/kg)	Affected (n=26)	107.07 ± 70.94	0.10	0.55
	Control (n=14)	138.18 ± 70.93		
	Low LiCu (n=16)	107.03 ± 40.71	0.82	0.07
	Adequate LiCu (n=24)	125.25 ± 86.47		
Cadmium (mg/kg)	Affected (n=26)	0.05 ± 0.03	0.70	0.13
	Control (n=14)	0.05 ± 0.02		
	Low LiCu (n=16)	0.05 ± 0.03	0.91	0.04
	Adequate LiCu (n=24)	0.05 ± 0.03		

*d** Cohen's d
** Welch test

6.4 Discussion

The results of this study indicate that in heifers with osteoporosis and humeral fractures low LiCu concentration is correlated with affected fracture status indicating there is significant mobilisation of liver Cu. This depletion has seemingly resulted from Cu being transported to extrahepatic tissues including bone, with bone Cu concentration being significantly higher in affected heifers than in control heifers and correlated with affected fracture status. To the best of our knowledge, this is the first time such a finding is described. Furthermore, these findings indicate that bone Cu concentrations explain a greater amount of

the variability in the presence of fracture in heifers compared with LiCu concentration (33.9% vs 28.2%) and increased bone Cu concentration is associated with a far greater risk of having an osteoporotic humeral fracture (56.1 times more) compared to low LiCu concentration (only 3.2 times more).

For the first time, we were able to measure the total collagen content and collagen crosslink content in bone from dairy cows in New Zealand which allowed us to establish important conclusions regarding the likely pathogenesis of spontaneous humeral fracture. In the case of collagen and its crosslinks, the total collagen content was significantly higher in bone from control heifers and heifers with adequate LiCu concentration consistent with the histologic findings of osteoporosis reported in chapter 4. Heifers with humeral fracture and low LiCu concentration have an increased concentration of total collagen crosslinks, DHLNL, and HYP in bone. Although only bone Cu concentration was positively correlated with DHLNL. The concentration of Cu in the liver does not correlate with any collagen crosslinks indicating no correlation between these two components.

Since the first reported case of humeral fracture, low LiCu or serum Cu concentration has been a common finding in cows and/or herds with spontaneous humeral fractures and has been suggested to be a potential cause of bone weakness leading to fracture.[36,11,37] This association comes from the fact that Cu deficiency, either alone or with other nutritional deficiencies, can result in musculoskeletal disorders (including lameness, enlargement of joints, radiographical loss of cortical bone index) and bone fractures in both animals

and humans.[18,21,32,22,33] The relationship between Cu and bone quality/strength is linked to the activity of the Cu-dependent lysyl oxidase (LOX) enzyme.[13,31] This enzyme has a central role in the control of the total amount of collagen crosslinking, an important determinant of bone quality.[31]

Like many previous reported case studies, most affected heifers in this study (16/26, 62%) had low LiCu concentration (<95 µmol/kg) at the time of fracture.[11,36,37,5,4,6,3,7] The concentration of Cu in the liver is defined as the Cu "storage pool" and indicates that in affected heifers there was inadequate Cu consumption for at least 30 days before testing, with subsequent mobilisation of stored Cu from the liver.[12]

What previous studies have not been able to describe, is whether the liver Cu deficiency equates to a whole-body Cu deficiency and the destination of the Cu that was stored in the liver. Serum Cu concentration, only measured in affected heifers, revealed that most animals (22/26, 85%) had adequate serum Cu concentration (>7.9 µmol/L) and furthermore, LiCu and serum Cu concentrations were significantly correlated within the affected group. This finding indicates that in affected heifers, although Cu storage is being depleted, there is still enough Cu to maintain adequate serum Cu concentrations (known as the Cu transportation pool).

The next step is to evaluate Cu concentration in the tissues where it is needed; this is regarded as the functional or tissue pool.[12] Experimental Cu deficiency in calves did not reduce bone Cu concentrations when compared with Cu-supplemented calves (measured in bone ash using atomic absorption

spectrophotometry).[33] In contrast, chicks on a Cu deficient diet had lower bone Cu content compared with chicks on a Cu-supplemented diet.[29] However, heifers with humeral fractures and heifers with low LiCu concentration had significantly higher bone Cu concentration compared with control heifers and heifers with adequate LiCu concentration. Although bone Cu and LiCu concentration are not significantly correlated in this study, the LiCu depletion appears to be linked to an increase in the bone Cu concentration (and possibly other tissues), probably in response to a stimulus from the bone. Additionally, increased bone Cu concentration (OR 56.1) and not decreased LiCu concentration (OR 3.2) has a greater association with humeral fracture. Therefore, it seems likely that the low LiCu concentration, so frequently described in heifers with humeral fractures is, at least in some part, due to mobilisation of Cu storage to bone and not indicative of a clinical Cu deficiency in these heifers. The increase in bone Cu concentration and not the decrease in LiCu concentration is more closely associated with humeral fracture in heifers in New Zealand.

Collagen stabilisation in the extracellular matrix is dependent on the formation of inter- and intramolecular crosslinks between collagen fibres.[35] This is known as the enzymatic pathway of collagen crosslinking and results in the formation of immature crosslinks (such as DHLNL, measured in this study).[13] The next step is the conversion of immature crosslinks to mature crosslinks, such as PYD and DPD (both measured in this study).[13]

In bone, the conversion of immature to mature crosslinks is described as a continuous, and independent process.[35,31] In normal bone, immature crosslink

content is higher than mature crosslinks and bone is considered to be one of the only tissues to contain a significant pool of immature crosslinks (2-4 times the content of mature crosslinks).[35,31] When comparing the content of immature (DHLNL) and mature (PYD) collagen crosslinks in bones from control heifers and heifers with adequate LiCu concentration, DHLNL was 3.1 and 2.9 times more than PYD, consistent with the ratio of immature to mature crosslinks reported in the literature for healthy bone.[35,31] Similar ratios are observed comparing DHLNL and PYD in bones from affected heifers and heifers with low LiCu concentration.

Although the conversion (immature to mature crosslinks) is continuous, bone growth and the bone turnover rate can affect the relative amounts of immature and mature crosslinks.[31] In Cu deficiency there is a reduction in the activity of LOX which impairs collagen crosslinking.[31] For example, in Cu-deficient chicks (due to low dietary Cu) low LOX activity was related to decreased immature crosslink concentration (mainly DHLNL) and a reduction in bone torsional strength.[26] In a study of osteoporotic avian bone, while there were no significant differences in the content of DHLNL compared with normal bone, decreased DHLNL was correlated with a decrease in breaking strength.[20] Finally, in postmenopausal osteoporosis in women there is a significant reduction in immature crosslink concentration that leads to a significant reduction in bone strength.[31]

Contrary to these previously mentioned studies, the current study found significantly higher content of immature (DHLNL) collagen crosslink in bones

from affected heifers (1.23 times more) and heifers with low LiCu concentration (1.20 times more) and additionally, bone Cu concentration was correlated with DHLNL amount. Notably, mechanical stress can accelerate the conversion of immature to mature crosslinks as an attempt to stabilise the collagen molecule and this is controlled by LOX.[31] This is likely to be occurring in the bones of heifers with humeral fractures considering the bone in these heifers is characterised by a reduction in trabecular density, abnormal trabecular architecture, and thinner cortex which can significantly compromise bone biomechanical strength.[27,1] Furthermore, high DHLNL concentration in bone is indicative of a higher collagen turnover rate and could lead to thinner newly synthesised collagen molecules that alter the properties of bone.[20]

Similarly, PYD (mature crosslink) was significantly higher in affected heifers (1.5 times more) and heifers with low LiCu (1.23 times more). As with DHLNL, reduced LOX activity inhibits the formation of PYD crosslinks which reduces bone strength.[35,31] The increased content of mature crosslinks (PYD and DPD) in bones from affected heifers and heifers with low LiCu concentration is likely in response to the higher concentration of DHLNL previously described and further support the hypothesis of a higher conversion rate of crosslinks to allow extra stabilisation of the collagen molecule in heifers with humeral fractures.

The total collagen content was also measured in heifers. In chicks on Cu-deficient diets, the content of soluble collagen in bone significantly increased compared with chicks on Cu-supplemented diets and this was thought to be related to decreased crosslinking (fewer crosslinks allowing greater ease of solubilisation)

and decreased mineralisation (demineralisation of bone before extraction solubilises collagen hence Ca may protect from solubilisation).[29] Similarly, an increase in collagen synthesis and production was found in the aorta of pigs on a Cu-deficient diet.[19] Our findings suggest that another factor may have influenced collagen synthesis in affected heifers causing a reduction in the production of collagen such as described here. In undernourished cattle, there is a significant reduction in serum albumin and creatinine concentration compared to adequately nourished animals.[2] We have previously shown that 69% of affected heifers have low creatinine concentrations (chapter 3) which was linked to low muscle mass and protein/calorie undernutrition. Likely, undernutrition and/or low muscle mass and/or low body condition score may explain the lower total collagen content in bone from affected heifers in this study.

