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Inherited Rickets

in

Corriedale Sheep

A thesis presented in partial fulfilment
of the requirements for the degree of
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Keren Elizabeth Dittmer
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ABSTRACT

Inherited rickets of Corriedale sheep is a newly discovered skeletal disease of sheep with simple autosomal recessive inheritance. The clinical signs resemble rickets in other species and include decreased growth rate, thoracic lordosis and angular limb deformities. Radiographic features include physeal thickening, blurred metaphyseal trabeculae and thickened porous cortices. Computed tomography scanning of long bones reveals increased bone mineral content and cortical area, but decreased bone mineral density. Gross lesions include segmental thickening of physes, growth arrest lines, collapse of subchondral bone of the humeral head, thickened cortices and enthesophytes around distal limb joints. Microscopically there is persistence of hypertrophic chondrocytes at sites of endochondral ossification, inappropriate and excessive osteoclastic resorption, microfractures and wide, unmineralised osteoid seams lining trabeculae and filling secondary osteons.

Affected sheep are persistently hypophosphataemic and hypocalcaemic. Normal serum 25-hydroxyvitamin D_3 concentration accompanied by a two-fold elevation in 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) suggested a defect in end-organ responsiveness to vitamin D as a likely mechanism, but this was not supported by *in vitro* studies using cultured skin fibroblasts. These studies revealed normal vitamin D receptor function and the presence of 24-hydroxylase mRNA in cells from affected sheep, even without induction by $1,25(OH)_2D_3$. Inappropriate overexpression of 25-hydroxyvitamin D_3-24-hydroxylase, the enzyme that breaks down active vitamin D, is therefore considered the probable cause of inherited rickets in Corriedale sheep. Such a

mechanism has not previously been described as a cause of inherited rickets in humans or other animal species. Treatment of affected sheep with high oral doses of vitamin D_3 weekly for 3 months showed a trend towards increased bone mineral density, thus supporting an intact vitamin D receptor. Preliminary studies on immune function revealed reduced numbers of CD4+ and CD8+ lymphocytes and reduced interferon-γ production by lymphocytes stimulated with parasite antigen.

This new form of inherited rickets may be widespread in the New Zealand Corriedale sheep population and has considerable potential as a model for studying aspects of vitamin D metabolism.

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Glossary

$1,25(OH)_2D$	1,25-dihydroxyvitamin D, active vitamin D, calcitriol
25(OH)D	25-hydroxyvitamin D, calcidiol
24-hydroxylase	25-hydroxyvitamin D-24-hydroxylase, CYP24, CYP24A1
$[^3H]$ $1,25(OH)_2D_3$	tritium labelled 1,25-dihydroxyvitamin D_3
ADHR	autosomal dominant hypophosphataemic rickets
AF2	ligand dependent activation function 2
alopecia	lack of hair or wool
ALP	alkaline phosphatase
ankylosis	fusion of joint
annexin-V	cell surface marker of apoptosis
ANOVA	analysis of variance
apoptosis	programmed cell death
ARHR	autosomal recessive hypophosphataemic rickets
basophilic	increase in blue staining
BMC	bone mineral content
BMD	bone mineral density
cAMP	cyclic adenosine monophosphate
CaSR	calcium sensing receptor
cholecalciferol	vitamin D_3, (3β,5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3-ol
ConA	*Concanavalia ensiformis* type IV-S
chondrocyte	cartilage cell
craniotabes	reduction in mineralisation of the skull, with abnormal softness of bone
CYP24	25-hydroxyvitamin D-24-hydroxylase, CYP24A1
CYP27B1	25-hydroxyvitamin D-1α-hydroxylase, 1α-hydroxylase
DEXA	dual energy x-ray absorptiometry
diaphysis	main or midsection (shaft) of a long bone
DMP1	dentin matrix protein 1
endosteum	tissue lining the medullary cavity of a bone
enthesophyte	calcification of a muscle or ligament attachment to bone
eosinophilic	increase in pink/red staining

epiphysis	the end of a long bone, separated from the shaft by the physis
ergocalciferol	vitamin D_2, (3β,5Z,7E,22E)-9,10-secoergosta-5,7,10(19),22-tetraen-3-ol
FGF	fibroblast growth factor
fibrous osteodystrophy	lesion where fibrous tissue replaces resorbed bone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
heterozygous	different alleles at one locus
HHRH	hereditary hypophoshataemic rickets with hypercalciuria
HVDRR	hereditary vitamin D-resistant rickets
hypercalcaemia	higher than normal serum calcium
hypercalciuria	excess of calcium in the urine
hyperphosphataemia	higher than normal serum phosphate
hyperplasia	increased formation of new cells
hypocalcaemia	lower than normal serum calcium
hypophosphataemia	lower than normal serum phosphate
hypoplasia	incomplete growth of a tissue
hypotonia	abnormally decreased strength
IFN-γ	interferon-gamma
lordosis	downward curvature of the spine
LPS	lipopolysaccharide
lymphopenia	lower than normal number of lymphocytes in the blood
MEPE	matrix extracellular phosphoglycoprotein
metaphysis	area of trabecular bone and thin cortex in between the epiphysis and diaphysis, consisting of primary and secondary spongiosa
monocytopenia	lower than normal number of monocytes in the blood
mRNA	messenger ribonucleic acid
myelofibrosis	replacement of bone marrow by fibrous tissue
nephrocalcinosis	deposition of calcium phosphate in the renal tubules
olsen P	test for soil phosphorus levels
OPG	osteoprotegerin
osteoblast	mesenchymal bone cell that makes osteoid
osteocalcin	non-collagenous bone protein
osteoclast	multinucleated bone cell that resorbs bone
osteogenesis imperfecta	inherited defect in type I collagen formation, leading to fragile bones

osteoid	matrix of collagen and non-collagenous bone proteins produced by osteoblasts
osteomalacia	impaired mineralisation of bone, leading to softening and accumulation of excess osteoid
osteopetrosis	hereditary disease that results in abnormally dense bone and retention of the primary spongiosa
osteoporosis	pathological loss of bone but the remaining bone is structurally normal
periosteum	connective tissue covering the outside of bones
phagocytosis	process of engulfing and destroying of bacteria and other foreign material
PHEX	phosphate-regulating gene with homologies to endopeptidases on the X-chromosome
phosphaturia	excess phosphate in the urine
physis	cartilaginous growth plate, plural=physes
pQCT	peripheral quantitative computed tomography
primary spongiosa	newly-formed bone beneath the physis with calcified cartilage core
PTH	parathyroid hormone
rachitic rosary	enlarged costochondral junctions
RANK	receptor activator of NF-κB
RANKL	receptor activator of the NF-κB ligand
recessive gene	gene that expresses itself in the homozygous state, but not in the presence of a dominant allele
rickets	bone disease due to a failure of endochondral ossification and lack of mineralisation of newly formed osteoid
RT-PCR	reverse transcriptase polymerase chain reaction
Runx2	runt related transcription factor 2
RXR	retinoid X receptor
sclerosis	hardening
secondary hyperparathyroidism	abnormally increased secretion of PTH as a result of either nutrtional deficiency of calcium or renal failure
secondary spongiosa	formed when the primary spongiosa undergoes remodelling
sFRP4	secreted frizzled-related protein 4
SMAD	mothers against decapentaplegic homolog
SSI	stress-strain index
SUG1	suppressor for gal, a regulatory component of the 26S proteosome

TGF-β	transforming growth factor-β
TRPV 5,6	transient receptor potential vanilloid 5, 6, calcium channels
valgus	deformity where the angulation is away from the midline of the body
varus	deformity where the angulation is towards the midline of the body
VDDR I	vitamin D-dependent rickets type I, also called pseudovitamin D-deficiency rickets, due to a defect in 1α-hydroxylase enzyme
VDDR II	vitamin D-dependent rickets type II, also called hereditary vitamin D-resistant rickets, due to a defect in vitamin D receptor
VDR	vitamin D receptor
VDRE	vitamin D-response element
VEGF	vascular endothelial growth factor
voxel	volume element, region in a tissue slice that corresponds to a pixel in an image
XLH	X-linked hypophosphataemic rickets
Wnt	wingless-ints

Chapter 1

Review of the Literature

1.1 Introduction

Metabolic bone diseases are a common and debilitating group of diseases that affect both humans and animals. Osteoporosis is by the far the most common metabolic bone disease of humans. It is thought that 30% of post-menopausal women may have osteoporosis and the societal cost of treating the disorder for men and women in New Zealand is likely to be around $NZ 3.4 million/year (Lane, 1996). Other metabolic bone diseases include secondary hyperparathyroidism, scurvy (vitamin C deficiency), osteomalacia and rickets.

Rickets is a classic metabolic bone disease of humans and animals, first described in the 1st and 2nd centuries (Pettifor, 2005; Rajakumar, 2003). With the discovery that vitamin D could prevent rickets, the prevalence of this disease in developed countries plummeted, however rickets still occurs, even in New

Zealand. In 1998 at one hospital in New Zealand, 18 children were diagnosed with rickets; of these 66% were of Indian ethnic origin, and 16% of Polynesian or Maori origin (Blok *et al*., 2000). Rickets in animals is only intermittently reported in New Zealand, with three reports in the last 20 years (Hill *et al*., 1994; Thompson and Cook, 1987; Thompson and Robinson, 1989). The prevalence of rickets and vitamin D insufficiency is increasing in people of all ages in the developed world, partially due to decreased sunlight exposure and widespread sunscreen usage (Holick, 2003).

Rickets is due to a failure of calcification of osteoid and the cartilage of the growth plate (Klein, 1999) . Osteomalacia is also caused by a failure of newly-formed osteoid to mineralise, but occurs in adults after closure of growth plates (Klein, 1999).

The most common aetiology of rickets is nutritional deficiencies of either dietary vitamin D or phosphorus (Thompson, 2007) . Other causes are genetic in nature and include hypophosphataemic rickets, vitamin D-dependent rickets type I (VDDR I) and hereditary vitamin D-resistant rickets (HVDRR) (Eicher *et al*., 1976; Fox *et al*., 1985; Godfrey *et al*., 2005; Whyte, 2002) .

A skeletal disease with features of rickets has recently been identified in Corriedale sheep. This thesis will present a detailed investigation of the genetic and phenotypic features of the disease and explore the disease mechanism. The discovery of this disease provides an opportunity to study a possibly new hereditary skeletal disorder of sheep.

This review of the literature will give an overview of bone formation and vitamin D metabolism, describe the different rachitic diseases in humans and animals, followed by an outline of the aims of the thesis.

1.2 Bone Formation

Bone formation occurs by one of two methods. The flat bones of the skull, part of the mandible and the clavicle form by intramembranous ossification, while most other bones form by endochondral ossification (McCarthy and Frassica, 1998a).

The developing skeleton is first apparent as condensations of undifferentiated mesenchymal cells in the approximate shape and location of the future skeleton (Olsen, 1999; Wagner and Karsenty, 2001). In intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts (Wagner and Karsenty, 2001) and start random deposition of type I, and some type II and III collagen fibrils. This is followed by secretion of collagen in a polarized manner (matrix secretion from one cell surface only) (Franz-Odendaal *et al*., 2006). Once the initial shape is formed, osteoblasts line up on the bone edge and deposit osteoid, increasing bone size appositionally (Franz-Odendaal *et al*., 2006; Hall, 2005).

In endochondral ossification the condensations of mesenchymal cells differentiate into chondrocytes, which form a primitive cartilage model (Wagner and Karsenty, 2001). Mesenchymal cells then form a bony sleeve around the centre of the cartilage model by intramembranous ossification,

called the perichondrial ring of Ranvier (Schwamm and Millward, 1995). At the same time, chondrocytes in the centre undergo hypertrophy followed by cell death and calcification (Milgram, 1990a). The combination of the bony sleeve, calcified cartilage matrix and primitive bone forms the primary ossification centre, and these occur within the cartilage model at genetically predetermined sites (McCarthy and Frassica, 1998a; Schwamm and Millward, 1995). As the cartilage model grows, the ossification centre also enlarges and the centre of the model is hollowed out by osteoclastic resorption to form the marrow cavity, with cartilage centres at either end (McCarthy and Frassica, 1998a; Milgram, 1990a). The physeal growth plate is formed in the region where the cartilage centres and bone meet (Milgram, 1990a). Interstitial growth occurs at the growth plates to increase the bone in length and width (Milgram, 1990a). At a genetically determined stage, secondary ossification centres form at the ends of tubular bones (Schwamm and Millward, 1995). The secondary ossification centre enlarges, until it is surrounded by a rim of cartilage comprising the articular cartilage and the growth plate (McCarthy and Frassica, 1998a; Schwamm and Millward, 1995). In humans, at birth only the proximal humerus, proximal tibia and distal femur contain secondary ossification centres (Milgram, 1990a). Apophyses or accessory centres of ossification do not contribute to growth in length, but form the bony protuberances such as the femoral trochanters (Milgram, 1990a).

The chondrocytes deposit an extracellular matrix, undergo proliferation forming parallel columns, then become hypertrophic and die (de Crombrugghe *et al.*, 2001). Other mesenchymal cells from the periosteum and perivascular tissue differentiate into osteoblasts, and with blood vessels and osteoclasts,

invade the zone of hypertrophic and dead chondrocytes (de Crombrugghe *et al*., 2001; McCarthy and Frassica, 1998a). Osteoblasts deposit a bony matrix (osteoid) consisting of 90% collagen (primarily type I collagen) and 10% non-collagenous proteins and proteoglycans using the cartilage matrix, partially resorbed by osteoclasts, as a framework (Baron, 1999; Wagner and Karsenty, 2001). There is a lag phase of approximately 10 days between matrix formation and calcification (Baron, 1999). The collagen in the matrix is initially arranged irregularly, and this is called woven bone (McCarthy and Frassica, 1998a). The new bone subsequently undergoes remodeling, the woven bone and cartilage remains being removed by osteoclasts and replaced with mature lamellar bone, where collagen is laid down in a regular, layered manner (Baron, 1999; McCarthy and Frassica, 1998a).

This progression of chondrocyte differentiation leads to the characteristic structure of the endochondral growth plate or physis, as shown in Figure 1.1, comprising the resting zone, proliferative zone, zone of hypertrophy and zone of provisional calcification (McCarthy and Frassica, 1998a; Schwamm and Millward, 1995). The primary spongiosa is located beneath the zone of provisional calcification, and contains newly formed bone with a calcified cartilage core (McCarthy and Frassica, 1998a). The secondary spongiosa is formed when the primary spongiosa undergoes remodeling (Baron, 1999) and together, the primary and secondary spongiosa form the metaphysis (McCarthy and Frassica, 1998a).