Finally, we measured the concentration of known Cu antagonists, Cd, Fe, Mo, and Zn, in the liver. As with Cu, the liver is the major organ of storage for these elements and reflects consumption levels.[10] Significant accumulation of Cd, Mo, Zn, and Fe were not found suggesting a minimal impact on low LiCu concentrations in heifers with humeral fracture.

6.5 Conclusion

This chapter was intended to determine the true role of collagen crosslinking and Cu as a determinant of bone strength in heifers with humeral fracture in New Zealand. The low liver Cu concentration found in most affected heifers is likely due to mobilisation of Cu to other tissues (including bone). The high content of immature (correlated with bone Cu concentration) and mature collagen

crosslinks is likely a response mechanism from a biomechanically stressed bone intended to improve stabilisation of the collagen molecule in heifers with osteoporosis.

In conclusion, Cu deficiency is not a major factor in the pathogenesis of humeral fractures in dairy heifers in New Zealand. The low total collagen content could indicate that inadequate feed quality/quantity is more important in the pathogenesis of this condition, thus increasing the incidence of spontaneous humeral fractures.

6.6 References

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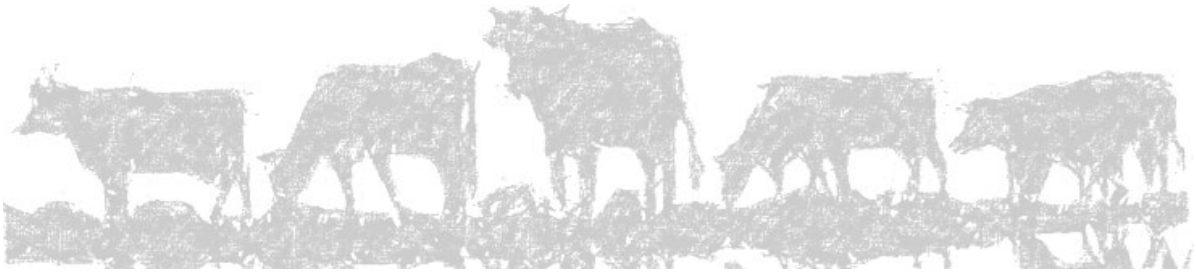
CHAPTER 7

GENERAL DISCUSSION



7.1 Introduction

For many years, outbreaks of spontaneous and catastrophic humeral fractures have been observed in New Zealand dairy cows. These outbreaks have severely impacted animal welfare (fractured animals are euthanised at diagnosis), affected the mental health of farmers and veterinarians, and resulted in major economic losses to the dairy industry.[12,5,6]

Any investigations into fracture outbreaks in production animals, no matter the cause, should focus on the environment, the diet, and the health of affected animals, to identify contributory causes or risk factors associated with the fracture. However, identification of causes/risk factors can be a challenge because changes in bone quantity and quality generally progress slowly and asymptomatically, with bone fractures representing the end-state of skeletal compromise.[10]

Previous small-scale studies and case reports on humeral fractures in dairy heifers in New Zealand have described potential risk factors and provided recommendations to farmers and veterinarians.[5,12,6] Even so, outbreaks still occur, and the definitive cause remains elusive. The main factors suggested to increase the risk of humeral fractures in New Zealand dairy cows include protein/calorie undernutrition during important growth periods and marked bone resorption associated with gestation and lactation.[5] Copper deficiency (diagnosed using liver Cu and/or serum concentration) was a relatively common clinical finding in many affected heifers (anecdotally, usually >50% of affected heifers have low liver and/or serum Cu concentration) when measured around the time of

euthanasia.[5,1-3] This suggested that Cu deficiency was another important contributory factor in the appearance of humeral fractures in dairy cows in New Zealand, although as previously stated, the bone changes progress slowly so there is no data on whether animals were Cu deficient when the bone was being formed (before humeral fracture).

The main aim of this thesis was to determine potential causes and/or risk factors associated with the pathogenesis of spontaneous humeral fractures in dairy heifers in New Zealand. A holistic approach to examining the various aspects of the occurrence of humeral fractures was planned and carried out. Each chapter focused on distinct aspects of the disease and included surveying farmers and their practices (for herd nutrition and calf rearing), determination of the energy and Cu status of affected cows at the point of euthanasia and evaluating bone structure and composition at the point of fracture. Different hierarchical levels of the bone structure were studied from macrostructure (gross findings) to microstructure (using histology and histomorphometry), and finally nanostructure of bone (using Raman and Fourier-transformed infrared spectroscopy (FTIR), high-performance liquid chromatography and inductively coupled mass spectrometry). Macroscopic and microscopic findings, as well as the chemical composition of the affected bones from a large cohort of 2-year-old heifers with spontaneous humeral fractures, were described and compared with age-matched control heifers. Figure 7.1 shows a compilation of steps taken and the methods used at each step in this study.

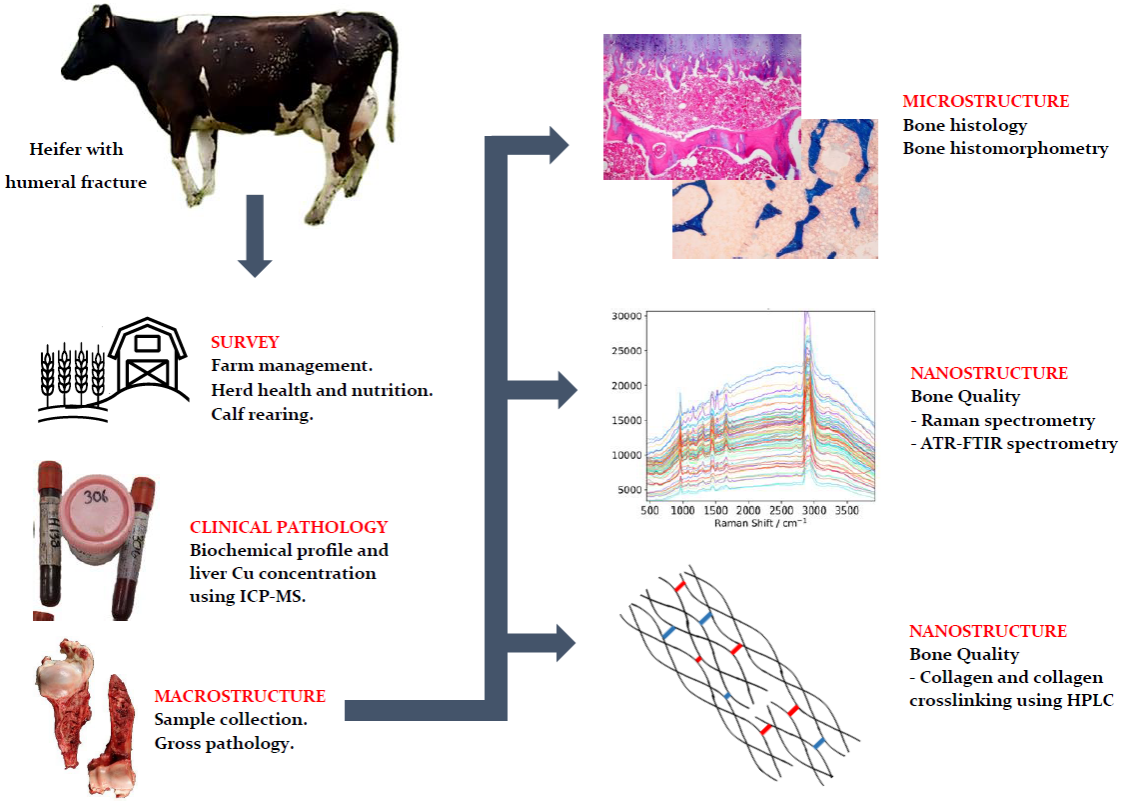


Figure 7.1. Flow chart showing the hierarchical approach and steps taken in the evaluation of spontaneous humeral fractures in dairy heifers in New Zealand with the end goal to provide pathogenesis of the disease. Cu: copper; ICP-MS: inductively coupled plasma mass spectrometry; ATR-FTIR: attenuated total reflectance-Fourier transform infrared; HPLC: high-performance liquid chromatography.

7.2 Heifers with spontaneous humeral fractures have osteoporosis

The World Health Organisation defines osteoporosis as a systemic disease characterised by low bone mass and microarchitectural deterioration of bone with a consequent increase in bone fragility and susceptibility to fracture.[9] The findings described in chapters 4 and 5 confirmed that heifers with humeral fractures have osteoporosis. Osteoporosis has several different causes, and it was an important step in the early part of this study to identify those associated with spontaneous humeral fracture. Macroscopic and microscopic evaluation of the humerus and ribs from affected heifers in Chapter 4 revealed that affected cows

had significant changes associated with two processes: impaired/irregular bone formation and increased bone resorption.

Affected heifers had thicker proximal humeral growth plates with abnormal chondrocyte architecture compared to control heifers. This finding and the presence of growth arrest lines in 21% of affected heifers are indicators of abnormal bone growth. Although abnormal growth plate width and architecture are microscopic findings associated with rickets/osteomalacia, this disease was ruled-out in chapter 4 (no significant differences in osteoid area and perimeter were found comparing affected and control cases) and in chapter 5 (no significant differences in absolute Ca and phosphorus concentration in bone comparing affected and control cases). In cases with humeral fractures, the presence of growth arrest lines revealed that bone growth had stopped for a period and then resumed. This re-stimulation of chondrocyte activity in the growth plate likely resulted in the changes in growth plate thickness and appearance, which was described in affected heifers in chapter 4. Decreased bone area, trabecular number, width, and perimeter are also consistent with decreased bone formation in affected heifers. All these findings suggest that the first cause of osteoporosis in affected cows is inadequate bone formation, probably due to periods of protein/calorie restriction (undernutrition or inadequate feed quality).

Although a specific time point (in months/days) when the period of undernutrition occurred cannot be ascertained the presence of growth arrest lines suggests that the changes which resulted in inadequate bone formation occurred recently, most likely in the heifers' second winter. A reduced growth

rate is observed in New Zealand heifers' during their second winter (between 20-22 months of age), which corresponds with a period of low pasture quality and/or quantity.[7] It is likely that most of the changes observed in the bones examined for this thesis coincide with this period, although changes occurring in the bones before this cannot be ruled out.

The second cause of osteoporosis in affected heifers was increased bone resorption. Bone changes consistent with this included decreased bone area, decreased trabecular number, trabecular width, trabecular perimeter, abnormal trabecular architecture, and increased resorption in the distal humerus. Although some bone resorption is expected in affected heifers (they were all primiparous cows within the first months of lactation), the amount of bone resorption observed in affected heifers was determined to be excessive and had occurred for a prolonged period. As such, it had a major detrimental factor impact on bone strength. Evidence for a prolonged period of resorption activity was found in the cortical bone (described in chapter 4). Increased abnormal cortical bone resorption was described in affected heifers, which resulted in a significant compromise of cortical bone strength. Normally, trabecular bone turnover is higher than in cortical bone and resorption of cortical bone to provide Ca for lactation should be a last resort but in affected heifers, this is the opposite. With increased cortical bone resorption, the cortex loses strength becoming more porous (as described in chapter 4).[8]

The discovery of macroscopic and microscopic findings in rib fractures (which was a novel finding for this condition) showed that the fractures were at

dissimilar stages (times) of healing, indicating a long course of the disease and that the observed changes are systemic. Likewise, the presence of significant additional woven bone formation in the humerus and ribs of affected heifers compared with control cases indicated a longer course of the disease.

Investigation into the nanostructure of bone in chapter 5 provided information that indicated decreased relative matrix and mineral content, reduced crystallinity, marked remodelling activity, and younger tissue in the cortical bone as opposed to the trabecular bone, in heifers with humeral fractures compared to control cases. Furthermore, reduced crystallinity and reduced total bone collagen content (described in chapter 6) were also identified as major contributors to bone fragility in heifers with humeral fracture. Changes described in chapters 5 and 6 further confirm the diagnosis of osteoporosis in affected heifers and additionally show the basic bone-building block (collagen fibril with hydroxyapatite crystals) is compromised, not only in quantity but in structure. Both the amount of mineral and its size/shape are important for the stabilization of the bone collagen molecule.[11]

7.3 Risk factors related to the occurrence of spontaneous humeral fractures in dairy heifers in New Zealand.

A key step in disease control programs, such as the outbreaks of spontaneous humeral fractures, is the identification of risk factors and/or causes associated with the disease. Accurate identification of risk factors is important to prevent further outbreaks and to formulate a list of recommendations for farmers and

veterinarians so that they can improve animal welfare and reduce the incidence of disease in the future.

In chapter 2 we identified that farms with Holstein-Friesian x Jersey crossbreed cows had a significantly higher risk of developing humeral fracture and the increase in the incidence of fractures could be related to the increase in the proportion of Holstein-Friesian x Jersey crossbreed cows in the total dairy cow population of New Zealand over the past two decades.[4] Furthermore, this rapid and extensive uptake in crossbreeding seems unique to New Zealand and could be a previously unidentified risk factor for humeral fractures in dairy cows in New Zealand. The association with crossbreeding is likely confounded by other co-factors such as herd size, farm management style, and animal nutrition (especially during reported periods of reduced growth rate) which will need to be carefully examined and controlled for in future studies.[7]

Interpretation of findings in samples from affected heifers in chapters 3, 4, 5, and 6 suggest that undernutrition, most likely due to protein/calorie deficiency is a crucial factor related to the appearance of humeral fractures in dairy heifers in New Zealand. For example, in chapter 3 low serum creatinine concentration was present in affected heifers which likely resulted from undernutrition and/or increased muscle catabolism for energy production. In chapter 4 diagnosis of osteoporosis with the presence of growth arrest lines and growth plate changes were suggestive of compensatory growth related to sub-optimum nutrition during important growth periods. Raman and FTIR spectroscopy in chapter 5 showed a significant reduction in bone quality in affected heifers with reduced

bone mineral content. Tissue age (using Raman and FTIR spectroscopy) was reduced in affected heifers compared with controls. This indicates that bone formation in affected heifers is more recent compared to controls and likely secondary to previous periods of inadequate bone production. Finally, chapter 6 revealed decreased collagen content in cortical bone from affected heifers. It can thus be suggested that nutrition during growth is inadequate (undernutrition) in affected heifers with severe consequences on animal health and welfare later in life such as outbreaks of humeral fractures.

Although low liver Cu concentration remains a common/typical clinical finding in heifers with humeral fracture, this low concentration is only indicative of the need for Cu supplementation in these animals, from a production point of view, rather than disease prevention. In chapter 6 we showed that in those heifers with humeral fractures, and in those that had low liver Cu concentration, bone Cu concentration was higher than that in control heifers and heifers with adequate liver Cu concentration. Furthermore, this increase in bone Cu was related to an increased quantity of collagen crosslinking in bones. This and the fact that other more common clinical signs of Cu deficiency are not described for any of the fracture cases submitted to this study suggest Cu deficiency does not contribute to the appearance of humeral fractures in dairy heifers in New Zealand.

7.4 Proposed pathogenesis of spontaneous humeral fractures in dairy heifers in New Zealand

After consideration of the results from this thesis, a proposed pathogenesis of spontaneous humeral fractures was developed and is shown in Figure 7.2.