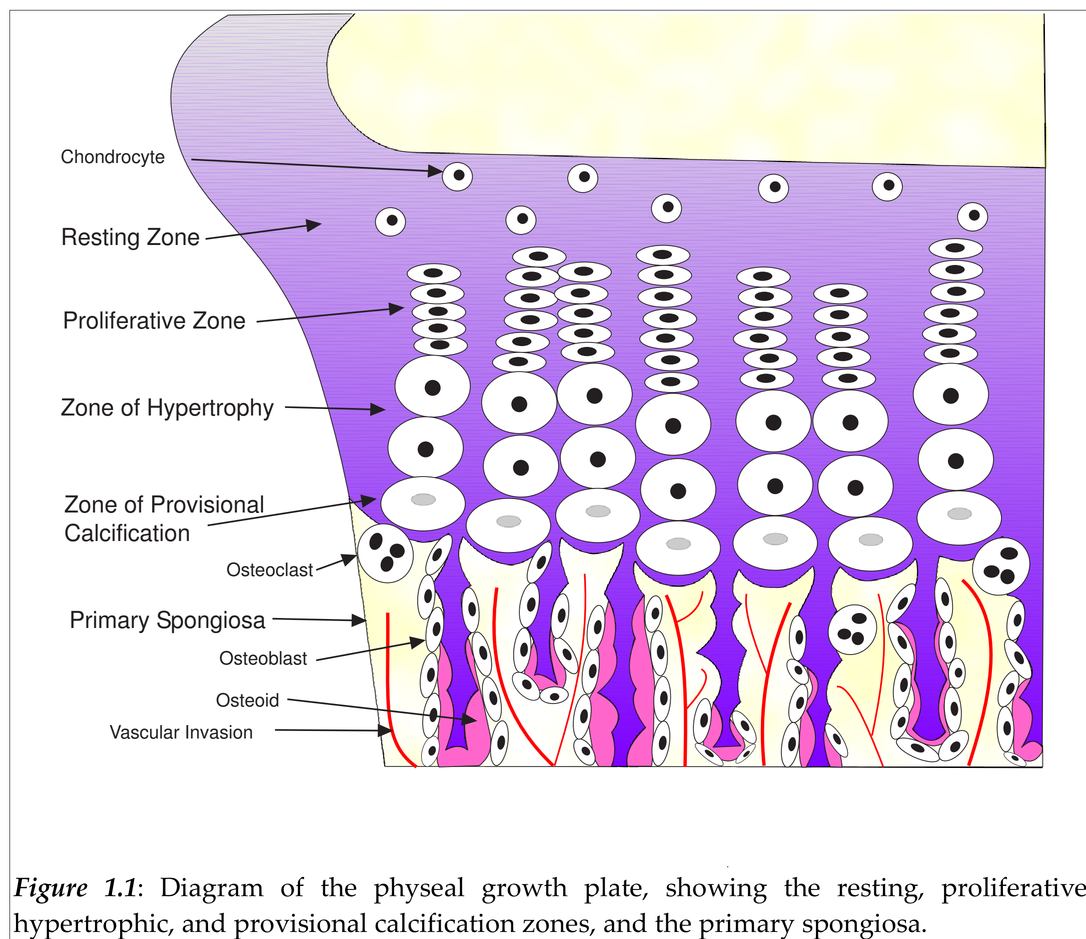


Figure 1.1: Diagram of the physeal growth plate, showing the resting, proliferative, hypertrophic, and provisional calcification zones, and the primary spongiosa.

At the edge of the growth plate is the ossification groove of Ranvier. Cells in this groove proliferate leading to an increase in diameter of the physis (Shapiro *et al.*, 1977). Growth in width of the diaphyseal cortex occurs by intramembranous ossification beneath the periosteum (Milgram, 1990a). Growth in bone length occurs by chondroblast proliferation, deposition of extracellular matrix, and chondrocyte hypertrophy (Hall, 2005; Woodard, 1997). Proliferation of chondrocytes only contributes a small amount to bone growth, 90% of growth in length being due to deposition of extracellular matrix and chondrocyte hypertrophy (Hall, 2005). Rates of growth vary between physes within a bone and between different long bones (Hall, 2005). Typically, the physes that grow the fastest also remain open longer, thereby contributing more

to the length of the bone (Farnum, 1994). In humans, 81% of humeral growth occurs at the proximal physis, whereas 75% of radial growth occurs at the distal physis (Farnum, 1994). Similarly in the sheep and goat, 75% of radial growth is from the distal physis, 82% of humeral growth is from the proximal physis, 64% of femoral growth is from the distal physis and 56% of tibial growth is from the proximal physis (Farnum, 1994). The fastest growing physes are most susceptible to damage from trauma or nutritional imbalances (Thompson, 2007).

In comparison to other species the physes of the sheep and rat remain active for a longer time after sexual maturity (Kilborn *et al*., 2002). The dog for example, reaches sexual maturity at 7-10 months, and growth plate closure (tibia and femur) occurs at 6-11 months (Kilborn *et al*., 2002). In the sheep, growth plate closure (metacarpal bone) does not occur until 17 months of age, while sexual maturity is reached at 5.5 months of age (Kilborn *et al*., 2002; Oberbauer *et al*., 1989). As a result, the bones of sheep remain susceptible to nutritional imbalances for a longer period than other species.

Throughout life, bone is continually being remodeled, removed and rebuilt in so-called bone metabolic units. It is estimated that resorption of bone matrix within the bone metabolic unit takes about 10 days, and subsequent deposition and repair takes approximately 3 months (McCarthy and Frassica, 1998a; Mundy, 1999; Pogoda *et al*., 2005). The bone metabolic units consist of two groups; osteoclasts that resorb bone, and osteoblasts that replace bone

(McCarthy and Frassica, 1998a). Remodeling of bone can be divided into five phases: quiescent, activation, resorption, formation and mineralisation.

Activation occurs when osteoblasts lining the bone surface retract and the endosteal membrane is digested by collagenase released from osteoclasts (Hernandez-Gil *et al*., 2006; McCarthy and Frassica, 1998a; Mundy, 1999). The exposed, mineralised bone surface attracts bone marrow macrophages, which differentiate into osteoclasts and form a resorption cavity as they remove the mineral and osteoid matrix (Hernandez-Gil *et al*., 2006; McCarthy and Frassica, 1998a). Osteoblasts contain a surface ligand called RANKL (receptor activator of NF-κB ligand) which can bind to osteoclasts via RANK (receptor) or a decoy receptor called OPG or OCIF (osteoprotegerin) (Suda *et al*., 2003). When RANKL binds to RANK it induces differentiation and maturation of osteoclast progenitor cells to osteoclasts (Dusso *et al*., 2005; Suda *et al*., 2003). RANKL is an absolute requirement for osteoclastogenesis (Zaidi, 2007). RANKL is stimulated by 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) via vitamin D-responsive elements (VDREs) on the RANKL promoter. OPG production is inhibited by $1,25(OH)_2D_3$. Stimulation of RANKL and inhibition of OPG leads to increased osteoclastic activity (Kitazawa *et al*., 2003; Kondo *et al*., 2004). Once resorption is complete, osteoclasts detach from the bone surface, and either relocate to a new resorption site or undergo apoptosis (Vaananen, 2005). After the departure of osteoclasts, the bone removal process is completed by macrophages (Hernandez-Gil *et al*., 2006; Mundy, 1999). Degradation of the matrix leads to the release of growth factors, including transforming growth factor-β (TGF-β), platelet derived growth factor and insulin-like growth factors I and II (Hernandez-Gil *et al*., 2006; Mundy, 1999). At the end of the resorption phase,

preosteoblasts secrete a collagen-poor and proteoglycan-rich substance that acts to "glue" new bone onto the surface of the resorption cavity; this remains visible in haematoxylin and eosin (H&E) stained sections as a deeply basophilic remodeling or cementing line (McCarthy and Frassica, 1998a; Mundy, 1999).

During the formation phase, preosteoblasts are attracted to the site by growth factors, which also stimulate cell proliferation (Hernandez-Gil *et al*., 2006). Three pathways regulate osteoblast maturation and bone formation (Zaidi, 2007). The major regulator of osteoblasts is Runt-related transcription factor-2 (Runx2), and this transcription factor is required for differentiation of an osteoprogenitor cell to an osteoblast (Bialek *et al*., 2004; Marie, 2008). Runx2 may be activated by Smad (mothers against decapentaplegic homolog) after its phosphorylation by bone morphogenic proteins or TGF-β (Zaidi, 2007). Runx2 may also be phosphorylated directly by parathyroid hormone (PTH) or fibroblast growth factors (FGFs) (Zaidi, 2007). Osterix is also required for osteoblast differentiation, and is activated by NFAT2 (nuclear factor for activated T cells 2) (Koga *et al*., 2005). The third pathway is Wnt-β-catenin (wingless-ints) signaling, where activated β-catenin stimulates osteoblast differentiation (Bodine *et al*., 2004). Active osteoblasts express alkaline phosphatase and are anchored by cadherin-11 and N-cadherin to newly formed osteoid (Zaidi, 2007). The active osteoblasts secrete osteoid, a matrix comprising type I collagen and non-collagenous matrix proteins, to fill the resorption cavities (Hernandez-Gil *et al*., 2006; Mundy, 1999; Zaidi, 2007). Once a resorption cavity is filled, osteoblast activity ceases, possibly due to the presence of TGF-β (Mundy, 1999). Mineralisation of the new osteoid then

begins, and once completed the bone re-enters the quiescent phase (Hernandez-Gil *et al*., 2006).

The synchronising of bone resorption and formation in the bone metabolic unit is called coupling and is thought to be due to local signaling, although the mechanism is unclear (Pogoda *et al*., 2005). Recent work has suggested that bone remodeling may be controlled centrally by the hormone leptin, which is produced by adipocytes (Pogoda *et al*., 2005; Takeda, 2005). Leptin is thought to be an inhibitor of bone formation, as well as being involved in prevention of obesity (Pogoda *et al*., 2005; Takeda, 2005). Experiments in mice indicate that leptin may act via the hypothalamus and the sympathetic nervous system (Ducy *et al*., 2000; Takeda *et al*., 2002). In support of this theory, catecholamine producing nerves have been found near osteoblasts, which also express noradrenaline responsive β_2-adrenergic receptors (Takeda *et al*., 2002). Neuropeptide Y and Y receptors in the hypothalamus and on osteoblasts are also thought to be involved in inhibition of bone formation (Elefteriou, 2008). It is likely that several pathways, both local and central, are involved in the control of bone remodeling (Pogoda *et al*., 2005).

Mineral accounts for 50-70% of adult mammalian bone, and provides mechanical stiffness and load bearing strength (Lian *et al*., 1999a). Early studies indicated that the mineral was a form of hydroxyapatite, but a number of researchers have failed to find hydroxy groups in the crystal (Loong *et al*., 2000; Rey *et al*., 1995). The basic crystal is now thought to be a carbonated apatite or dahllite, $[Ca_5(PO_4,CO_3)_3]$ (Nyman *et al*., 2005; Weiner and Wagner, 1998). Often

the crystals contain impurities such as magnesium, fluoride, sodium and citrate (Lian *et al*., 1999a; McCarthy and Frassica, 1998a).

Mineralisation may start in extracellular matrix vesicles (in woven bone) or directly within collagen (in lamellar bone)(Nyman *et al*., 2005). Initially, water fills the spaces between the collagen molecules, and when crystals form the water is displaced (Nyman *et al*., 2005). The collagen fibrils contain gaps or hole zones where the initial accumulation of crystals occurs (Glimcher, 1989). As they grow, the crystals move into the overlap zone, and then start to compress the collagen triple helix, and eventually join together to form extended sheets (Weiner and Wagner, 1998). Non-collagenous proteins, such as bone sialoprotein, osteonectin, osteopontin and osteocalcin, are deposited by osteoblasts onto collagen fibrils and are involved in the control of calcification (Wiesmann *et al*., 2005). Bone sialoprotein and osteonectin are thought to be initiators of calcification, while osteopontin may control mineralisation by inhibiting crystal formation, and osteocalcin by delaying nucleation and crystal maturation (Hunter and Goldberg, 1993; Hunter *et al*., 1996; Termine *et al*., 1981).

Matrix vesicles are extracellular membrane bound particles 100 nM in diameter (Anderson, 2003). Calcium ion channels such as annexin, and phosphatases such as alkaline phosphatase are present in the matrix vesicle membrane and are involved in crystal nucleation (Anderson, 2003; Balcerzak *et al*., 2003). Apatite crystals initially build up on the inner membrane of the matrix vesicles, and then internally (Anderson, 1989). The mineralisation then advances beyond

the matrix vesicle membrane, and into the extracellular fluid, where crystal propagation continues (Anderson, 2003; Wiesmann *et al*., 2005). The extracellular calcium (Ca^{2+}) and phosphate (PO_4^{3-}) levels are generally adequate to allow continuous crystal proliferation, with the pre-formed crystals acting as a template (Anderson, 2003).

At least three vitamin K-dependent proteins or γ-carboxyglutamate (Gla) proteins are present in bone, osteocalcin (bone Gla protein), matrix Gla protein and protein S (Bugel, 2003). Osteocalcin is produced solely by osteoblasts, odontocytes and hypertrophic chondrocytes and is the main non-collagenous protein present in bone matrix, while matrix Gla protein and protein S are also produced in other soft tissues (Bugel, 2003; Calvo *et al*., 1996). Vitamin K is a co-enzyme for glutamate carboxylase, which converts glutamic acid residues in the proteins to Gla (Bugel, 2003; Lian and Friedman, 1978). The Gla residues attract Ca^{2+} and assist with hydroxyapatite formation; however due to the structure of osteocalcin and its weak dissociation constant, the calcium remains available for other interactions (Bugel, 2003; Hauschka *et al*., 1989). Osteocalcin is also dependent on $1,25(OH)_2D_3$. The binding of $1,25(OH)_2D_3$ to vitamin D-response elements (VDREs) in the *osteocalcin* gene promotes transcription of the gene and synthesis of osteocalcin (Lian *et al*., 1999b). Vitamin D is therefore required for osteocalcin synthesis, while vitamin K is needed for its activation (Bugel, 2003).

While the vast majority of osteocalcin is incorporated into bone, a small amount makes it into the circulation (Calvo *et al*., 1996). Serum osteocalcin may be

considered a biomarker of osteoblast function as its presence in serum is a result of bone matrix production, not bone resorption (Price *et al*., 1981). Serum osteocalcin is greater in children than in adults and it peaks at puberty, due to the speed of growth at that time (Calvo *et al*., 1996). A circadian rhythm is found with serum osteocalcin levels, which peak after midnight, and hit a nadir after midday (Calvo *et al*., 1996; Hauschka *et al*., 1989). Serum osteocalcin concentrations are elevated in diseases with high bone production, such as Paget's disease, hyperthyroidism, hyperparathyroidism, high turnover osteoporosis; and decreased in low bone turnover diseases, such as hypoparathyroidism, hypothyroidism and low turnover osteoporosis (Hauschka *et al*., 1989). Serum osteocalcin in rickets may be low normal or elevated, possibly due to differences in the degree of vitamin D depletion (Baroncelli *et al*., 2000; Daniels *et al*., 2000; Scariano *et al*., 1995; Wharton and Bishop, 2003). Concentrations of osteocalcin are particularly elevated during the treatment of rickets (Baroncelli *et al*., 2000; Daniels *et al*., 2000). The cells of patients with hereditary vitamin D-resistant rickets (HVDRR) experimentally show an inability to transcribe the *osteocalcin* gene, even at high vitamin D doses (Kristjansson *et al*., 1993).