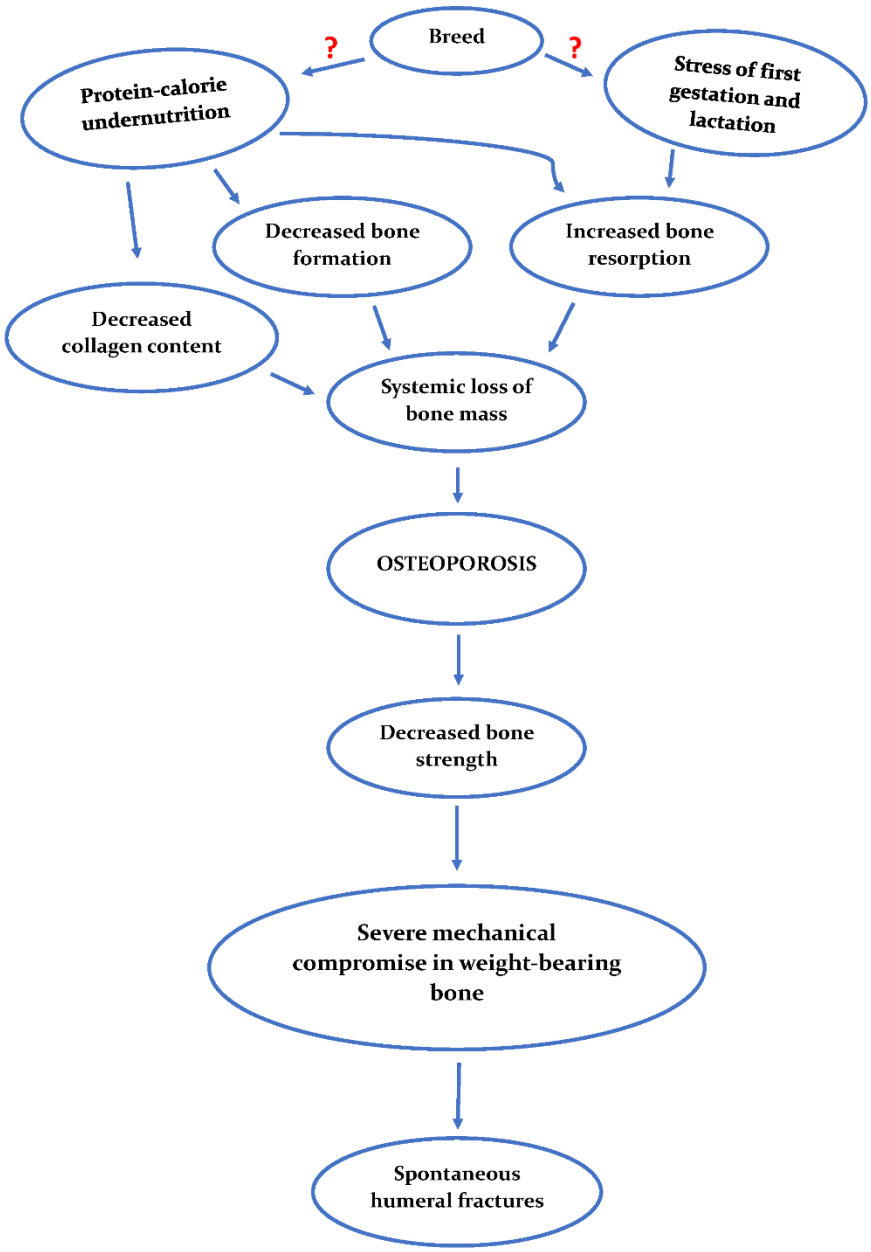


Figure 7.2. Proposed pathway for the pathogenesis of humeral fractures in dairy heifers in New Zealand.

7.5 Limitations

Some limitations are identified for the different chapters of this thesis. In chapter 2 an important limitation was the small number of respondents. Even when diverse methods of delivery of questionnaires were used, the response rate was 0.6% of dairy farms in New Zealand. The only way to address this issue is to use in-person and/or telephone surveys to collect quality data, with a random selection of case and control farms across the whole of New Zealand. This will be expensive but there is a real urgency to get meaningful data on this condition.

When interpreting findings in chapter 3, a major difficulty was the lack of control samples, meaning that interpretation of the results was against the reference ranges provided by the same laboratory that analysed the samples. It was presumed that values from control animals, had they been available, would have fallen within these references' ranges. However, similarities or differences between true control (unfractured) animals and affected animals could not be determined. Additionally, the provided reference ranges did not consider the age of the affected cows and the time of the year the sample was taken. Another limitation was the number of affected animals for which blood was available for biochemical analysis. Unfortunately, budgeting constraints limited the number of tests performed and the number of animals evaluated.

In chapters 4, 5, and 6 the main limitation was again sample size, especially the number of control cases. For example, most control cases had liver Cu concentration within the reference range and only a few had low liver Cu. The small sample size means that this finding may not be representative of all cows

unaffected by spontaneous humeral fractures. In chapter 6 a similar problem was encountered, none of the control cases had low liver Cu concentration. In general control cases for humeral fractures were difficult to source for this study since we needed to control for age, season, and stage of lactation, and healthy lactating 2-year-old heifers are not commonly euthanised.

The chemical composition of bone was analysed in chapters 5 and 6, and the concentration of different biochemistry analytes and minerals in blood and liver samples were described in chapters 3, 4, 5, and 6. Details about sample transportation, storage time, and conditions between sample collection and sample preparation were not collected and not considered. It is possible that these factors could influence the reported values on the chemical composition of bones and serum analytes.

Finally, the Cu concentration in the liver and bone was measured at one point in time, the time of fracture, and this does not reflect changes in Cu concentration during cow growth, gestation, and lactation. As illustrated by chapter 6, liver Cu concentration at the time of fracture is not necessarily reflective of Cu concentration in other tissues, such as bone.

7.6 Future work

The researchers aimed to investigate the aetiology and pathogenesis of spontaneous humeral fractures in dairy heifers in New Zealand. Potential mechanisms of disease have been identified and reported in the different chapters and provide valuable new information on this catastrophic disease.

Other mechanisms that were previously believed to be associated with the occurrence of the condition (low liver Cu concentration for example) appear to be less important determinants of humeral fractures in New Zealand dairy heifers than previously thought.

The research into the mechanism of humeral fracture described in this thesis has resulted in several important and novel research findings which will require further investigation, these include:

- Investigating the relationship and/or the consequences of breed selection and heterosis of dairy cows in New Zealand and the appearance of humeral fractures in New Zealand. Although all breeds were affected by fractures and breed is not the sole risk factor for developing bone fractures there was a significant association with crossbreeds found. Could the heterosis achieved by crossbreeding in New Zealand put a Friesian udder on a Jersey skeleton, leading to a faster and more severe demineralisation of bone in Holstein-Friesian x Jersey cows? Studies looking into metabolic drain per kg of live weight for the different breeds in New Zealand, differences in the skeletal composition between breeds or from crossbreeding, differences in the mobilisation of Ca and other important minerals from bone, and changes in bone composition during skeletal growth, through gestation and lactation in dairy cows in New Zealand, should be evaluated and described based on breed. Additionally, analysis of potentially genetic links to humeral fracture should also be investigated.

- Investigating the effect of different feeding systems in New Zealand on skeletal growth. For example, a considerable proportion of affected heifers were grazing on fodder beet prior to fracture occurrence and there was a trend for heifers that grazed on fodder beet as their main winter feed to fracture earlier (August peak) compared with heifers that grazed on pasture (October peak). Further work on the effect of fodder beet on skeletal integrity is required in New Zealand.
- Investigating the change in liver, serum, and bone Cu concentrations over the full life of the animal up to 2 years of age, particularly in the second winter, will improve understanding of the disease. Currently, we are making recommendations based on measurements made at one-time point. As this thesis has shown this has led to spurious associations.
- Running randomised control trials aimed at testing specific hypotheses to define the cause-effect of humeral fractures in dairy cows in New Zealand are required.

7.7 Conclusion

Periods of protein/calorie undernutrition during growth, particularly in the second winter, plus marked bone resorption (associated with gestation and lactation) in a poorly formed bone have led to substantial changes in bone quantity and architecture that significantly compromised bone strength leading to spontaneous and catastrophic humeral fractures in dairy cows in New Zealand.

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APPENDICES

Appendix A

Control of bone growth

Growth plate cartilage and bone growth are dependent on numerous local and systemic genes, hormones, growth factors, environmental conditions, and adequate nutrition.[3,17,16] Listed in Table A.1 are the main substances that control bone growth.

Table A.1 List of some factors that control bone growth.

Hormones	**Effect**
Growth hormone (GH)	Stimulates transition from resting chondrocytes to proliferative chondrocytes and increases osteoblast activity either directly or through the action of IGFI and II.[14,4,8,17] Stimulates periosteal bone formation.[17] Increases intestinal Ca absorption. [10]
Thyroid hormones, triiodothyronine (T3) and thyroxine (T4)	Stimulates resting and proliferative chondrocytes directly or stimulating GH secretion.[14] Thyroxine also induces expression of collagen type II and X and increases ALP activity.[14] Decreases intestinal Ca absorption.[10]
Glucocorticoids	Stimulates osteoclast longevity and osteoblast and osteocyte apoptosis by suppression of RUNX-2 and BMPs signalling pathways.[17] Decreases intestinal Ca absorption.[10] Inhibition of the Wnt signalling pathway.[9]
Oestrogen	Increased plasma concentration is associated with growth plate fusion seen in puberty.[14,17] Bone resorption: Impedes osteoclastogenesis and promotes osteoclast apoptosis by inhibiting RANKL production – by osteoblasts, T and B lymphocytes.[12,6,13] Increases secretion of OPG by osteoblasts and decreases IL-1, -6, M-CSF, prostaglandins, and TNF-α by bone marrow cells.[17,11] Bone formation (relative): increases osteoblast apoptosis, amount of reactive oxygen species, and increased activity of nuclear Factor kappa-light-chain-