1.3 Biology of Vitamin D

1.3.1 Activation of Vitamin D

Vitamin D is available from two sources, isomerisation of 7-dehydrocholesterol (7-DHC) in the skin to vitamin D_3 following exposure to ultraviolet light, or from ingestion of vitamin D_2 or D_3 in the diet. Only a few foods, such as cod liver oil, and fatty fish like salmon and sardines, naturally contain high

concentrations of vitamin D_3 but many foods now have vitamin D added artificially (Dusso *et al*., 2005). Vitamin D_2 is present in certain plants due to conversion of ergosterol to vitamin D_2 by ultraviolet light (DeLuca, 2004).

Ultraviolet light in the 270-315 nm range is required for the conversion of 7-DHC to previtamin D_3, which then undergoes thermal isomerisation to vitamin D_3 over the course of three days (Holick, 1981; Holick, 1987; Jones *et al*., 1998). Both vitamin D_2 and D_3 are inactive. Figure 1.2 shows the steps involved in conversion of vitamin D to its active form, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). Vitamin D_3 preferentially binds to vitamin D-binding protein in the capillaries of the dermis and is transported to the liver (Holick, 1981). If there is excessive or prolonged sun exposure, previtamin D_3 photoisomerises to biologically inert tachysterol and lumisterol, which are sloughed off during normal skin turnover (Holick, 1981).

One of the factors that influences vitamin D_3 formation is the amount of pigment in the skin. Melanin vies with 7-DHC for ultraviolet photons, therefore a longer time in sunlight is required for maximum previtamin D_3 formation in dark-skinned individuals (Holick, 1981). Latitude is also a factor. Ultraviolet radiation is less at higher latitudes, particularly during winter when daylight hours are low. When the suns' altitude is less than 35°, there is insufficient penetration of ultraviolet light to convert 7-DHC to previtamin D_3 (Best and Taylor, 1939). In northern and southern areas of the globe, not only are there fewer sunshine hours, but the sun is closer to the horizon and more rays are absorbed by the thicker atmosphere at that point (Best and Taylor,

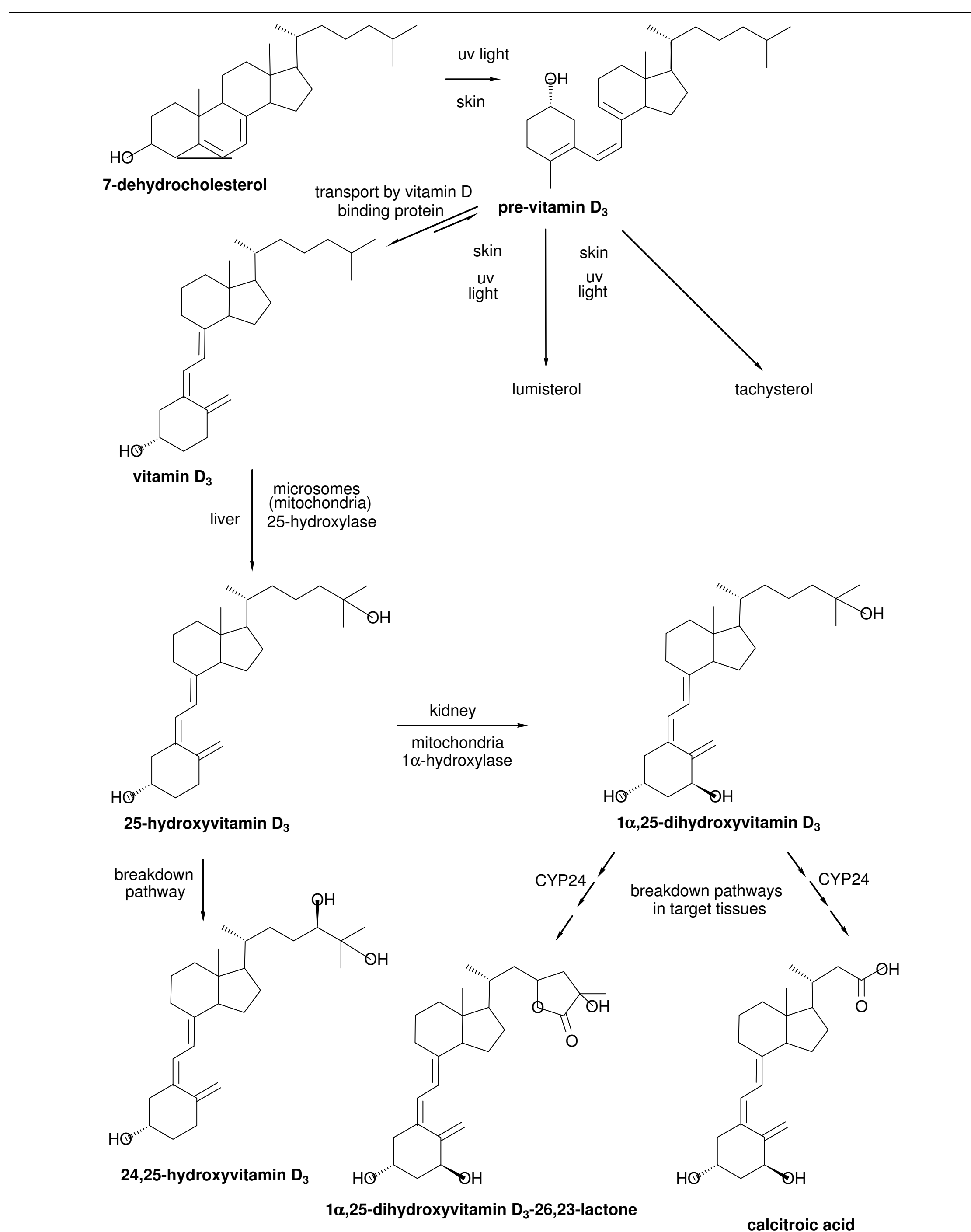


Figure 1.2: **Vitamin D synthesis, activation and breakdown**. Exposure of 7-dehydrocholesterol in the skin to ultraviolet irradiation leads to thermal isomerisation to vitamin D_3, which is transported by vitamin D-binding protein to the liver. Vitamin D_3 25-hydroxylation occurs in the liver by a cytochrome P450. This is followed by transport to the kidney and 1α-hydroxylation in the proximal convoluted tubules, to produce $1{,}25(OH)_2D_3$, the active form of vitamin D. Also included are the breakdown pathways catalysed by CYP24 cytochromes. Isomerisation of 7-dehydrocholesterol also produces tachysterol and lumisterol, which are biologically inert, and are sloughed off during normal skin turnover. (Holick, 1987)(Jones *et al.*, 1998)(Prosser and Jones, 2004)(Dusso *et al.*, 2005)

1939). Regions with a sun altitude of 35° or less in winter lie along latitude 31° and higher. Most regions of New Zealand lie within this zone. The differences between latitudes is illustrated by a study in South Africa, where in Cape Town during winter, formation of vitamin D at midday was one third of that formed in Johannesburg (Pettifor *et al*., 1996).

Not all animals have the ability to form vitamin D_3 in their skin. Cats and dogs have only 10% of the 7-DHC in their skin that rats do due to the presence of a 7-DHC-Δ^7-reductase, as such irradiation with ultraviolet light does not cause an increase in serum vitamin D concentration (Morris, 1999). Dogs and cats must therefore gain their vitamin D requirements from the diet. In their natural state, carnivores were able to meet this demand from the fat, liver and blood of their quarry (How *et al*., 1994). Pet dogs and cats now rely on being fed a complete and balanced diet to supply their vitamin D requirements. Herbivores on the other hand have evolved the ability to produce vitamin D_3 in response to ultraviolet irradiation of the skin (Hidiroglou *et al*., 1985). As would be expected, shorn sheep show greater increases in serum vitamin D_3 concentrations after ultraviolet radiation than unshorn sheep (Chaudhary and Care, 1985).

1.3.2 Hepatic 25-Hydroxylation

Circulating vitamin D is either stored in fat, or undergoes 25-hydroxylation in the liver. A number of hepatic cytochrome P450s have been postulated as carrying out 25-hydroxylation of vitamin D. CYP27A1, CYP3A4, CYP2R1 and CYP2J3 are possible candidates (Prosser and Jones, 2004). CYP2R1 is likely, as a mutation in the gene for *CYP2R1* has been found in a person with VDDR I

(Cheng *et al*., 2004). This step in vitamin D metabolism is not closely regulated. Blood 25(OH)D concentration is proportional to dietary intake and is therefore a useful measure of dietary consumption and/or skin production (Holick, 1981). Due to its strong association with vitamin D-binding protein, 25(OH)D_3 is very stable and is subsequently the main form of vitamin D in circulation (Jones *et al*., 1998). The next step in the process of vitamin D activation depends on the ionised calcium concentration. If ionised calcium concentration is low, renal 1α-hydroxylation of 25(OH)D occurs to produce the active form of vitamin D, but if adequate, 24-hydroxylation to an inactive metabolite occurs (Jones *et al*., 1998).

1.3.3 RENAL 1α-HYDROXYLATION

The enzyme responsible for 1α-hydroxylation is CYP27B1 (also known as CYP1a or 1α-hydroxylase), which contains a cytochrome P450, a ferredoxin and a ferredoxin reductase (Ghazarian *et al*., 1974; Jones *et al*., 1998; Prosser and Jones, 2004). Renal 1α-hydroxylase regulation is tightly controlled by the extracellular concentration of ionised calcium, which acts directly on 1α-hydroxylase activity and mRNA production, and indirectly via PTH (Dusso *et al*., 2005; Jones *et al*., 1998; Murayama *et al*., 1999; Prosser and Jones, 2004). Table 1.1 summarises the factors involved in regulation of 1α-hydroxylase activity. PTH itself regulates 1α-hydroxylase activity and mRNA production by cAMP pathways, and by directly stimulating the *1α-hydroxylase* gene promoter (Brenza *et al*., 1998; Murayama *et al*., 1998; Rost *et al*., 1981). Low serum phosphate concentration induces renal 1α-hydroxlyase activity independent of any changes in PTH or calcium concentrations. High serum phosphate concentration has its effect via the phosphatonins: fibroblast growth factor 23

(FGF23) and secreted frizzled-related protein 4 (sFRP4), which inhibit renal phosphate reabsorption and $1,25(OH)_2D_3$ formation (Berndt *et al.*, 2005). The klotho gene product is thought to be a negative regulator of *1α-hydroxylase* gene expression (Tsujikawa *et al.*, 2003). Feedback inhibition by $1,25(OH)_2D_3$ also occurs, mostly due to inhibition of PTH and cAMP and by inducing klotho gene product (Brenza *et al.*, 1998; Tsujikawa *et al.*, 2003).

***Table 1.1:* Regulation of 1α-hydroxylase**

Suppression	Stimulation
high serum calcium concentration	low serum calcium concentration
$1,25(OH)_2D_3$	low serum phosphate concentration
high serum phosphate concentration	parathyroid hormone
via FGF23, sFRP4, MEPE	via cAMP, gene transcription
klotho gene product	

$1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; FGF23 = fibroblast growth factor 23; sFRP4 = secreted frizzled-related protein 4; MEPE = matrix extracellular phosphoglycoprotein.

1.3.4 Breakdown Pathways

There are two main pathways responsible for the breakdown of active vitamin D, one of which involves C-24 oxidation to calcitroic acid (Jones *et al.*, 1998). CYP24 is the enzyme responsible for 24-hydroxylation and is capable of catalyzing multiple steps in the breakdown pathway (Beckman *et al.*, 1996). The *CYP24* gene contains two VDREs in its promoter, which allow $1,25(OH)_2D_3$ to up-regulate its expression (Zierold *et al.*, 1995). Increased serum phosphate also increases CYP24 activity and expression, and PTH suppresses it (Tanaka and DeLuca, 1973; Tanaka *et al.*, 1975). Calcitroic acid is the main end product and is excreted in the bile (Esvelt *et al.*, 1979). The other pathway involves conversion

of $1,25(OH)_2D_3$ to $1,25(OH)_2D_3$-26,23-lactone via a 23-hydroxylation, also catalysed by CYP24 (Prosser and Jones, 2004).

1.3.5 THE VITAMIN D RECEPTOR

The vitamin D receptor (VDR) is a member of the family of type II nuclear receptors for steroid hormones (Barsony and Prufer, 2002; Dusso *et al*., 2005). The VDR protein contains four main domains: ligand binding, heterodimerisation with the retinoid X receptor (RXR), binding of VDREs and enlistment of co-regulators (Dusso *et al*., 2005). The VDR is considered to be localised predominantly in the nucleus, even in the unoccupied state (Berger *et al*., 1988; Zanello *et al*., 1997). However, a recent study using a fluorescent-labelled ligand found that approximately 50% of the VDRs are present in the cytoplasm (Barsony *et al*., 1997). VDRs are present in most tissues, but their concentration is greatest in target tissues such as bone, kidney and intestine (Jones *et al*., 1998).

The gene for the human VDR is situated on chromosome 12 and consists of 11 (1A, B, C, and 8 other) exons (Faraco *et al*., 1989; Miyamoto *et al*., 1997a). Alternative splicing of exons 1A, B or C leads to at least three different VDR mRNAs (Miyamoto *et al*., 1997a). In exon 2, a polymorphism (FokI polymorphism) exists that leads to an alternative translation start site (Miyamoto *et al*., 1997a). These *VDR* gene polymorphisms exist naturally in the human population, possibly resulting in changes in susceptibility to various diseases. Some researchers have found changes in bone density in human patients with different *VDR* genotypes, while others have found no significant

differences (Alonso *et al*., 1998; Eisman, 1999; Ferrari *et al*., 1995; Hustmeyer *et al*., 1994). Similarly, there are inconsistent results in studies investigating possible links between *VDR* genotype and susceptibility to hyperparathyroidism (Alonso *et al*., 1998; Fernandez *et al*., 1997; Gomez *et al*., 1997; Howard *et al*., 1995).

Type II nuclear receptors require heterodimerisation with RXR (Figure 1.3) (Barsony and Prufer, 2002). Prior to heterodimerisation the VDR has low affinity for 1,25$(OH)_2D_3$. Dimerisation induces a conformational change that increases the VDR-RXR affinity for its ligand (Quack and Carlberg, 2000). Once 1,25$(OH)_2D_3$ (the ligand) binds, it induces a conformational change due to relocation of AF2 (ligand dependent activation function 2) helix 12 in the C-terminus end of the ligand binding domain (Dusso *et al*., 2005). This allows transport of cytoplasmic VDR along microtubules into the nucleus (Barsony and McKoy, 1992; Racz and Barsony, 1999).

In the nucleus, VDR-RXR binds to specific sequences called vitamin D-response elements (VDREs) present in the promoter area of the target gene. The DNA binding domain contains two zinc fingers that interact with the VDREs, causing a 55° bend in the DNA, the reason for which is unclear (Jehan-Kimmel *et al*., 1999). The most common VDRE, DR3, has a consensus sequence of AGGTCA separated by three nucleotides and then repeated; the RXR binds to the 5' site, and the VDR to the 3' site (Haussler *et al*., 1998).