	enhancer signalling pathway. On the other hand, it has been shown to increase osteoblast lifespan and inhibition of cell apoptosis.[6] Increases intestinal Ca absorption.[10]
Androgens	Stimulates longitudinal bone growth and decreases bone resorption.[14] Stimulates periosteal bone formation (is the cause of gender differences in human bones).[17] Increases intestinal Ca absorption.[10]
$1,25(OH)_2D_3$	According on the degree of differentiation of osteoblast can promote bone resorption or stimulate extracellular matrix production.[15,1] When formed within osteoblasts can promote the controlled formation of hydroxyapatite crystals stimulating ALP activity in osteoblasts and inhibiting ectonucleotide pyrophosphatase phosphodiesterase 1, ankylosis protein, and osteopontin.[15]
Leptin	Increases osteoblast lifespan.[17]
Glucagon-like peptide 2	It may cause the decreased resorption of bone observed after feeding.[17]
Calcitonin	Decreases osteoclast lifespan and secretory activity (especially TRAP), hence is known as a hypocalcaemic factor.[2]
PTH	Ca homeostasis: increases bone resorption (by increasing RANKL expression) in response to hypocalcaemia. Also increases renal reabsorption of Ca and renal hydroxylation of vitamin D (for gastrointestinal Ca absorption).[17] Regulates the expression of VDR in osteoblasts.[15]
Growth Factors	
Insulin-like growth factor-1	Most abundant growth factor present in the bone matrix.[11] Stimulates migration of mesenchymal stem cells during fracture healing.[5] Necessary for osteoblast differentiation, survival, and proliferation.[2] Promotes osteoclast differentiation.[11]
Indian hedgehog factor (Ihh)	Secretion by prehypertrophic chondrocytes in the growth plate is associated with inhibition of chondrocyte hypertrophy and hence bone formation.[16]

Parathyroid hormone-related peptide (PTHrP)	Secreted from the perichondrium and diffuses to the cells of the prehypertrophic zone where it inhibits their differentiation.[16]
Fibroblast growth factors (FGF)	FGF2 regulates bone and cartilage growth.[11] FGF18 and FGFR3 regulate the proliferation and differentiation of hypertrophic chondrocytes, ossification, and expression of osteogenic markers.[14] FGF23 functions are described in section 1.5.1.3.4. of chapter 1
BMPs and TGF-β	Stimulators of bone formation, early cartilage differentiation, and chondrocyte apoptosis.[13,7] TGF-β can stimulate early osteoblast differentiation and inhibit late osteoblast differentiation.[11]
Vascular endothelial growth factor (VEGF)	Required for the vascular invasion to the mineralised cartilaginous matrix and later trabecular bone formation. Both hypoxia-inducible factor 1α and core-binding factor alpha-1 are necessary for VEGF expression.[13]
Wingless-type MMTV integration site (Wnt)	Necessary for osteoblast differentiation.[11] Important for chondrocyte survival, proliferation, and hypertrophy.[7]
Epidermal growth factor (EGF)	Regulates the expression of VDR in osteoblasts.[15] Suppresses expression of RUNX2, osterix, ALP, bone sialoprotein, and osteocalcin.[11]

IGF, insulin-like growth factor; ALP, alkaline phosphatase; RUNX-2, runt-related transcription factor 2; BMPs, bone morphogenetic proteins; Wnt, wingless/integrated; RANKL, receptor activator of nuclear factor kappa-B ligand; OPG, osteoprotegerin; IL. Interleukin; M-CSF, macrophage colony-stimulating factor; TNF-α, Tumour necrosis factor-α; TRAP, tartrate-resistant acid phosphatase; VDR, vitamin D receptor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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Appendix B

Humeral fracture questionnaire

Humeral Fracture Questionnaire

Contact's person name			
Farm address			
Nearest town/city		**LIC No**	
Phone/Mobile No		**Post code**	

A- Farm Information

1. What is the predominate breed of cow?	Friesian ☐	Jersey ☐	Kiwi cross ☐	Other ☐
2. How could you best describe your farm enterprise (select only once choice)				
Spring calving ☐	Autumn calving ☐	Split calving ☐		
3. What was the average herd production for the 2018-2019 season?	MS/cow			
4. Do you milk once or twice daily	All once a day ☐	All twice a day ☐	Mix of once and twice daily ☐	
5. Have you ever had broken shoulders in your herd?	Yes ☐	No ☐		
6. How many years have you had cases?				

B- Herd health and nutrition

7. Has copper deficiency been diagnosed in your herd?	Yes ☐	No ☐		
8. Is copper supplemented on farm?	Yes ☐	No ☐		
9. If yes, what type(s) of copper was used?	Fertiliser ☐	Bolus ☐	Injection ☐	Other ☐
10. What age groups were given copper?	Milkers ☐	R1s ☐	All ☐	
11. Was lime supplemented at any time through lime flour?	Yes ☐	No ☐		
12. If yes, what product and when was it used?				
13. Do you feed any fodderbeet to the herd?	Yes ☐	No ☐		
14. If yes, when and to what age groups and for how long?				
15. Were there any growth checks or health issues in general on farm? If yes, please explain				

C- Calf rearing

16. Apart from colostrum, did you use whole milk or milk powder to feed calves?	Whole milk ☐	Milk powder ☐	Both ☐
17. On average how much milk was fed per calf per day?		L/per calf/day	
18. Was a supplement meal fed pre and/or post weaning	Prewean ☐	Postwean ☐	Both ☐
19. At what age were calves allowed access to pasture pre-weaning?			

D. Additional information:

CONSENT

PLEASE READ AND SIGN THE FOLLOWING:

I understand that the information collected will be used as part of a PhD research project investigating Heifer fractures in dairy cows. Data and conclusions from the study may be published, but no identifiable information about respondents, their farms or animals will be shared at any time.

SIGNED: _____ **DATE:** _____

Please send finished questionnaire to Keren Dittmer at K.E.Dittmer@massey.ac.nz or post to Keren Dittmer, School of Veterinary Science Massey University Private Bag 11222 Palmerston North 4410.

If you are interested in contributing to this research and answering a longer more in-depth survey on heifer rearing please let Keren know you availability at K.E.Dittmer@massey.ac.nz

Appendix C

Pro forma submission form for cases with humeral fractures

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

SCHOOL OF VETERINARY SCIENCE

School of Veterinary Science
Private Bag 11222, Palmerston North 4442, New Zealand T 06 350 4525 F 06 355 7957 www.massey.ac.nz

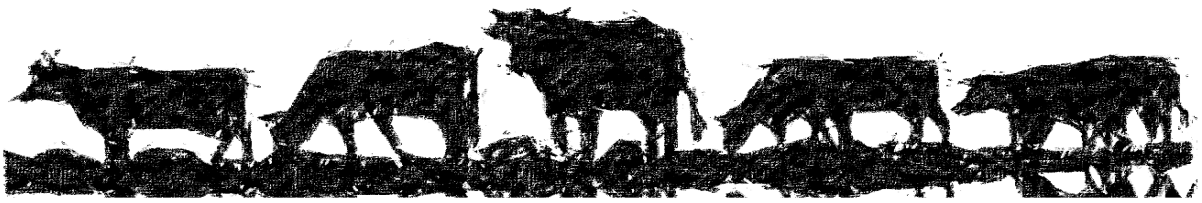
BOVINE CONNECTIVE TISSUE DISEASES RESEARCH GROUP
SAMPLE SUBMISSION FORM

FARM INFORMATION	
Farm/er name	
LIC No	
Farm address	
Town/City	

ANIMAL INFORMATION				
ID No / Name				
Breed	Friesian ☐	Jersey ☐	Kiwi cross ☐	Other ☐
Sex	Male ☐	Female ☐		
DOB or Age	R1/yearling ☐	R2/2-year-old ☐	R3/3-year-old ☐	
Predominant winter feed	Fodder beet ☐	Other crop ☐	Pasture ☐	

CLINICAL HISTORY /OBSERVATIONS	Eg number of affected cows, fractured just walking in paddock, Cu results etc.

SAMPLES SUBMITTED		
Bone	Fractured humerus ☐	Contralateral (nonfractured) humerus ☐
	Metacarpus (cannon bone) ☐	Rib (costochondral juntion) ☐
	Ear and ear tag ☐	
Liver	Yes ☐	
Blood	Serum ☐	EDTA ☐



Project contact: Keren Dittmer: K.E.Dittmer@massey.ac.nz

Appendix D

Individual results of the concentrations of biochemistry analytes, serum copper and liver copper measured in heifers with humeral fractures.