As can be seen in Figure 1.3, nuclear co-activators interact with the RXR heterodimerisation domain and the AF2 domain to augment 1,25(OH)$_2$D$_3$ gene transcription activation (Dusso *et al.*, 2005). The conformational shift at AF2 after ligand binding, allows interaction with RNA polymerase II and transcription factors like transcription factor II B (Dusso *et al.*, 2005). Steroid receptor co-activator 1/p160 and CREB (cAMP response element binding) protein/p300 are VDR-nuclear co-activators with histone acetyl transferase activity (Rachez and Freedman, 2000). These co-activators loosen chromatin and expose the DNA, allowing recruitment of the vitamin D receptor interacting proteins-thyroid receptor hormone associated proteins (DRIP-TRAP) complex, which enhances ligand-dependent VDR-RXR transcription

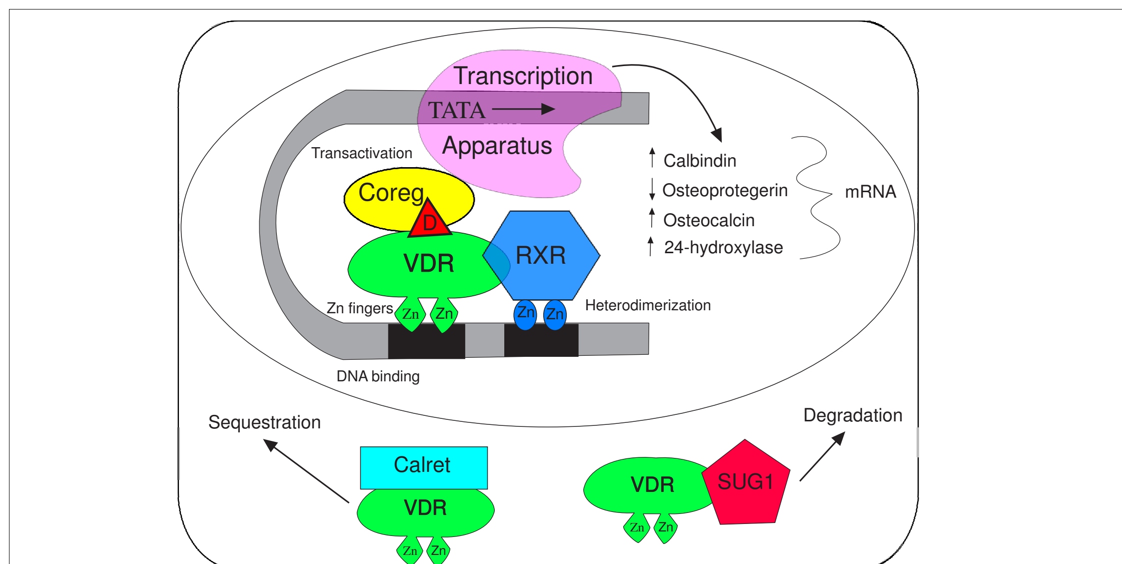


Figure 1.3: **Control of the Vitamin D receptor (VDR).** The 1,25(OH)$_2$D$_3$ (D) -VDR-retinoid X receptor (RXR) heterodimer binds to specific sequences in the promoter region of the DNA. The heterodimer attracts co-regulators (CoReg) and transcription factors that change the rate of transcription, leading to increased/decreased mRNA from vitamin D responsive genes like osteoprotegerin (OPG) and calbindin. The ligand-VDR interacts with SUG1 (suppressor for gal 1), leading to proteolysis. Calreticulin (Calret) interaction with VDR prevents transactivation. Revised from (Dusso *et al.*, 2005).

(Rachez and Freedman, 2000). Co-activators may work in combination or sequentially (Rachez and Freedman, 2000).

The VDR-RXR complex may also bind to negative VDREs, which engage co-repressors (Rachez and Freedman, 2000). Some, like silencing mediator for retinoid and thyroid receptors and nuclear receptor co-repressor (NCoR) may stabilise chromatin; although no direct evidence exists for the VDR and results have been extrapolated from thyroid receptors (Rachez and Freedman, 2000). Others, like ribosomal protein L7a, inhibit VDR-RXR heterodimerisation and VDR-RXR binding to VDREs (Berghofer-Hochheimer *et al.*, 1998). Ski-interacting protein may act as a co-repressor or co-activator depending on the relative levels of NCoR and CREB protein/p300 (Leong *et al.*, 2004). The VDR-ligand complex may also be targeted for degradation by interaction of AF2 with suppressor for gal (SUG1 - a regulatory component of the 26S proteasome), which leads to proteasome-mediated breakdown and down-regulation of transcription (Masuyama and MacDonald, 1998).

1.3.6 Functions of Vitamin D

The main target organs for vitamin D are: the intestine, bone, kidney and the parathyroid glands (Dusso *et al.*, 2005). Together with PTH, the primary function of vitamin D is to maintain blood calcium and phosphate concentrations within physiological limits, as illustrated in Figure 1.4. In the intestine, $1,25(OH)_2D_3$ promotes active intestinal uptake and intra-cellular transport of calcium. Intestinal VDR concentration is highest in the duodenum (Holick, 1999). The epithelial calcium channel, transient receptor potential

vanilloid 6 (TRPV6), transports calcium into the cell, where it binds to calbindin D and is transported across the cell (Suzuki *et al.*, 2008). A Ca^{2+} ATPase (PMCA1b) and Na^{+}/Ca^{2+} exchanger (NCX1) then discharge calcium into the bloodstream (Bouillon *et al.*, 2003). TRPV6 and calbindin are up-regulated by $1{,}25(OH)_2D_3$ (Bouillon *et al.*, 2003; Hoenderop *et al.*, 2005). There is also paracellular transport of calcium down an electrochemical gradient (van Abel *et al.*, 2003). Blood calcium concentrations provide negative feedback on the numbers of TRPV6 channels (Hoenderop *et al.*, 2005). TRPV6 and calbindin may also be induced, independent of vitamin D, by high dietary and intestinal calcium concentrations (van Abel *et al.*, 2003). Active phosphate absorption is also increased by $1{,}25(OH)_2D_3$ through increased expression of the $Na\text{-}P_i$

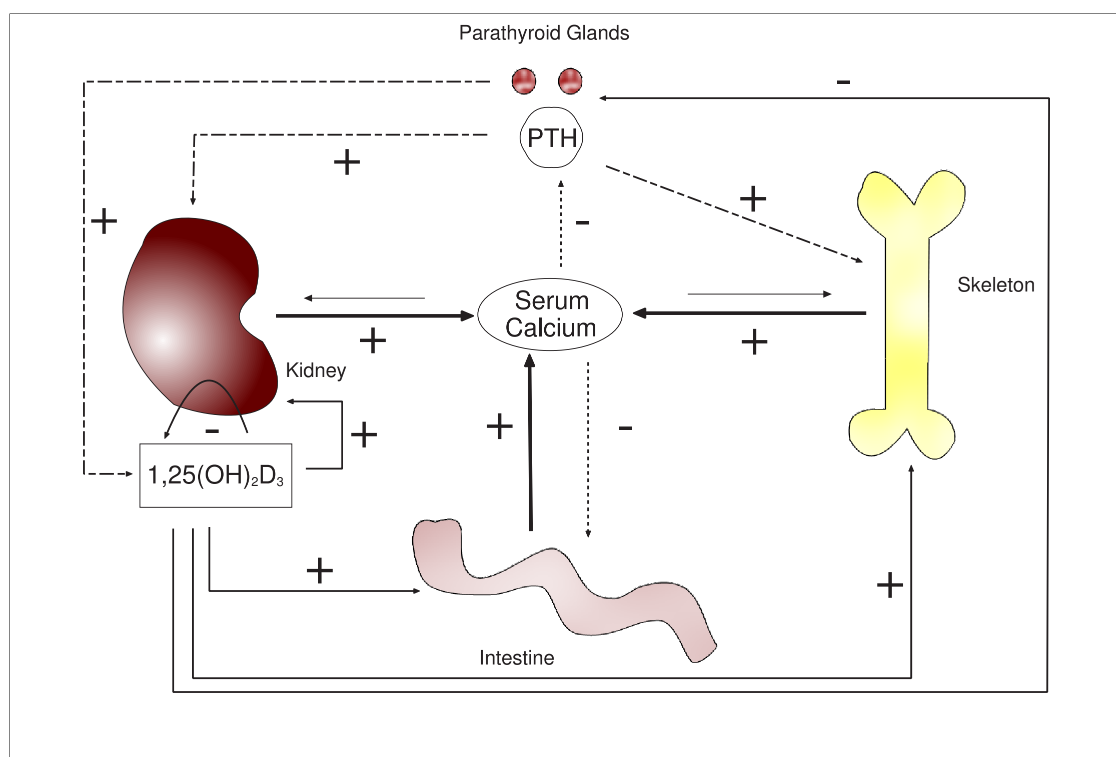


Figure 1.4: **The vitamin D endocrine system for calcium homeostasis.** Active vitamin D ($1{,}25(OH)_2D_3$) is produced by 1α-hydroxylation in the kidney, working directly and in partnership with PTH, induces calcium mobilisation from bone, calcium absorption from the intestine, inhibits PTH function, and increases calcium reabsorption from the kidney to keep serum calcium within physiological limits. PTH = Parathyroid hormone. Revised from (Dusso *et al.*, 2005)

transporter and altered intestinal cell plasma membrane lipid composition (Kurnik and Hruska, 1985; Yagci *et al*., 1992).

In the skeleton, $1,25(OH)_2D_3$ in association with PTH, promotes mobilisation of calcium from bone stores to maintain ionised blood calcium concentration within a narrow range (Broadus, 1999; Holick, 1999). It is questionable as to whether vitamin D induces mineralisation of bone directly, or by altering calcium and phosphate concentrations in the extracellular fluid via intestinal uptake of calcium (Suda *et al*., 2003). The latter is more likely. If calcium and phosphate concentrations are within the physiological range, it appears that $1,25(OH)_2D_3$/VDR is not essential for mineralisation of osteoid (Panda *et al*., 2004). However, both calcium and $1,25(OH)_2D_3$, independent of the VDR, are required for normal growth plate development (Panda *et al*., 2004). Osteoblasts and chondrocytes express both 25-hydroxyvitamin D-1α-hydroxylase and VDR (St-Arnaud, 2008). VDR-mediated signaling in chondrocytes leads to increased RANKL and vascular endothelial growth factor (VEGF). These lead to vascular invasion and osteoclastogenesis (St-Arnaud, 2008). Juvenile mice, with inactivation of the VDR in chondrocytes, showed normal growth plate development, but vascular invasion was impaired and osteoclast numbers were decreased, leading to a transient increase in the density of the primary spongiosa (Masuyama *et al*., 2006). Inactivation of the VDR in chondrocytes also led to a decrease in FGF23, and an increase in both renal 1α-hydroxylase and Na-P_i transporter, leading to increased serum phosphate and $1,25(OH)_2D_3$ concentrations (Masuyama *et al*., 2006).

Vitamin D also has effects on the parathyroid gland. A deficiency of vitamin D results in hypocalcaemia which leads to parathyroid hyperplasia and secondary hyperparathyroidism (Milgram, 1990c) Treatment with 1,25(OH)$_2$D$_3$ shuts down parathyroid cell growth and PTH production by suppressing the transforming growth factor/epidermal growth factor receptor growth loop and increasing known inhibitors of cell growth (cyclin dependent kinase inhibitors p21 and p27) (Dusso *et al*., 2004). Active vitamin D also directly suppresses *PTH* gene transcription (Demay *et al*., 1992; Silver *et al*., 1986). VDR and 1,25(OH)$_2$D$_3$ are not essential for controlling PTH production but work in co-operation with calcium (Dusso *et al*., 2005). Active vitamin D has been shown to up-regulate calcium sensing receptor (CaSR) expression by binding to VDREs on the *CaSR* gene promoter, leading to increased sensitivity of the parathyroid gland to blood ionised calcium, and decreased PTH secretion (Brown *et al*., 1996; Canaff and Hendy, 2002).

The main role of 1,25(OH)$_2$D$_3$ in the kidney is to control its own production by inhibition of 1α-hydroxylase and stimulation of 24-hydroxylase (Dusso *et al*., 2005). The direct effects of 1,25(OH)$_2$D$_3$ on the renal filtration of calcium and phosphate are controversial, as its effects are clouded by its actions on PTH and intestinal calcium and phosphate absorption (Dusso *et al*., 2005). The epithelial calcium channel (TRPV5) is present in the distal convoluted tubule of the kidney (Suzuki *et al*., 2008) and 1,25(OH)$_2$D$_3$ has been shown to increase expression of TRPV5 via VDREs in the human *TRPV5* promoter, leading to increased renal calcium reabsorption (Hoenderop *et al*., 2001). PTH-dependent calcium transport in distal convoluted tubules is also accelerated by 1,25(OH)$_2$D$_3$ (Friedman and Gesek, 1993). Renal tubular reabsorption of

phosphate is increased by $1,25(OH)_2D_3$ when PTH is present, but this is unlikely to be a direct effect on the kidney (Dusso *et al*., 2005).

Vitamin D has also been shown to have other endocrine and autocrine/paracrine roles in multiple cell types and organs. It has been suggested that vitamin D may protect against cancer, and possibly even treat cancer by suppression of cell growth (Dusso *et al*., 2005; Zittermann, 2003). Serum vitamin D concentration and the incidence of some cancers may be inversely related, although many confounding factors were present (Davis, 2008). Vitamin D may also have a role in the immune system, be involved in prevention of cardiovascular disease, hypertension and diabetes mellitus, and aid in treatment of rheumatoid arthritis, inflammatory bowel disease, psoriasis and multiple sclerosis (Dusso *et al*., 2005; Jones *et al*., 1998; Muller and Bendtzen, 1991; Zittermann, 2003).

In spite of its many beneficial effects, an excess of $1,25(OH)_2D_3$ is toxic, causing widespread soft-tissue mineralisation secondary to persistent hypercalcaemia and hyperphosphataemia (Murphy, 2002). Affected animals show severe gastrointestinal signs, hypertension, heart rhythm abnormalities, neurological signs such as seizures and eventually death (Murphy, 2002; Parton *et al*., 2001). This underlies the use of cholecalciferol as a rodenticide, and for possum control in New Zealand.

1.4 Pathology of Rickets

The characteristics of rickets are similar in all species. Lesions are typically most severe in the fastest growing bones, including the radius, tibia and the metacarpal/metatarsal bones (Fitch, 1943; Nisbet *et al*., 1966). On radiographic and post-mortem examination widening of the physeal growth plate is the most archetypal change (Fitch, 1943; Klein, 1999; Malik *et al*., 1997; McCarthy and Frassica, 1998b; Pepper *et al*., 1978; Spratling *et al*., 1970). Other abnormalities seen radiographically may include, metaphyseal flaring, thinning of the cortex, poor mineralisation of the skeleton and pathological fractures (Duckworth *et al*., 1961; Klein, 1999; Malik *et al*., 1997; McCarthy and Frassica, 1998b; Pepper *et al*., 1978; Spratling *et al*., 1970). In addition, post-mortem examination may reveal irregular thickening of the physeal cartilage, erosion of articular cartilage due to collapse of subchondral bone, and spontaneous fractures (Bonniwell *et al*., 1988; Fitch, 1943; Nisbet *et al*., 1966; Pepper *et al*., 1978). Enlargement of the costochondral junctions, the so-called "rachitic rosary", is also a classic lesion of rickets that may be seen on radiographic or post-mortem examination (Fitch, 1943; McCarthy and Frassica, 1998b).

Impaired provisional calcification of the growth plate leads to the accumulation of hypertrophic chondrocytes, resulting in thickened and irregular growth plates with islands and tongues of chondrocytes extending into the metaphyses (Bonniwell *et al*., 1988; Fitch, 1943; Nisbet *et al*., 1966; Theiler, 1934; Thompson and Cook, 1987). Other microscopic changes may include thick osteoid seams lining trabeculae, and disorganisation or absence of the primary spongiosa (Bonniwell *et al*., 1988; Fitch, 1943; Nisbet *et al*., 1966; Pitt, 1995; Thompson and Cook, 1987). One of the early pioneers of research into rickets, Sir Arnold

Theiler, regarded the pathognomonic change as "...the presence of osteoid tissue in quantities surpassing normal physiological limits" (Theiler, 1931, p1145). Haemorrhage and signs of trauma may be seen in the metaphysis and primary spongiosa due to damage to weakened trabeculae of poorly mineralised bone (Bonniwell *et al*., 1988; Fitch, 1943; Nisbet *et al*., 1966; Thompson and Cook, 1987).

1.5 RICKETS IN HUMANS

1.5.1 NUTRITIONAL RICKETS

In the developed world, nutritional rickets has been re-emerging as a disease problem; in third world countries it never went away and is thought to be one of the five most common diseases of children (Al-Jurayyan *et al*., 2002; Hatun *et al*., 2005b; Pettifor, 2002; Wharton and Bishop, 2003). Medical writings from the 1st and 2nd centuries described the classical bone deformities associated with rickets, and in the mid 17th century Daniel Whistler and Francis Glisson wrote the first detailed medical reports (Pettifor, 2005; Rajakumar, 2003). It was not until the industrial revolution however, that rickets became an epidemic (Pettifor, 2005; Rajakumar, 2003). In 1909, histological evidence of rickets was an incidental finding in 96% of children who died at less than 18 months of age (Rajakumar, 2003). At that time rickets was associated with heavy air pollution, urbanisation, overcrowding, and inadequate diet (Pettifor, 2005). Experimental work in the early 20th century confirmed the efficacy of the traditional remedy, cod liver oil, for treating rickets, and eventually vitamin D was named in 1922

as the agent responsible for preventing the disease (Pettifor, 2005; Rajakumar, 2003).

Rickets in humans is seen most commonly either during infancy or at puberty, corresponding to periods of maximal growth (Wharton and Bishop, 2003). The common clinical signs in humans include; craniotabes, skeletal deformities such as bowed or knock-kneed legs, rachitic rosary, delayed eruption of teeth and enamel hypoplasia (Prince and Glendenning, 2004; Wharton and Bishop, 2003). Low to normal calcium, low to normal phosphate, high PTH, high alkaline phosphatase, low 25(OH)D_3 and normal to high 1,25$(OH)_2D_3$ concentrations are the common changes seen in the serum chemistry of children affected with nutritional rickets (Prince and Glendenning, 2004; Wharton and Bishop, 2003).

In developed countries, nutritional rickets is most often associated with exclusive breastfeeding (Chesney, 2003; Gessner *et al*., 2003; Pettifor, 2005; Pettifor, 2002; Thomson *et al*., 2004). Although vitamin D passes readily into breast milk, breast milk is low in vitamin D (reported values of 4-40 IU/L) (Lammi-Keefe, 1995; Reeve *et al*., 1982) and does not supply an infant's daily requirements (200-400 IU/L) (Lammi-Keefe, 1995; Weisberg *et al*., 2004). The vitamin D metabolites, 25(OH)D_3 and 1,25$(OH)_2D_3$, pass poorly into breast milk. Most breast-fed infants acquire vitamin D through sunlight exposure, however this is affected by such factors as: skin pigmentation (Holick *et al*., 1981), the amount of skin exposed to sunlight (Matsuoka *et al*., 1992), air pollution (Agarwal *et al*., 2002), latitude (Pettifor *et al*., 1996; Webb *et al*., 1988), cloud cover (Weisberg *et al*., 2004) and sunscreen usage (Matsuoka *et al*., 1987). In a review of reports on nutritional rickets from 1986-2003 in the USA, 83% of children

with rickets were African-American or other dark-skinned races, and 96% were breast-fed (Weisberg *et al*., 2004).

In the UK and Northern Europe, rickets is most common in infants and children of either Asian or Afro-Caribbean origin (Hannam *et al*., 2004; Holvik *et al*., 2005; Huh and Gordon, 2008; Pedersen *et al*., 2003; Pettifor, 2005). In these communities, risk factors include: latitude, vegetarianism, consumption of chapattis, traditional clothing and social customs with limited exposure to sunlight (Batchelor and Compston, 1983; Pedersen *et al*., 2003; Pettifor, 2005). Vegetarian diets and chapattis typically contain high concentrations of phytates and dietary fibre which, when combined with low calcium intake and limited exposure to the sun, may lead to vitamin D deficiency (Batchelor and Compston, 1983; Pettifor, 2005).

The elderly and housebound are also at risk, and although clinical osteomalacia is not common in this age group, their 25(OH)D_3 concentrations are frequently low (Brock *et al*., 2004; McKenna, 1992; Weisman, 2003). Elderly people generally spend more time indoors and aged human skin has reduced ability to produce vitamin D in response to ultraviolet light (MacLaughlin and Holick, 1985).

Although rickets is usually associated with locations of high latitude, the disease also occurs in tropical areas where sunshine hours are not a limiting factor. Some cases in Nigeria and South Africa, are thought to be due to a dietary deficiency of calcium (Oginni *et al*., 2003; Thacher *et al*., 1999). Children

in these countries typically have a diet high in phytates with limited or no dairy products (Pettifor, 2004). The daily dietary calcium intake was low in both rachitic and normal children, and calcium absorption was found to be normal in both (Graff *et al*., 2004; Thacher *et al*., 1999). However, serum 25(OH)D_3 concentrations were also low in affected individuals indicating that vitamin D deficiency may be involved (Thacher *et al*., 1999). Affected individuals had a better response to treatment with a combination of calcium and vitamin D, than either calcium or vitamin D alone (Thacher *et al*., 1999). The possibility of differences in dietary levels of phytates was considered a possible reason for the occurrence of rickets in some of these children but not others (Graff *et al*., 2004). Similar cases have been seen in South Africa in children fed diets of maize and vegetables without dairy products (Marie *et al*., 1982), and in Bangladesh with diets predominantly based on cereals and starchy vegetables (Combs and Hassan, 2005). Experimental evidence is needed to confirm that calcium deficiency is indeed the cause of rickets in these cases.

Cultural practices may also lead to rickets in tropical areas. Rickets and osteomalacia are seen in women and their children in the Middle East and India due to traditional body covering clothing, limited sun exposure and diets high in phytates (Dawodu *et al*., 2005; Fida, 2003; Hashemipour *et al*., 2004; Hatun *et al*., 2005a; Hatun *et al*., 2005b; Narchi *et al*., 2001; Sachan *et al*., 2005; Weisman, 2003). Women in these populations also tend to have high pregnancy rates, which likely further depletes vitamin D stores (Weisman, 2003).

Food allergies are considered a possible risk factor for nutritional rickets (Fox *et al*., 2004; Imataka *et al*., 2004). Allergies to hen eggs, cow's milk and peanuts are the most common, and if unsupervised may lead to parents giving their children diets that are nutritionally unbalanced (Fox *et al*., 2004; Noimark and Cox, 2008). Soy milk is a common substitute for cow's milk in allergic individuals but is low in calcium, phosphorus, magnesium and vitamin D (Imataka *et al*., 2004). Such a diet is unsuitable as a complete food for infants and may lead to the development of rickets (Fox *et al*., 2004; Imataka *et al*., 2004). Gastrointestinal disease or cholestatic liver disease may also lead to the development of rickets and osteomalacia due to malabsorption, impaired 25-hydroxylase activity, changes in enterohepatic circulation, intestinal unresponsiveness to vitamin D and excessive faecal loss of vitamin D (Mankin, 1994; Sitrin *et al*., 1978).

1.5.2 Hereditary Rickets

A number of heritable causes of rickets and osteomalacia have been identified and are listed in Table 1.2. Heritable rickets is most commonly due to a problem in either vitamin D metabolism or renal tubular function (Whyte, 2002).

1.5.2.1 Hypophosphataemic Rickets

Heritable diseases associated with renal phosphate wasting include, X-linked hypophosphataemic rickets (XLH), autosomal dominant hypophosphataemic rickets (ADHR), autosomal recessive hypophosphataemic rickets (ARHR) and hereditary hypophosphataemic rickets with hypercalciuria (HHRH) (Feng *et al*.,

2006; Ritz *et al*., 2003). These diseases, as well as tumour-induced osteomalacia are characterised by hypophosphataemia, renal inorganic phosphate wasting, rickets and inappropriately low to normal 1,25$(OH)_2D_3$ concentrations (Bielesz *et al*., 2004; Ritz *et al*., 2003; Whyte, 2002).

Table 1.2: **Heritable causes of rickets and osteomalacia** (Whyte, 2002).

Disorders of vitamin D bioactivation
Vitamin D-dependency, type I (1α-hydroxylase deficiency)
Hereditary vitamin D-resistant rickets
Renal tubular acidosis, distal (classic, type I)
Primary
Galactosaemia
Hereditary fructose intolerance with nephrocalcinosis
Fabry disease
Phosphate depletion from impaired renal tubular reabsorption
X-linked hypophosphataemic rickets
Syndrome of lipoatrophic diabetes, vitamin D-resistant rickets, persistent Müllerian ducts
X-linked hypercalciuric nephrolithiasis
McCune-Albright syndrome
General renal tubular disorders (Fanconi syndrome)
Primary idiopathic
Cystinosis
Fanconi-Bickel syndrome
Lowe syndrome
Wilson disease
Tyrosinaemia
Primary mineralisation defect
Hypophosphatasia
Miscellaneous
Axial osteomalacia
Osteopetrosis (osteopetrorickets)
Neurofibromatosis

XLH has an X-linked dominant mode of inheritance, and with one in 20,000 people affected, is the most common form of hereditary rickets in humans (Bielesz *et al*., 2004). In addition to the microscopic changes of rickets, "halos" of unmineralised osteoid surround individual osteocytes (hypomineralized periosteocytic lesions), a lesion considered pathognomonic for this disease (Glorieux, 1999; Whyte, 2002). The genetic mutation that causes XLH has been isolated to the *PHEX* gene on chromosome 22 in the human genome (Du *et al*., 1996). *PHEX* stands for Phosphate-regulating gene with Homologies to Endopeptidases on the X-chromosome, and produces a protein that has homology with membrane bound metalloendopeptidases (Du *et al*., 1996; Rowe, 2004). No single predominant mutation in *PHEX* causes XLH, and in a mutational analysis of patients with XLH, 18 different mutations in *PHEX* were found (Ichikawa *et al*., 2008). PHEX is expressed by osteoblasts, odontoblasts and chondrocytes, but is not expressed in the kidney (Bielesz *et al*., 2004).

The mechanism by which *PHEX* mutations cause XLH has yet to be elucidated (Bielesz *et al*., 2004; Ichikawa *et al*., 2008). One hypothesis is that a circulating factor that is degraded or processed by PHEX influences phosphate reabsorption in the kidney. Candidates for the circulating factor include fibroblast growth factor 23 (FGF23), matrix extracellular phosphoglycoprotein (MEPE) and secreted frizzled-related protein 4 (sFRP4) (Berndt *et al*., 2005; Bielesz *et al*., 2004; Ritz *et al*., 2003). FGF23 is thought to inhibit the NPT-2a co-transporter (a NaP_i co-transporter), thereby altering tubular phosphate transport (Ritz *et al*., 2003). *FGF23* gene transcription is up-regulated in XLH (Liu and Quarles, 2007). MEPE is increased in XLH, possibly due to the defect in PHEX leading to impaired PHEX sequestration of MEPE (Rowe, 2004). This

makes MEPE available for proteolysis, which leads to the formation of ASARM (Acidic-Serine-Aspartate-Rich-MEPE-associated motif) peptide (Rowe, 2004). ASARM locally inhibits mineralisation of osteoid, and inhibits renal phosphate uptake by binding to NPT-2a (Rowe, 2004). Recent work has shown that MEPE or phosphorylated ASARM, inhibit PHEX activity, lead to increased FGF23 production, and phosphaturia (Liu *et al*., 2007). The mechanism of vitamin D dysfunction is unknown, although phosphatonins like FGF23 do inhibit 1,25$(OH)_2D_3$ production (Bielesz *et al*., 2004; Shimada *et al*., 2004). The mechanism of the impaired bone mineralisation is also equivocal as it is only partly restored by restoring PHEX function in knock-out mice (Liu *et al*., 2002).

Autosomal dominant hypophosphataemic rickets (ADHR) is less common than XLH and its variable age of onset and incomplete penetrance leads to difficulties in diagnosis (Ritz *et al*., 2003; Tenenhouse and Econs, 2001). Mutations in the gene for FGF23 on chromosome 12 are believed to be the cause of ADHR (The ADHR Consortium, 2000). Such mutations induce loss of a cleavage site of FGF23, leading to an increase in its activity and consequent phosphaturia, hypophosphataemia and rickets (Bai *et al*., 2003; Liu and Quarles, 2007; Shimada *et al*., 2002).

A disease with autosomal recessive inheritance similar to ADHR and XLH has been identified in three families (Ramasamy, 2008). The patients had renal phosphate wasting and elevated FGF23 concentrations (Lorenz-Depiereux *et al*., 2006). Mutations were found in the gene for dentin matrix protein 1 (DMP1), a non-collagenous bone protein (Feng *et al*., 2006; Lorenz-Depiereux *et al*., 2006).

Hereditary hypophosphataemic rickets with hypercalciuria is characterised by autosomal recessive inheritance and was first described in a Bedouin family (Tieder *et al*., 1985). In contrast to other hypophosphataemias, 1,25$(OH)_2D_3$ is elevated, possibly explaining the hypercalciuria (Ritz *et al*., 2003; Tieder *et al*., 1985). The disease occurs as a result of a single nucleotide deletion in the gene coding for the NaP_i-IIc protein (a renal Na-P_i co-transporter), truncating the protein in patients homozygous for the deletion (Bergwitz *et al*., 2006).