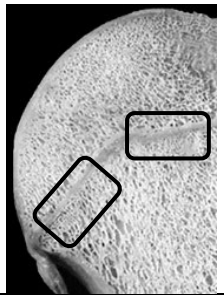
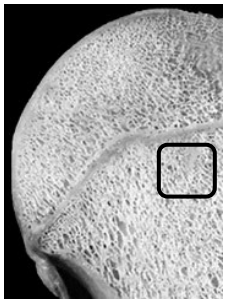
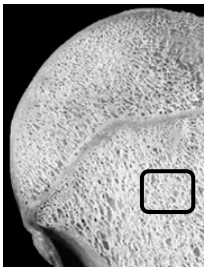
ID	Breed	Alb[a]	Cr[b]	PO_4[c]	Ca[c]	Mg[c]	BHB[c]	NEFA[c]	SeCu[b]	LiCu[d]	Diet	Farm[e]
1	HFXJ	29	38	1.96	2.32	0.55	1.2	0.2	16	33	FB	A
2	HF	33	51	2.56	2.64	0.93	1.2	0.4	5	34	FB	B
3	HF	28	62	2.8	2.74	0.73	1.2	0.3	23.1	1540	FB	B
4	HFXJ	32	59	2.3	2.27	0.78	1.2	0.5	26.7	774	FB	C
5	HF	29	36	2.4	2.36	0.91	1	0.2	15.4	139	PT	D
6	HF	32	42	1.81	2.21	0.83	1.1	n/a	3.2	56	PT	E
7	HFXJ	32	55	2.65	2.55	1.05	1.4	0.3	25.1	884	FB	C
8	HFXJ	30	51	1.79	2.15	0.92	0.8	1.1	7	33	FB	A
9	HFXJ	32	51	1.57	2.34	0.77	0.8	0.2	22.1	49	PT	F
10	HFXJ	31	67	1.91	2.21	0.85	1.1	1.4	18.8	15	MX	G
11	HF	29	51	1.61	2.33	0.81	0.9	0.6	3.8	27	FB	B
12	HF	35	36	1.45	n/a	n/a	0.4	0.4	9.7	19	PT	H
13	HF	33	43	2.37	2.47	0.99	0.9	n/a	n/a	141	PT	D
14	HF	34	56	2.1	2.35	0.87	0.9	0.4	7	21	FB	B
15	HF	35	68	1.64	2.16	0.88	2.1	1.4	12	25	FB	B
16	HF	29	40	2.27	2.42	0.95	1.2	0.2	12.7	35	MX	I
17	HF	35	64	2.24	2.38	1.22	1.5	0.6	12.6	38	PT	J

18	HF	30	61	2.47	2.3	1.2	1.2	0.4	9	28	FB	B
19	HF	33	44	2.32	2.53	1.03	1.2	0.2	19.7	32	MX	I
20	HFXJ	32	57	2.93	2.47	1.01	1.3	0.3	9.6	27	PT	K
21	HFXJ	29	38	0.78	2.12	0.5	0.8	0.2	15.4	105	PT	F
22	HFXJ	32	73	2.03	2.24	0.75	1.6	1	22.3	763	FB	C
23	HF	31	42	2.57	2.58	0.6	1	0.5	13.1	38	PT	L
24	HFXJ	38	48	2.19	2.52	1	1.3	0.5	14.1	368	PT	M
25	n/a	36	39	1.49	2.46	1.32	1.1	1.3	17.4	145	MX	N
26	HFXJ	35	42	1.98	2.46	0.92	1.2	0.5	13.9	40	MX	O
27	HF	31	53	1.97	2.29	0.9	0.9	0.6	31.4	23	FB	B
28	HF	36	44	2.53	n/a	n/a	0.5	0.3	5.2	21	PT	H
29	HF	34	39	1.79	2.45	0.64	0.7	0.3	5.5	34	PT	H
30	HF	37	37	1.66	2.42	0.93	0.7	0.7	26.1	22	PT	H
31	HFXJ	35	38	1.8	2.57	0.75	1	0.2	19	44	FB	A
32	HFXJ	31	36	2.64	2.38	0.93	1.3	n/a	n/a	41	PT	P
33	HF	32	38	2.4	2.45	0.87	0.9	0.4	12	60	PT	L
34	HFXJ	29	55	2.87	2.51	1.19	1.2	0.2	12.2	115	MX	Q
35	HFXJ	34	49	2.76	2.45	1.18	1.1	0.5	22.1	790	FB	F

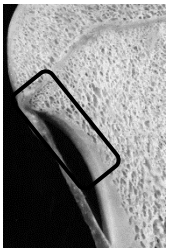
[a] in g/L, [b] in µmol/L, [c] in mmol/L, [d] in µmol/kg wet weight, [e]farms are identified A to Q; n/a, not applicable; Alb: albumin, Cr: creatinine, PO_4: phosphate, Ca: calcium, Mg: magnesium, BHB: beta-hydroxybutyrate, NEFA: non-esterified fatty acids, SeCu: serum copper, LiCu: liver copper, HFXJ: Holstein-Friesian Jersey crossbreed, HF: Holstein-Friesian, FB: fodder beet and PT: pasture, MX: mixed diet.

Appendix E

Selected locations (▭) and histological parameters evaluated in sections of the humerus from affected and control cows. In brackets () the dichotomous parameters and in square bracket [] the microscopic changes considered for the category.

Location	Parameter Evaluated
Growth Plate 	a) measurement of the proximal growth plate thickness (in µm 200x lens) from the zone of proliferative chondrocytes to the zone of hypertrophic chondrocytes measured at 4 different sites for the humerus, and the mean value used. b) evaluation of the growth plate appearance (0=normal [growth plate with organized chondrocyte columns] or 1=abnormal [segmental widening, architectural disarray, extension of cartilage into the primary spongiosa]).
Primary spongiosa 	a) evaluation of the trabecular density (0= normal [abundant] or 1= abnormal [a decrease of ~30% compared with normal]). b) appearance of the trabecular architecture (0=normal [interconnected trabeculae] or 1=abnormal [long and thin or short and stout trabeculae]). c) growth arrest lines (0=absent or 1=present). d) additional bone between trabeculae (0=absence or 1=presence). e) additional bone formation in the cut-back zone (0=absent or 1=present).
Proximal metaphysis 	a) evaluation of additional bone between trabeculae (0=absence or 1=presence).

Cortical bone

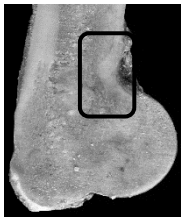


a) thickness was measured at a consistent skeletal distance from the growth plate (600 μm at 400x).

b) evaluation of cortical resorption (0= normal [periosteal/endosteal surface] or 1= abnormal [transverse]).

c) periosteal reaction (0=absent or 1=present).

Distal humerus



a) evaluation of additional bone between trabeculae (0=absent or 1=present).

b) the number of resorption cavities was counted and the mean for each case was calculated.

Appendix F

Distribution of cases for the dichotomous parameters evaluated and comparing affected vs control cases, fodder beet vs pasture, and low/marginal vs adequate liver copper concentration.

	Affected (n=80)	**Control (n=22)**	**Fodder beet (n=33)**	**Pasture (n=28)**	**Low/marginal (n=45)**	**Adequate (n=43)**
Trabecular architecture						
Normal	18 (22%)	20 (91%)	8 (24.2%)	7 (25%)	12 (27%)	19 (44%)
Abnormal	62 (78%)	2 (9%)	25 (75.8%)	21 (75%)	33 (73%)	24 (56%)
Trabecular density						
Normal	15 (19%)	20 (91%)	8 (24%)	4 (14.3%)	11 (24%)	21 (49%)
Abnormal	65 (81%)	2 (9%)	25 (76%)	24 (85.7%)	34 (76%)	22 (51%)
Growth plate appearance						
Normal	31 (39%)	19 (86%)	4 (12.1%)	19 (67.9%)	22 (49%)	22 (51%)
Abnormal	49 (61%)	3 (14%)	29 (87.9%)	9 (32.1%)	23 (51%)	21 (49%)
Growth arrest lines						
Absent	63 (79%)	22 (100%)	29 (87.9%)	20 (71.4%)	36 (80%)	37 (86%)
Present	17 (21%)	0 (0%)	4 (12.1%)	8 (28.6%)	9 (20%)	6 (14%)
Additional bone PS						
Normal	27 (34%)	14 (64%)	11 (33.3%)	11 (39.3%)	14 (31%)	22 (51%)
Abnormal	53 (66%)	8 (36%)	22 (66.7%)	17 (60.7%)	31 (69%)	21 (49%)