While not inherited, tumour-induced osteomalacia is a renal phosphate wasting syndrome (Bielesz *et al*., 2004; Ritz *et al*., 2003), most often associated with slow-growing mesenchymal tumours (Folpe *et al*., 2004; Weidner and Santa Cruz,1987). The most common is phosphaturic mesenchymal tumour mixed connective tissue type (which resembles haemangiopericytoma) and accounts for 70-80% of tumours causing tumour-induced osteomalacia (Folpe *et al*., 2004). Affected individuals develop osteomalacia with bone and muscle pain, hypophosphataemia and low to normal 1,25$(OH)_2D_3$ concentrations (Bielesz *et al*., 2004). Surgical removal of the tumour leads to resolution of the clinical signs (Kumar, 2000; Ritz *et al*., 2003; Ward *et al*., 2004). Tumours causing tumour-induced osteomalacia have been found to produce phosphatonins such as FGF23, MEPE, sFRP4, and FGF7 (Berndt *et al*., 2005; Carpenter *et al*., 2005; Rowe *et al*., 2000; Shimada *et al*., 2001).

1.5.2.2 Vitamin D-Dependent Rickets Type I (VDDR I)

VDDR I or pseudovitamin D-deficiency rickets is an autosomal recessive disorder caused by a failure to convert 25(OH)D_3 to 1,25$(OH)_2D_3$ (Fraser *et al*., 1973); most likely due to a defect in renal 25-hydroxyvitamin D_3-1α-hydroxylase (Fraser *et al*., 1973; Kitanaka *et al*., 1999). This has yet to be confirmed, as assays for renal 1α-hydroxylase activity in human patients have not been performed, although renal 1α-hydroxylase activity in renal homogenates of pigs with VDDR I are negligible (Fox *et al*., 1985; Kitanaka *et al*., 2001).

Children with VDDR I are normal at birth but develop clinical signs of rickets from 2 to 24 months of age, together with muscle weakness, reduced growth rate and occasionally tetany and convulsions (Kitanaka *et al*., 2001; Liberman and Marx, 2001). Affected individuals have normal 25(OH)D_3 concentrations and low 1,25$(OH)_2D_3$ concentrations (Mankin, 1994). The disease can be successfully treated with high doses of cholecalciferol or physiologic doses of 1,25$(OH)_2D_3$ (Fraser *et al*., 1973).

VDDR I is particularly common in French Canadians in the Saguenay-Lac-St-Jean region of north-eastern Quebec, with an estimated carrier rate of 1/27 people (De Braekeleer and Larochelle, 1991). This is probably due to the founder effect, when a small number of people emigrated to Quebec from France (Yoshida *et al*., 1998). The defect in VDDR I involves the *CYP27B1* gene (encoding 25-hydroxyvitamin D_3-1α-hydroxylase) on chromosome 12q13.3 and a number of different mutations in the gene have been detected in affected individuals (Fu *et al*., 1997; Kitanaka *et al*., 1998; Wang *et al*., 1998; Yoshida *et al*.,

1998). In the French Canadian population, nine out of ten individuals with VDDR I that were tested, had a one base pair deletion in the *CYP27B1* gene (Wang *et al*., 1998). In other individuals, different substitutions, duplications, and multiple deletions have been found (Fu *et al*., 1997; Kim *et al*., 2007; Kitanaka *et al*., 1998; Wang *et al*., 1998).

1.5.2.3 Hereditary Vitamin D-Resistant Rickets (HVDRR)

In 1978, the first three cases of a new type of rickets, HVDRR, or vitamin D-dependent rickets type II, were described (Brooks *et al*., 1978; Marx *et al*., 1978). These patients had hypocalcaemia, secondary hyperparathyroidism, and high serum $1,25(OH)_2D_3$ concentrations (Brooks *et al*., 1978; Marx *et al*., 1978). The high concentrations of $1,25(OH)_2D_3$ were thought to indicate target organ unresponsiveness to active vitamin D (Brooks *et al*., 1978; Marx *et al*., 1986).

Patients were normal at birth due to transplacental calcium flux between the mother and fetus, but skeletal changes and hypocalcaemia were evident from 2 to 8 months of age (Hochberg, 2002; Liberman and Marx, 2001; Malloy and Feldman, 1999). Late onset of HVDRR has occurred occasionally in teenagers, and one case is reported to have developed in an adult at the age of 45, however these cases have not been confirmed with molecular studies (Brooks *et al*., 1978; Fujita *et al*., 1980; Kudoh *et al*., 1981). Approximately 75% of affected individuals are born to consanguineous parents (Malloy *et al*., 1999).

Clinical signs of HVDRR include severe dental caries or dental hypoplasia, retarded growth, bone pain, muscle weakness, hypotonia, and occasionally

convulsions due to hypocalcaemia (Balsan *et al.*, 1983; Brooks *et al.*, 1978; Liberman *et al.*, 1980; Malloy *et al.*, 1999; Tsuchiya *et al.*, 1980). Approximately 70-80% of affected individuals have partial to total alopecia, including eyebrows, and in some cases eyelashes (Liberman and Marx, 2001; Malloy and Feldman, 1999; Malloy *et al.*, 1999). The hair loss may be obvious at birth, or develop over the first few months of life, and in families with HVDRR can be used to presumptively diagnose the disease prior to the development of other signs (Al-Khenaizan and Vitale, 2003; Malloy *et al.*, 1999). There is no improvement in the alopecia with either $1,25(OH)_2D_3$ or calcium treatment, even if the rickets is successfully treated (Liberman and Marx, 1999; Marx *et al.*, 1986). Skin biopsies from individuals with alopecia have shown normal follicles, with epithelium similar to that seen in the telogen stage, but complete absence of hair shafts (Al-Khenaizan and Vitale, 2003; Malloy *et al.*, 1999). VDR are present in nucleii of the epidermis and in the external root sheath of hair, therefore it is likely that the VDR plays an important role during a crucial stage of hair follicle development (Malloy *et al.*, 1999; Stumpf *et al.*, 1979).

Experiments using cultured skin fibroblasts have been useful in determining the type of defect present in the VDR of human patients with HVDRR. This followed the discovery that the VDR was present in fibroblasts and it was shown that a patient with HVDRR had no specific binding of $[^3H]1,25(OH)_2D_3$ to the VDR (Feldman *et al.*, 1982; Feldman *et al.*, 1980). Peripheral blood mononuclear cells (Koren *et al.*, 1985) and various types of lymphocytes also contain the VDR and may be used instead of fibroblasts (Hughes *et al.*, 1988; Koeffler *et al.*, 1990; Malloy *et al.*, 1989; Takeda *et al.*, 1986). The experiments commonly performed to determine the defect present in the VDR include,

$1,25(OH)_2D_3$ induction of 24-hydroxylase activity, $[^3H]1,25(OH)_2D_3$ binding studies, DNA cellulose chromatography and western blot analysis of the VDR (Hirst *et al*., 1985; Hughes *et al*., 1988; Liberman *et al*., 1983; Malloy *et al*., 1989; Malloy *et al*., 1990).

From these studies in cultured cells, three main categories of defect have been identified. The first, and most common, is a hormone binding defect or ligand binding-negative phenotype (Liberman and Marx, 1999; Liberman and Marx, 2001; Malloy *et al*., 1999). This category includes those with no, or negative ligand binding (Chen *et al*., 1984; Liberman *et al*., 1983; Liberman and Marx, 1999; Liberman and Marx, 2001), decreased maximal capacity of hormone binding (Balsan *et al*., 1983), and decreased hormone binding affinity (Malloy *et al*., 1997). The second group involves defective receptor binding to DNA (ligand binding-positive phenotype) (Hirst *et al*., 1985; Liberman *et al*., 1986; Malloy *et al*., 1989). The third group was originally thought to comprise defective receptor translocation to the nucleus, with normal binding affinity and capacity for $[^3H]1,25(OH)_2D_3$ (Hewison *et al*., 1993; Whitfield *et al*., 1996), but recent work has shown that this defect is the result of constitutive overexpression of heterogenous nuclear riboprotein competing with the VDR-RXR complex for binding to the VDRE (Chen *et al*., 2006; Chen *et al*., 2003).

Patients with HVDRR tend to have normal serum concentrations of $25(OH)D_3$, high concentrations of $1,25(OH)_2D_3$, and low concentrations of $24,25(OH)_2D_3$ in comparison to controls (Yokota *et al*., 1991). In one study, the heterozygous parents were shown to have serum concentrations of $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ that were significantly different from normal unaffected

individuals and homozygous affected HVDRR patients (Yokota *et al*., 1991). In this study, the ability to induce 25-hydroxyvitamin D-24-hydroxylase in cultured skin fibroblasts from the parents was between 50-82% of controls and the binding capacity of $[^3H]1,25(OH)_2D_3$ to the nucleus was 38-54% of controls, indicating a gene dosage effect (Yokota *et al*., 1991). This correlates with the finding that the parents of HVDRR individuals contain both normal and defective VDR (Hughes *et al*., 1988; Malloy *et al*., 1989). However, while some heterozygous parents show a reduced binding capacity, others have normal binding of $1,25(OH)_2D_3$ (Malloy *et al*., 1990).

Treatment with high doses of oral calcium and pharmalogical doses of $1,25(OH)_2D_3$ has met with limited success in individuals with HVDRR (Gardezi *et al*., 2001). A small number of patients however have been successfully treated with oral 1α-OH-D (3-6µg/kg/day) (Takeda *et al*., 1987), and high oral doses (3-20µg/day) of either $1,25(OH)_2D_3$ (Castells *et al*., 1986; Kruse and Feldmann, 1995), or vitamin D_2 (50,000IU) (Takeda *et al*., 1989). Long term intravenous calcium infusion has been the most successful treatment option for patients resistant to all forms of vitamin D and is used routinely by some clinics if children with HVDRR do not respond to oral calcium and vitamin D (Hochberg *et al*., 1985; Malloy and Feldman, 1999). Once healing of the bone lesions has occurred, some patients can be maintained on high-dose oral calcium therapy (Hochberg *et al*., 1992). With intravenous calcium treatment, bone pain is eliminated, growth is increased, and healing of rachitic lesions occurs within 5 months (Balsan *et al*., 1986; Bliziotes *et al*., 1988; Hochberg *et al*., 1992; Weisman *et al*., 1987). The success of this treatment may be due to intravenous calcium

bypassing defective VDR-mediated calcium absorption in the intestine (Malloy and Feldman, 1999).

Since the *VDR* was sequenced in 1988 (Baker *et al*., 1988) progress has been rapid in determining the DNA mutations present in individuals with HVDRR. The mutations can be grouped into seven categories: those causing missense mutations in the DNA-binding domain (Hughes *et al*., 1988), mutations causing premature termination of the VDR (Ritchie *et al*., 1989; Weise *et al*., 1993), missense mutations in the ligand-binding domain (Arita *et al*., 2008; Kristjansson *et al*., 1993; Malloy *et al*., 1997), mutations in the ligand-binding domain that affect RXR heterodimerization (Malloy *et al*., 2001; Whitfield *et al*., 1996), mutations in the ligand-binding domain that affect co-activator binding (Malloy *et al*., 2002a), splice site mutations (Cockerill *et al*., 1997), and gene deletion (Malloy and Feldman, 1999). HVDRR may also occur without mutations in the *VDR*, as in the case of constitutive overexpression of heterogenous nuclear riboprotein (Chen *et al*., 2006; Chen *et al*., 2003).

1.6 Rickets in Animals

1.6.1 Animal Models of Rickets

A number of mouse models have been discovered or created in order to study some of the skeletal diseases present in humans. Some of these are summarised in Table 1.3, together with a selection of other models that have been created in order to study the function of different hormones and proteins involved in skeletal homeostasis.

Table 1.3: **Selected models of metabolic bone disease in mice and rats.**

Mouse Model	Human Disease Equivalent	Abnormalities	Reference
Hyp	X-linked hypophosphataemic rickets	hypophosphataemia, rickets, dwarfism, normal $1,25(OH)_2D_3$, high fractional excretion of phosphate	(Eicher *et al*., 1976)
Gy	X-linked hypophosphataemic rickets	hypophosphataemia, rickets, circling, inner ear abnormalities, increased $1,25(OH)_2D_3$, increased renal phosphate loss	(Lyon *et al*., 1986)
FGF23 knock-out		growth retardation, shortened life span, hyperphosphataemia, increased renal phosphate reabsorption, elevated $1,25(OH)_2D_3$, focal osteomalacia	(Shimada *et al*., 2004)
1α-hydroxylase knock-out	Vitamin D-dependent rickets type I	rickets, growth retardation, hypocalcaemia, low $1,25(OH)_2D_3$, high $25(OH)D_3$, hyperparathyroidism	(Dardenne *et al*., 2001) (Panda *et al*., 2001)
VDR null or *VDR*$^{-/-}$	Hereditary vitamin D-resistant rickets	alopecia, rickets, hypocalcaemia, hypophosphataemia, hyperparathyroidism	(Li *et al*., 1997) (Bula *et al*., 2005)
VDR/RXR$_{\gamma}$ knock-out		growth retardation, rickets, more severe bone abnormalities then *VDR*$^{-/-}$, hypocalcaemia, alopecia	(Yagishita *et al*., 2001)
Cyp24a1 (*24-hydroxylase*) knock-out		50% of progeny show death at less than 3 weeks due to high $1,25(OH)_2D_3$ and hypercalcaemia, accumulation of unmineralised osteoid at sites of intramembranous ossification	(St-Arnaud *et al*., 2000)
24-hydroxylase transgenic rats		constitutive expression of 24-hydroxylase, albuminuria, hyperlipidaemia, atherosclerosis, urine loss of vitamin D-binding protein and 25(OH)D, decreased plasma $24,25(OH)_2D_3$	(Kasuga *et al*., 2002)
DMP-1 knock-out	Autosomal recessive hypophosphataemic rickets	rickets, osteomalacia, elevated FGF23, hypocalcaemia, hypophosphataemia, inappropriately normal $1,25(OH)_2D_3$, mineralisation defects, defective osteoblast to osteocyte differentiation and maturation	(Feng *et al*., 2006)
Vitamin D-binding protein knock-out		on a vitamin D-replete diet mice are normal, on a vitamin D-deficient diet mice developed secondary hyperparathyroidism and rickets, while on a diet excessive in vitamin D mice were resistant to the development of hypercalcaemia.	(Safadi *et al*., 1999)
Ca^{2+}-sensing receptor (*CaSR*) knock-out		rickets, dwarfism, hypercalcaemia, hypophosphataemia, hyperparathyroidism	(Garner *et al*., 2001)

Two naturally occurring models of hypophosphataemic rickets due to renal phosphate wasting have been described in mice: the *Gy* mouse and *Hyp* mouse (Eicher *et al*., 1976; Lyon *et al*., 1986). Both models are characterised by hypophosphataemia, rickets, normal to high 1,25(OH)$_2$D$_3$, and increased renal phosphate loss (Eicher *et al*., 1976; Lyon *et al*., 1986). The *Gy* mouse also has inner ear abnormalities that lead to circling (Lyon *et al*., 1986). The phenotype observed in the *Hyp* mouse is the result of excessive FGF23 concentrations due to inactivating mutations in *PHEX* (Liu *et al*., 2006b).

Four strains of *VDR* null mice have been developed. The Tokyo strain, has exon 2 (which encodes the first of the two zinc fingers involved in DNA binding) of the *VDR* gene removed (Bula *et al*., 2005), and the Boston strain has exon 3 (which encodes the second zinc finger) removed (Li *et al*., 1997). Two other strains also have a missing first zinc finger (Erben *et al*., 2002; Van Cromphaut *et al*., 2001). The disease in all strains is phenotypically similar to HVDRR of humans (Bula *et al*., 2005). Affected mice are normal at birth, but become hypocalcaemic, hypophosphataemic and develop hyperparathyroidism by 21 days of age (Li *et al*., 1997). The *VDR* null mice then develop rickets and osteomalacia by day 35 (Li *et al*., 1997).

VDR null mice develop a progressive alopecia that starts from 4 weeks of age, and is complete by 100 days (Demay, 2006; Erben *et al*., 2002; Li *et al*., 1997; Van Cromphaut *et al*., 2001). Examination of the skin of *VDR* null mice has shown that the alopecia is due a defect in anagen initiation in epidermal keratinocytes that is not dependent on the presence of 1,25(OH)$_2$D$_3$ (Sakai *et al*., 2001). In a

strain of *VDR* null mice with the first zinc finger deleted, it has been shown that VDR DNA-binding is required to prevent alopecia (Erben *et al*., 2002). Recent work has shown that VDRs with mutations in the ligand-binding domain were able to prevent alopecia (Demay *et al*., 2007). In addition, absence of the VDR impairs canonical Wnt signaling and leads to decreased keratinocyte stem cell colony formation and alopecia (Cianferotti *et al*., 2007).

By feeding a diet high in calcium (2%), phosphorus (1.25%) and lactose to *VDR* null mice from day 18 (prior to changes in serum biochemical values), hypocalcaemia, hyperparathyroidism, rickets and osteomalacia can be prevented (Li *et al*., 1998). The alopecia however remains unchanged (Li *et al*., 1998). The absence of biochemical abnormalities and clinical signs when *VDR* null mice were on the high calcium, phosphorus and lactose diet, indicates that the VDR is not required to prevent hyperparathyroidism or for skeletal homeostasis (Demay, 2006). Similar results are seen by feeding a diet low in phosphorus (0.25%) and containing a normal concentration of calcium (0.5%); mice fed the restricted diet had fewer osteoid seams, and higher bone mineral content and density than mice on the control diet (Masuyama *et al*., 2001).

Studies performed in knock-out mice have focused on attempting to separate the actions of the VDR from the metabolic disturbances caused by a lack of VDR function. A number of researchers have examined the growth plate in order to determine a cause for the enlargement of the hypertrophic chondrocyte zone seen in rickets. Vitamin D has been shown to promote chondrocyte differentiation, as has calcium, implying that either vitamin D deficiency or

hypocalcaemia may have a role in the growth plate abnormalities seen in rickets (Demay, 2006). Experiments in *VDR* null and wild-type mice however, have shown that the VDR is not required for normal growth plate maturation (Demay, 2006; Donohue and Demay, 2002). Instead, a marked decrease in apoptosis of late hypertrophic chondrocytes is seen in *VDR* null mice, and it has been shown that phosphate induces chondrocyte apoptosis, possibly via activation of the caspase-9 mitochondrial pathway (Adams *et al*., 2001; Donohue and Demay, 2002; Meleti *et al*., 2000; Sabbagh *et al*., 2005). The presence of calcium potentiates phosphate-dependent apoptosis (Adams *et al*., 2001). Other studies on *VDR* null mice have shown that maintenance of osteoblastogenesis and maximal PTH-induced osteoclastogenesis require both $1,25(OH)_2D_3$ and the VDR (Panda *et al*., 2004). A lack of either results in uncoupling of bone turnover due to abnormal control of RANK and RANKL, therefore the increase in thickness of the growth plate seen in rickets may also be due to impaired growth plate remodelling (Dusso *et al*., 2005; Panda *et al*., 2004).

Other researchers have examined the effects of the VDR on fetal mineralisation. *VDR* null mice tend to produce fetuses that are less viable and lower in body weight than wild-type mice (Kovacs *et al*., 2005). Supplementation with calcium, corrected these reproductive deficiencies (Kovacs *et al*., 2005; Rummens *et al*., 2003). One study found that the calcium content of the fetal skeletons did not differ between wild-type, heterozygotes and *VDR* null fetuses of heterozygote mothers or *VDR* null mothers, when corrected for relative fetal weight (Kovacs *et al*., 2005). Contradicting this is a study where fetal skeletons from *VDR* null mice were found to have a lower calcium content (Rummens *et al*., 2003). Hypercalcaemia was found in fetuses from *VDR* null mice by some

researchers, but others found that fetuses from *VDR* null mice had normal ionised calcium concentrations (Kovacs *et al*., 2005; Rummens *et al*., 2003). In both studies, $1,25(OH)_2D_3$ was increased in *VDR* null fetuses (Kovacs *et al*., 2005; Rummens *et al*., 2003). Similarly, placental calbindin-D_{9k} (a calcium-binding protein required for placental calcium transfer) was found to have a similar location and expression level in *VDR* null and wild-type placentas in both investigations (Kovacs *et al*., 2005; Rummens *et al*., 2003).

New world monkeys may also be used as a model for HVDRR. The marmoset (*Callithrix jacchus*) in particular requires a diet high in vitamin D_3 for normal growth. It has $1,25(OH)_2D_3$ concentrations that are four to ten times higher than those seen in rhesus monkeys or humans, with no evidence of hypercalcaemia (in fact normocalcaemia or hypocalcaemia may be seen) suggesting that the marmoset has relative end-organ resistance to $1,25(OH)_2D_3$ (Shinki *et al*., 1983). On an x-ray and histological examination of 20 marmosets, three were found to have changes consistent with osteomalacia. These monkeys also had lower 25(OH)D concentrations than non-osteomalaciac marmosets (Yamaguchi *et al*., 1986). Recent evidence has suggested that the resistance of marmosets to $1,25(OH)_2D_3$ is not due to the VDR, but instead due to overexpression of a VDR-independent vitamin D-response element binding protein, which interferes with vitamin D-regulated transactivation (Chen *et al*., 2000; Chun *et al*., 2001). Marmosets also have high circulating glucocorticoid concentrations, and are resistant to high levels of glucocorticoids, indicating that these monkeys may be resistant to a variety of steroid hormones, not just $1,25(OH)_2D_3$ (Shinki *et al*., 1983).

The *DMP1* knock-out mouse is a model for autosomal recessive hypophosphataemic rickets. These mice have renal phosphate wasting, elevated FGF23 and inappropriately normal serum $1,25(OH)_2D_3$ concentrations (Feng *et al*., 2006; Lorenz-Depiereux *et al*., 2006). DMP1 is thought to promote mineralisation of osteoid, and may be required for differentiation of osteoblasts into osteocytes (Feng *et al*., 2006; Schiavi, 2006). DMP1 also leads to up-regulation of FGF23, by an unknown pathway (Liu *et al*., 2008; Schiavi, 2006).

A transgenic rat model with constitutive expression of 24-hydroxylase has been developed, but it showed an unexpected phenotype (Kasuga *et al*., 2002). An increase in $24,25(OH)_2D_3$ levels was expected but the transgenic rats had decreased $24,25(OH)_2D_3$ levels and normal $1,25(OH)_2D_3$ levels when compared with wild-type rats (Kasuga *et al*., 2002). These rats developed glomerulonephritis leading to loss of albumin, vitamin D-binding protein and $25(OH)D_3$ into the urine (Hosogane *et al*., 2003; Kasuga *et al*., 2002). The transgenic rats also developed hyperlipidaemia and aortic atherosclerosis (Kasuga *et al*., 2002). The development of the glomerulonephritis and renal insufficiency, was thought to be due to constitutive expression of CYP24 during glomerulogenesis (Kasuga *et al*., 2002), however no evidence was presented to support this hypothesis. The renal protein wasting may therefore override the effects of constitutive expression of 24-hydroxylase, suggesting that these transgenic rats may not be a credible model for constitutive expression of 24-hydroxylase.

1.6.2 SHEEP AND GOATS

Rickets is uncommon in sheep in New Zealand and all reported cases have been nutritional in origin (Fitch, 1943). The first report of metabolic bone disease in sheep in New Zealand was a condition called osseous cachexia diagnosed on phosphorus deficient properties (Reakes, 1912; Reid and Aston, 1910). Fitch and Ewer both performed extensive investigations into rickets in sheep in New Zealand in the 1940s and 1950s (Ewer and Bartrum, 1948; Fitch, 1943; Fitch, 1944; Fitch and Ewer, 1944). In 1953, Ewer stated that there were three factors that influenced the vitamin D requirements of sheep: the amount of sunshine, the type of grazing and the level of calcium and phosphorus in the diet (Ewer, 1953). Five hours of winter sunshine at Wallaceville, New Zealand (41°S) was shown to be insufficient to prevent rickets in sheep (Ewer, 1953). Rickets occurred most commonly in the South Island, particularly Canterbury and Otago, in association with the feeding of greenfeed oats to hoggets during winter months. Calcium and phosphorus levels in the crops were adequate (Ewer and Bartrum, 1948), and the cause of rickets in these sheep was shown to be due to high concentrations of available carotene (Grant and O'Hara, 1957). This has been confirmed with experiments in rats and humans, where vitamin A was shown to antagonise the action of vitamin D on the intestine and bone (Johansson and Helhus, 2001; Rohde *et al*., 1999). While rickets is most common in sheep fed cereal crops, the disease may also occur in lambs grazing pasture, Italian ryegrass, choumoellier, and turnips (Ewer and Bartrum, 1948). Such feeds also have high concentrations of carotenes, however cereal crops may be more rachitogenic due to the higher availability of carotene (Grant and O'Hara, 1957).

Naturally occurring rickets is also uncommon in sheep in the United Kingdom (Bonniwell *et al*., 1988; Ewer, 1950; Mearns *et al*., 2008; Nisbet *et al*., 1966) but outbreaks associated with a high latitude, low sunshine hours and poor quality pasture low in phosphorus have occurred in Scotland (Bonniwell *et al*., 1988; Nisbet *et al*., 1966). In Ireland, rickets has occurred in sheep fed oats in winter months, and in northern England in sheep grazing lush ryegrass swards over winter, most likely due to the rachitogenic effect of carotenes (Crowley, 1961; Mearns *et al*., 2008).

Clinical signs of rickets in sheep include stiff gait, lameness, enlarged joints, particularly the radio-carpal, bowed or bent legs and loss of condition or poor weight gain (Bonniwell *et al*., 1988; Crowley, 1961; Duckworth *et al*., 1961; Ewer, 1953; Fitch, 1943; Nisbet *et al*., 1966; Van Saun, 2004). Rickets is most commonly seen in weaned animals, generally hoggets, because of the rapid growth at this age and their dependence on pasture or crops (Bonniwell *et al*., 1988; Duckworth *et al*., 1961; Duckworth *et al*., 1943; Ewer, 1953; Nisbet *et al*., 1966; Van Saun, 2004).

Sheep have a seasonal low in serum 25(OH)D concentrations in late winter, due to fleece cover and reduced exposure to ultraviolet light, which is exacerbated by the demands of pregnancy (Smith and Wright, 1981; Smith *et al*., 1987). The serum vitamin D concentration of lambs has been shown to be closely related to the vitamin D concentration of the dam (Smith and Wright, 1981; Smith *et al*., 1987). Lambs at 3-4 weeks are predominantly feeding on milk which may have

low vitamin D concentrations if the vitamin D status of the dam is inadequate (Van Saun, 2004).

Numerous experiments have been performed in an effort to better understand the relationship between dietary calcium, phosphorus and vitamin D, and the development of rickets in sheep. Several authors have described the effect of different concentrations of dietary phosphorus on skeletal development, but comparison of their results is difficult due to inconsistencies over the concentration of phosphorus in deficient and control diets (Duckworth *et al*., 1943; Martin and Peirce, 1934; Stewart, 1935). The general trend however is similar, with low phosphorus diets leading to hypophosphataemia, normal to increased levels of serum calcium and bones with lower total ash content and lower bone density than controls (Ewer, 1951; Field *et al*., 1975; Hemingway, 1963; Martin and Peirce, 1934; Stewart, 1935; Young *et al*., 1966). Once again, inconsistencies in the concentrations of dietary calcium in deficient and control diets, make comparison between studies difficult (Auchinachie and Fraser, 1932; Duckworth *et al*., 1943; Martin and Peirce, 1934). Diets low in calcium led to low serum calcium concentrations, increased blood inorganic phosphorus concentrations and decreased total bone ash content (Auchinachie and Fraser, 1932; Field *et al*., 1975).

The ratio of calcium to phosphorus may also be a factor in the development of rickets. In one study, a dietary Ca:P ratio of approximately 29:1 produced severe hypophosphataemia, and clinical and radiographic signs of rickets (Ewer, 1951). However, the diet also had a low vitamin D concentration, the

experimental group was small, and the diagnosis of rickets was not confirmed histologically.

Various forms and dose rates of supplementary vitamin D have been tested in sheep fed rachitogenic diets (Ewer, 1950; Fitch, 1944; Fitch and Ewer, 1944; Green, 1953). Despite this variation, vitamin D supplementation has been shown to reduce both the incidence and severity of rickets (Fitch, 1944; Fitch and Ewer, 1944).

An unusual outbreak of rickets was described in lambs of 3-4 weeks of age in Oregon, USA (Van Saun, 2004). Since ewes' milk may be low in vitamin D, vitamin D deficiency was considered the most likely cause of the outbreak. While rickets was not confirmed histologically, the clinical signs, low 25(OH)D concentrations and positive response to treatment with cholecalciferol indicate that vitamin D deficiency was the likely cause of the bone deformities (Van Saun, 2004).

Skeletal lesions resembling rickets have been reported in goat kids on a large commercial goat farm in The Netherlands. The kids had been fed artificial milk intended for calves (Dercksen and Berger, 1992).

1.6.3 CATTLE

The earliest published investigations on rickets and osteomalacia in cattle were conducted by Theiler and colleagues in South Africa. In their studies,

phosphorus deficiency was shown to be the basis of a syndrome characterised by osteophagia, stiff gait, lameness, swollen joints and sometimes spontaneous fracture (Theiler, 1931). Gross and microscopic lesions of rickets and/or osteomalacia were seen at necropsy (Theiler, 1931). Vitamin D deficiency was not considered to be a contributing factor due to the abundant sunshine available in South Africa (Theiler, 1931).