Additional bone CB						
Absent	56 (70%)	22 (100%)	22 (66.7%)	19 (67.9%)	31 (69%)	34 (79%)
Present	24 (30%)	0 (0%)	11 (33.3%)	9 (32.1%)	14 (31%)	9 (11%)
Cortical resorption						
Normal	15 (19%)	17 (77%)	7 (21.2%)	4 (14.3%)	9 (30%)	20 (46%)
Abnormal	65 (81%)	5 (23%)	26 (78.8%)	24 (85.7%)	36 (80%)	23 (54%)
Periosteal reaction						
Present	20 (25%)	21 (95%)	11 (33.3%)	3 (10.7%)	14 (31%)	23 (54%)
Absent	60 (75%)	1 (5%)	22 (66.7%)	25 (89.3%)	31 (69%)	20 (46%)
Additional bone PM						
Present	20 (25%)	15 (68%)	13 (39.4%)	4 (14.3%)	15 (33%)	13 (30%)
Absent	60 (75%)	7 (32%)	20 (60.6%)	24 (85.7%)	30 (64%)	30 (70%)
Additional bone DH						
Present	61 (76%)	15 (100%)	22 (73.3%)	18 (66.7%)	33 (73%)	37 (86%)
Absent	19 (24%)	0 (0%)	8 (26.7%)	9 (33.3%)	12 (27%)	6 (14%)

PS, primary spongiosa; CB, cut-back zone; PM, proximal metaphysis; DH, distal humerus.

Appendix G

Comparison of histomorphometric results between affected vs control and fodder beet vs pasture. Results are presented as mean ± SD.

	Groups compared					
	Affected (*n*=20)	**Control (*n*=10)**	***P***	**Fodder beet (*n*=10)**	**Pasture (*n*=10)**	***P***
Measurements						
Bone area, µm²[a]	1508714.9 ± 402099.8	2243136.4 ± 562806.4	<0.005*	1599136.8 ± 388244.0	1418292.9 ± 415248	0.33
Bone area/total area, µm²[a]	0.151 ± 0.04	0.225 ± 0.05	<0.005*	0.16 ± 0.03	0.14 ± 0.04	0.38
Mean trabecular perimeter, µm[b]	651.7 ± 154	790.0 ± 149.1	0.027*	603.1 ± 107.7	700.4 ± 182.2	0.16
Mean trabecular width, µm	50.8 ± 6.0	60.1 ± 9.9	0.003*	50.2 ± 6.9	51.3 ± 5.4	0.69
Mean osteoid area, µm²	98.8 ± 78.1	106.2 ± 77.7	0.809	77.9 ± 45.9	119.7 ± 45.9	0.24
Mean osteoid perimeter, µm	73.7 ± 35.5	77.9 ± 32.5	0.753	63.5 ± 34.8	85.1 ± 27.9	0.18

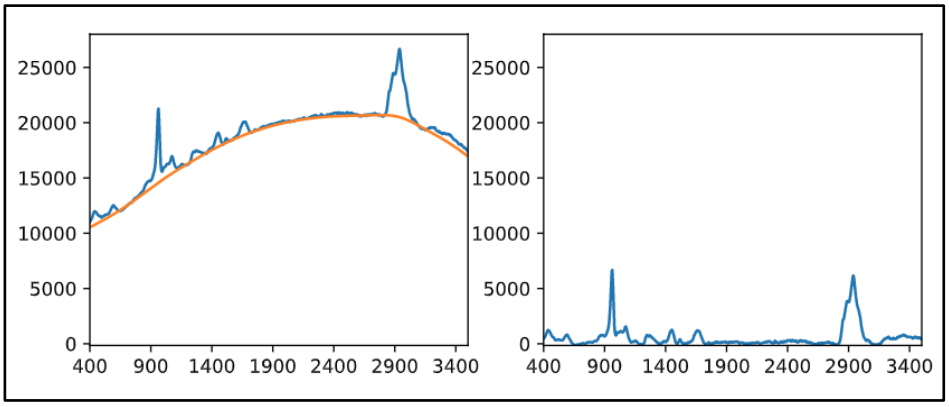
[a]Square micrometre.
[b]Micrometre.
* *P* value <0.05.

Appendix H

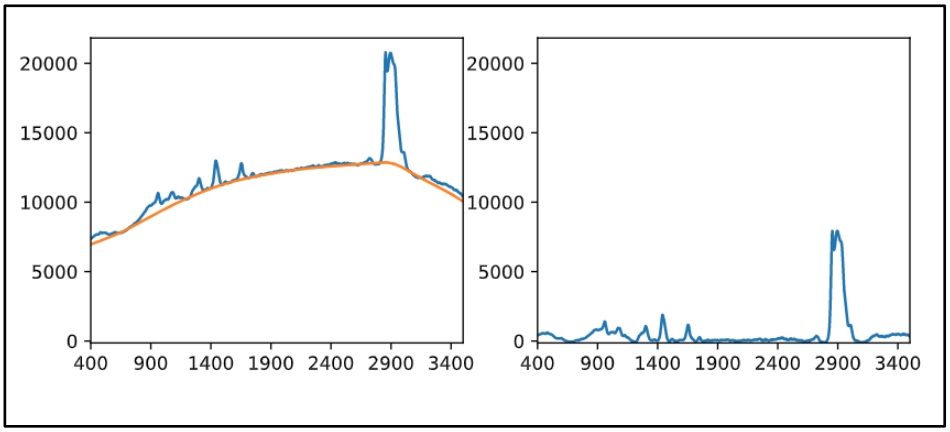
Pre-processing of spectral data.

Individual examples of the baseline correction utilised for normalisation of spectra. A, cortex; B, primary spongiosa; C, proximal metaphysis. Blue line, raw data; Orange line, average baseline. The right figure shows the converted spectrum.

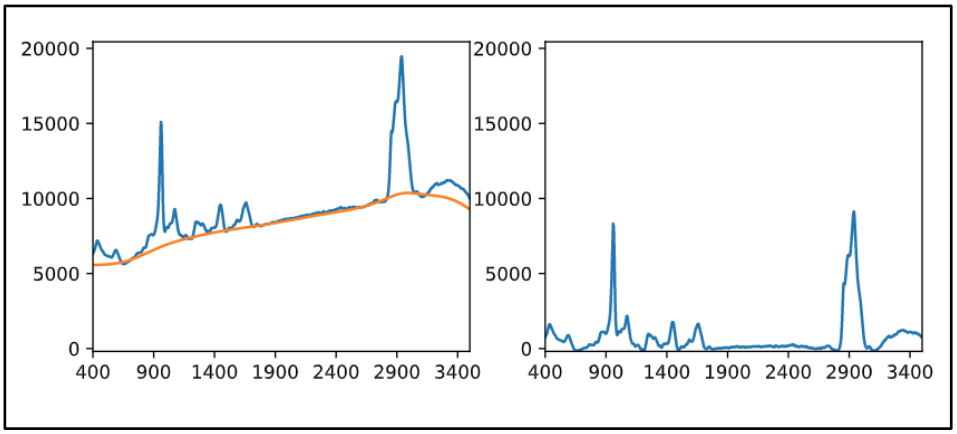
A



B



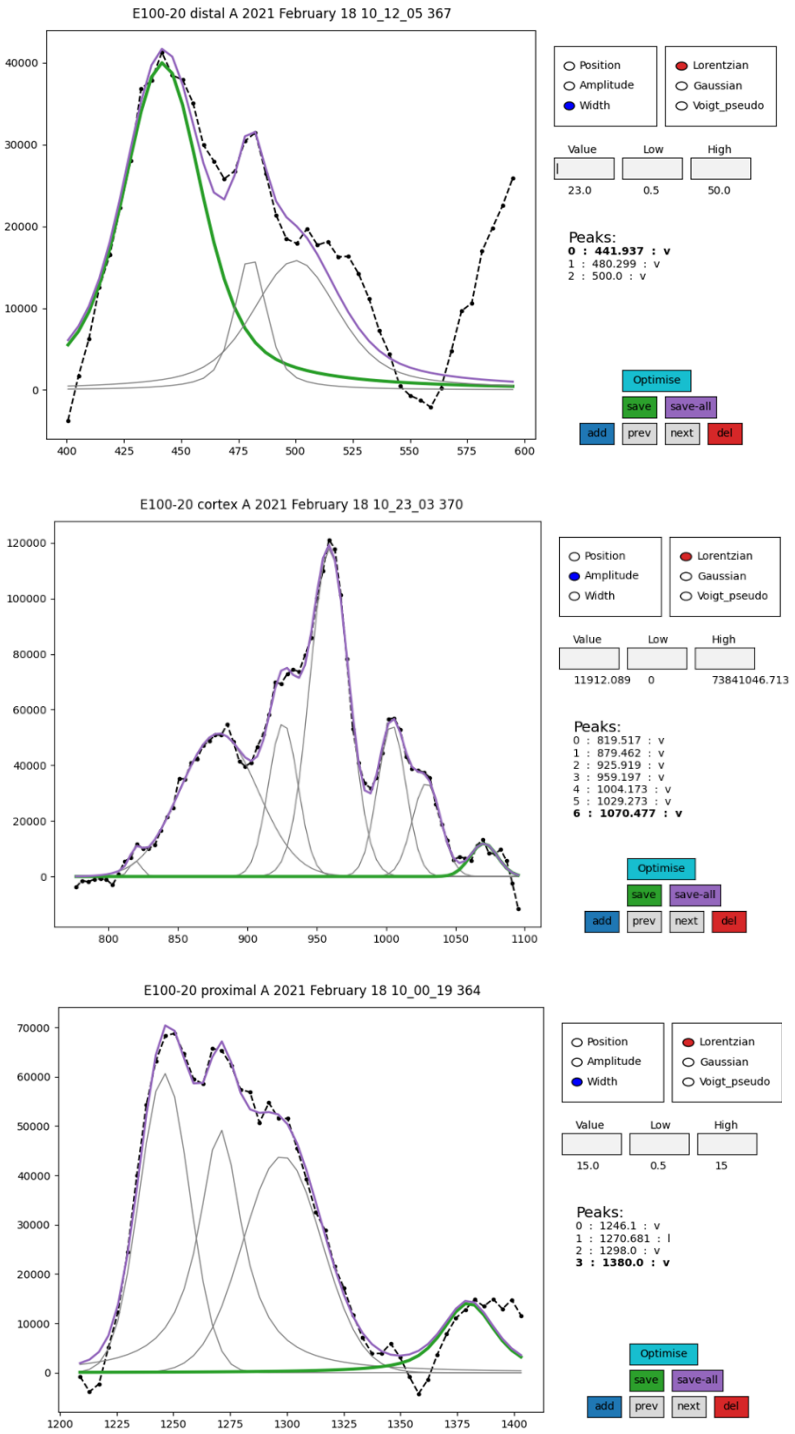
C



Appendix I

Peak-fitting of spectra.

Three working spectral ranges are shown that include at least one of the peaks selected for study with the most significant spectra in the working region. Top figure: spectral range from ~400 to 650 cm^{-1}, centre figure : from ~800 to 1100 cm^{-1}, and bottom figure: from ~1200 – 1~~400~~ ~~c~~m^{-1}. : spectral amplitude for each peak included in the working range, least square optimisation line ( ).



Appendix J

Table J.1 Instrument configuration details and settings for collagen crosslink analysis.

LC system	Dionex UltiMate™ LPG-3400RS Rapid Separation Quaternary Pump (ThermoFisher Scientific, USA).
Mass spectrometer	Q Exactive™ Focus (ThermoFisher Scientific, Bremen, Germany)
Ionisation source	HESI-II (ThermoFisher Scientific, USA)
Analytical column	Cogent Diamond Hydride™ HPLC column, 2.2 µm particle size, 2.1 mm inner diameter, 150 mm length, 100 Å pore size (PM Separations NZ Ltd, Hamilton, New Zealand)
Flow rate	Analytical column: 0.4 mL/min
Column oven temperature	40°C
Gradient	80 - 10 % B for 3 mins, 2.5 min hold at 10 % B, 10 - 80 % B for 1 min, 3 mins equilibration at 80 % B
Buffers	A: 0.1 % Formic acid/water B: 0.1 % Formic acid/acetonitrile

Table J.2 Mass spectrometer source settings for collagen crosslinks analysis and collagen crosslink detection.

Crosslink analysis		**Crosslink detection**	
Capillary temperature	350 °C	Resolution	35,000
S-Lens RF level	50%	Isolation window	1.0 m/z
Polarity	Positive	Default charge	1
Source voltage	4.0 kV	AGC target	$2x10^4$
Sheath gas flow rate	35 L/min	Maximum injection time	Auto
Aux gas flow rate	6 L/min	Minimum AGC target	$8x10^3$
Aux gas heater temperature	300 °C	Number of micro-scans	1
		Spectrum data type	Profile

Table J.3 Inclusion list for parallel reaction monitoring analysis. The table shows accurate masses for all crosslinks.

Crosslink	Mass (m/z)	CS [z]	Polarity	Start (min)	End (min)	CE
DHLNL	308.1816	1	Positive	5.0	7.0	24
HLNL	292.1867	1	Positive	5.0	7.0	24
HHL	223.1237	1	Positive	3.0	7.5	20
HHMD	287.6635	2	Positive	5.0	8.5	12
DPD	413.2030	1	Positive	5.0	8.5	27
PYD	429.1979	1	Positive	5.0	8.0	30

DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HHL, histidinohydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; DPD, deoxypyridinoline; PYD, pyridinoline.

Appendix K

Individual results of the concentration of Cu in liver, serum and bone, and liver concentration of Mo, Zn, Fe, and Cd in 26 heifers with humeral fractures (affected) and 14 unaffected heifers (control).

Case	Fracture status	LiCu[a]	SeCu[b]	BoCu[c]	Mo[c]	Zn[c]	Fe[c]	Cd[c]
1	Affected	774	26.7	0.40	1.70	86.16	125.78	0.12
2	Affected	763	22.3	0.72	0.89	97.08	379.40	0.10
3	Affected	884	25.1	1.01	0.57	87.85	52.91	0.09
4	Affected	23	31.4	0.39	0.77	51.42	178.14	0.05
5	Affected	1540	23.1	0.86	1.06	62.99	55.13	0.02
6	Affected	56	3.2	0.43	1.07	78.92	89.89	0.05
7	Affected	19	9.7	0.64	0.75	50.31	119.52	0.03
8	Affected	21	5.2	0.64	0.71	42.17	125.22	0.03
9	Affected	22	26.1	0.46	1.09	68.54	115.12	0.04
10	Affected	139	15.4	0.38	1.03	58.62	67.64	0.05
11	Affected	34	5.5	0.67	0.97	71.68	143.45	0.04
12	Affected	38	13.1	0.69	0.68	52.91	73.60	0.02
14	Affected	60	12	0.83	0.82	70.47	66.54	0.02
15	Affected	145	17.4	0.78	1.06	42.20	49.94	0.04
16	Affected	105	15.4	1.19	1.00	87.84	41.89	0.05
17	Affected	368	14.1	0.61	1.06	37.60	48.13	0.02
18	Affected	35	12.7	0.72	0.83	70.33	114.83	0.04
19	Affected	32	19.7	0.75	1.03	88.54	33.26	0.09
20	Affected	40	13.9	0.40	0.91	52.32	109.93	0.04
21	Affected	33	7	0.67	1.01	33.06	71.21	0.07
22	Affected	33	16	0.77	1.08	81.07	129.62	0.07
23	Affected	44	19	1.57	1.23	50.75	181.26	0.06
24	Affected	27	9.6	0.41	1.50	49.91	62.55	0.09
25	Affected	38	12.6	0.79	1.23	48.36	98.35	0.10
26	Affected	115	12.2	0.58	1.24	44.67	186.98	0.04
27	Control	790	n/a	0.72	1.31	35.44	63.65	0.04

28	Control	485	n/a	0.25	0.73	40.41	54.18	0.05
29	Control	323	n/a	0.26	0.76	104.61	133.73	0.08
30	Control	108	n/a	0.22	1.58	49.97	106.30	0.05
31	Control	893	n/a	0.41	0.89	49.50	179.37	0.03
32	Control	399	n/a	0.41	1.26	77.95	232.80	0.05
33	Control	168	n/a	0.49	0.47	99.31	197.42	0.03
34	Control	365	n/a	0.33	1.35	44.64	178.40	0.02
35	Control	482	n/a	0.57	0.78	162.66	299.47	0.04
36	Control	883	n/a	0.45	1.75	44.08	80.40	0.06
37	Control	635	n/a	0.61	1.31	56.39	83.92	0.06
38	Control	722	n/a	0.58	0.68	65.44	136.55	0.08
39	Control	260	n/a	0.45	0.87	58.77	87.36	0.02
40	Control	163	n/a	0.40	1.28	37.85	68.16	0.04

[a]µmol/kg, micromoles per kilogram

[b]µmol/L, micromoles per litre

[c]mg/kg, microgram per kilogram

LiCu, liver Cu concentration; SeCu, serum Cu concentration; BoCu, bone Cu concentration; n/a, not applicable.