A similar syndrome reported in the Northern Territory of Australia, was referred to as “stiffs”, “creeps” or “peg-leg”. The disease occurred during periods of drought, due to a lack of access to surface water, swamps, flood-outs and red ground (Rose, 1954). Cattle were in poor condition, stiff-legged and weaners sometimes had enlargement of the carpal joints (Rose, 1954). Osteophagia was a feature, and phosphorus deficiency was considered the cause (Rose, 1954).

Cattle are considered more susceptible than sheep to phosphorus deficiency (Thompson, 2007). In a 10 year trial investigating the effect of phosphorus deficiency in cattle, the main skeletal changes were osteoporosis and osteomalacia (Shupe *et al*., 1988). When dietary phosphorus was less than 6g per day, loss of body condition occurred, as did lameness, abnormal stance, spontaneous fractures, reproductive failure, and hypophosphataemia. Clinical recovery occurred within 6 months once the dietary phosphorus deficiency was corrected (Shupe *et al*., 1988).

There are few published reports of naturally occurring rickets in cattle, and no

inherited forms have been described. One report involves four yearling bullocks housed inside and fed on oats, sugar-beet pulp, barley, hay and raw potatoes (Spratling *et al*., 1970). These animals showed clinical signs consistent with rickets. The cows on the farm showed obvious signs of fluorosis, and this may have contributed to the development of rickets, as rickets has been reported in children in areas of India with water containing high fluoride levels (Khandare *et al*., 2005). Fluoride stimulates bone formation, thus increasing skeletal demand for calcium and exacerbating vitamin D deficiency (Khandare *et al*., 2005; Kurland *et al*., 2007). Plasma vitamin D levels were one-sixth those of a normal yearling (Spratling *et al*., 1970).

A similar skeletal disease was reported in Sweden, in fattening bulls kept indoors and fed a concentrated diet with high levels of phosphorus (Jonsson *et al*., 1972). The clinical, biochemical and radiological changes were consistent with rickets and histologically there were features of both fibrous osteodystrophy and rickets. This combination would be expected in vitamin D deficiency, which leads to low calcium and subsequent secondary hyperparathyroidism. Treatment with vitamin D led to resolution of clinical signs (Jonsson *et al*., 1972).

In New Zealand, rickets was reported in yearling Angus steers fed on a swede crop over winter. Affected cattle had poor growth rate, and lameness that became more severe when moved from the crop onto hill country grazing land (Thompson and Cook, 1987). Steers developed vertebral fractures leading to posterior paralysis, and rickets was confirmed histologically (Thompson and Cook, 1987). The phosphorus concentration of the crop, when combined with

the low dry matter content, was inadequate to supply daily phosphorus requirements (Thompson and Cook, 1987). Once transferred to pasture with the addition of bone flour, mildly affected animals recovered (Thompson and Cook, 1987).

1.6.4 LLAMAS AND ALPACAS

Since being transported from the Andes to other parts of the world, llamas and alpacas have been found to be highly susceptible to rickets. In the high Andes, the South American camelid's natural setting, solar irradiation is intense. Consequently, the llama and alpaca may have evolved a thick hair coat and pigmentation to protect them from strong sunlight (Bryant and Farfan, 1984; Hill *et al*., 1994; Van Saun *et al*., 1996). In areas of high latitude, and low altitude, solar irradiation is much lower, and vitamin D levels in llamas and alpacas in these areas decline over winter (Smith and Van Saun, 2001). In one study, crias born in autumn/winter had lower vitamin D concentrations, and were more likely to develop rickets, than those born in summer (Van Saun *et al*., 1996). A peak in the incidence of rickets in crias occurs from January to March in the Northern Hemisphere (Van Saun *et al*., 1996). This may be because these animals receive less vitamin D via the placenta or colostrum due to the low level of solar radiation at that time of year (Smith and Van Saun, 2001), and a young cria's diet consists substantially of milk, which is low in vitamin D (Van Saun *et al*., 1996). As in other animals, the disease in camelids can be easily treated and prevented with either oral or injectable vitamin D (Judson and Feakes, 1999).

1.6.5 Horses

Reports of rickets in horses are rare, and rickets appears to occur less frequently than in other species (El Shorafa *et al.*, 1979). Experimentally, Shetland ponies deprived of sunlight and dietary vitamin D showed irregularly widened physes on radiographs, consistent with rickets (El Shorafa *et al.*, 1979). Horses have higher serum calcium concentrations, and lower serum 25(OH)D and $1,25(OH)_2D_3$ concentrations than other animal species (Breidenbach *et al.*, 1998; Maenpaa *et al.*, 1988; Smith and Wright, 1984). In fact, the vitamin D metabolite concentrations (25(OH)D and $1,25(OH)_2D_3$) in horses are lower than those at which rickets occurs in other animals (Breidenbach *et al.*, 1998), suggesting a species variation and perhaps reflecting a different mechanism for calcium metabolism in horses.

1.6.6 Pigs

Piglets grow rapidly and are weaned early, making them susceptible to the development of rickets if their diet contains inadequate vitamin D (Thompson, 2007). Due to intensive pig farming and the housing of pigs indoors, vitamin D requirements of the pig must be supplied by the diet (Thompson, 2007).

An outbreak of rickets affecting 82 pigs was described on a small fattening unit in Scotland (Pepper *et al.*, 1978). The pigs were emaciated, had difficulty rising, bowed forelegs, joint swellings and pain on moving (Pepper *et al.*, 1978). Folding and erosion of articular cartilage and enlargment of long bone metaphyses and costochondral junctions were seen radiographically and during post-mortem examination (Pepper *et al.*, 1978). Histologically, there were

changes consistent with both rickets and nutritional secondary hyperparathyroidism (Pepper *et al.*, 1978). A similar case was reported on a semi-intensive pig farm in New Zealand, where the pigs were housed indoors and fed a diet with no vitamin supplement (Thompson and Robinson, 1989). Affected pigs had a shifting lameness, were reluctant to rise, but had no joint swellings (Thompson and Robinson, 1989). A combination of nutritional secondary hyperparathyroidism and rickets were seen histologically, with myelofibrosis, and multifocal thickening of physes (Thompson and Robinson, 1989). No further cases were seen once fat soluble vitamins (including vitamin D) were added to the diet (Thompson and Robinson, 1989).

Of particular interest in pigs is the presence of a genetic form of rickets similar to VDDR I in humans. The condition was originally described in pigs in 1962, and a strain (the Hannover) was bred with the syndrome. Pigs affected with VDDR I are used as a model for the same disease in humans (Fox *et al.*, 1985).

From 3 to 8 weeks of age piglets with VDDR I develop progressive hypocalcaemia, and hypophosphataemia, leading to secondary hyperparathyroidism, and rickets (Fox *et al.*, 1985; Wilke *et al.*, 1979). Concentrations of $1,25(OH)_2D_3$ in the blood were low but significantly above zero, while concentrations of $25(OH)D_3$ and PTH were high (Fox *et al.*, 1985; Kaune and Harmeyer, 1987; Wilke *et al.*, 1979). Calcium-binding protein concentrations in the plasma and intestine were reduced and no 25-hydroxyvitamin D_3-1α-hydroxylase and 24-hydroxylase activity was found in renal homogenates (Fox *et al.*, 1985). A single high dose of cholecalciferol

produced a temporary elevation in serum 1,25$(OH)_2D_3$ and short-term healing of the clinical signs of rickets, possibly by extra-renal production of 1,25$(OH)_2D_3$ (Kaune and Harmeyer, 1987). The disease can be treated with lifelong pharmacological doses of cholecalciferol or physiological doses of 1,25$(OH)_2D_3$ (Fox *et al*., 1985; Kaune and Harmeyer, 1987).

Pregnant sows with VDDR I had low serum concentrations of 1,25$(OH)_2D_3$ during pregnancy (Lachenmaier-Currle *et al*., 1989). Serum concentrations of 25$(OH)D_3$, calcium and phosphate increased during pregnancy, until the last 3 weeks, when calcium and phosphate concentrations declined sharply (Lachenmaier-Currle *et al*., 1989). Fetal calcium and phosphorus concentrations however were normal, suggesting that the placenta of pigs may transfer calcium and phosphorus without the need for vitamin D (Lachenmaier-Currle *et al*., 1989).

At the molecular level, VDDR I in the Hannover pig is associated with one of two different deletions in the *P450C1* coding region (Chavez *et al*., 2003). One involves a deletion of 173 base pairs (bp); the other a deletion of 329 bp, both of which lead to a frame-shift mutation, and a premature stop codon (Chavez *et al*., 2003). The result produces conformational changes that make the enzyme ineffectual due to loss of the heme-binding region and other domains (Chavez *et al*., 2003). The authors hypothesised that a mRNA processing error may have caused the deletions as they occurred at mRNA processing sites (Chavez *et al*., 2003).

1.6.7 DOGS

There are few reports of naturally occurring rickets in dogs and cats and the disease is considered rare (Bennett, 1976). This may be due to the extensive feeding of commercially formulated food. The most common unsuitable diets fed to pups and kittens comprises meat or offal, which are more likely to cause nutritional secondary hyperparathyroidism due to their low Ca:P ratio (Malik *et al*., 1997). As mentioned previously, cats and dogs require a dietary source of vitamin D as ultraviolet radiation does not increase plasma 25(OH)D_3 concentrations (How *et al*., 1994; Morris, 1999).

Over the last 20 years, three reports of rickets have occurred in Australia. Two involved litters of greyhound pups, and the other a 12-week-old collie dog (Lavelle, 1988; Malik *et al*., 1997). The collie, and one litter of greyhounds were reared on a diet of milk and meat. The other greyhound litter had an unreliable dietary history (Lavelle, 1988; Malik *et al*., 1997). The age at presentation ranged from 10 weeks for the collie, to 14 weeks for one of the greyhound litters (Lavelle, 1988; Malik *et al*., 1997). Clinical signs and radiographic changes in all dogs were consistent with rickets. Serum 25(OH)D concentrations were measured in one greyhound litter and found to be low (Malik *et al*., 1997). The low concentration of vitamin D in milk, together with low maternal vitamin D status and the rapid growth of greyhound pups may have predisposed to rickets in these litters (Malik *et al*., 1997).

Recently, rickets has been reported in a Shetland sheepdog diagnosed with acute renal failure at 10 weeks of age, and put on to a low phosphate renal failure diet (McMillan *et al*., 2006). Ten weeks later, the dog presented with varus deformities of the forelimbs, failure to grow, prominent metaphyses, and radiographically it had widened growth plates (McMillan *et al*., 2006). The renal failure diet had low protein and phosphate levels, which were inadequate for growth. Replacement of the diet with one specific for growing puppies led to resolution of clinical signs (McMillan *et al*., 2006).

A possible inherited form of VDDR I was diagnosed in a Saint Bernard dog in Australia (Johnson *et al*., 1988). The female pup, fed commercial dog food and supplemented with powdered calcium carbonate and multivitamin drops was presented at 12 weeks of age with forelimb abnormalities. Serum calcium concentration was within normal limits, phosphorus was low, and plasma PTH concentration was elevated. Radiographs showed changes consistent with rickets. Treatment consisted of calcium carbonate and dihydrotachysterol, a synthetic vitamin D analogue, which is hydroxylated in the liver to a $1,25(OH)_2D_3$ analogue (Johnson *et al*., 1988). The authors considered a 25-hydroxyvitamin D-1α-hydroxylase enzyme deficiency to be the most likely cause of the clinical signs seen in this dog (Johnson *et al*., 1988), but serum 25(OH)D and $1,25(OH)_2D_3$ concentrations were not measured and a diagnosis of VDDR I was not confirmed.

1.6.8 CATS

Naturally occurring rickets is rare in cats. Experimentally, cats fed a vitamin D-deficient diet developed clinical signs of rickets after 4-5 months (Anonymous, 1958). The diagnosis of rickets was not confirmed histologically, and was based on radiographic changes and increased serum alkaline phosphatase concentrations, which are not specific for rickets.

There have been several reports of possible HVDRR in cats (Godfrey *et al*., 2005; Henik *et al*., 1999; Schreiner and Nagode, 2003; Tanner and Langley-Hobbs, 2005), but the diagnosis has not always been supported by adequate biochemical data or histopathology. Confirmation of a diagnosis of HVDRR using skin biopsies for fibroblast culture to examine $1,25(OH)_2D_3$ receptor binding was only obtained in one case (Godfrey *et al*., 2005). Other reported cases do however appear similar. Affected kittens usually presented at around 4 months of age, perhaps reflecting the time required for kittens born with normally mineralised bones to develop signs of vitamin D deficiency (Hochberg, 2002). This assumes that transplacental calcium transport in cats does not require vitamin D. The kittens showed different responses to treatment, as seen in humans with HVDRR, presumably due to different defects in the VDR (Malloy *et al*., 1999).

In one report, a male, 4-month-old cat was presented with reduced mobility and bowing of the front legs (Godfrey *et al*., 2005). PTH and $1,25(OH)_2D_3$ concentrations were elevated, while calcium and phosphorus concentrations were low (Godfrey *et al*., 2005). Skin biopsies for fibroblast cell culture were

taken when the cat was 4 years of age, to assess the binding of 1,25(OH)$_2$D$_3$ to the VDR (Godfrey *et al*., 2005). The cat's VDR showed an inability to bind active vitamin D, supporting a diagnosis of HVDRR. At 5 years of age the cat developed degenerative joint disease and required euthanasia at 9 years of age due to hip pain (Godfrey *et al*., 2005).

In another report, a 4-month-old kitten was presented with bilateral forelimb swelling, reluctance to jump and stiff gait (Tanner and Langley-Hobbs, 2005). Serum phosphate concentration was normal, however the ionised calcium concentration was low (Tanner and Langley-Hobbs, 2005). Bone density was decreased on radiographs, and there was widening and flaring of the growth plate. Serum PTH concentration was increased, 25(OH)D$_3$ was within normal limits, and 1,25(OH)$_2$D$_3$ was significantly elevated leading to a presumptive diagnosis of vitamin D-dependent rickets type II (Tanner and Langley-Hobbs, 2005). Treatment with calcium and calcitriol was not successful, ionised calcium concentration remained low, the kitten failed to grow and died at 13 months of age (Tanner and Langley-Hobbs, 2005). The diagnosis of rickets was not confirmed by histology and vitamin D-binding assays were not performed.

1.7 AIMS OF THE THESIS

During 2003 and 2004, a commercial Corriedale sheep breeding and fattening property in Marlborough had up to 20 lambs per year affected with a syndrome characterised by reduced growth rate and skeletal deformities. Initial investigations suggested the skeletal deformities were consistent with rickets,

and a genetic aetiology was considered likely. The primary objectives of this study were to:

a) Test the hypothesis that this disease of Corriedale sheep is inherited and if so, to determine the mode of inheritance.

b) Describe in detail the phenotypic characteristics of the disease using imaging techniques, serum chemistry measurements, macroscopic and microscopic pathology.

c) Determine the disease mechanism.

CHAPTER 2

DISEASE INHERITANCE

2.1 INTRODUCTION

Although most cases of rickets in humans and animals are caused by either reduced exposure to sunlight or dietary deficiencies of vitamin D or phosphorus, inherited forms of the disease are also reported (Whyte, 2002). These hereditary rachitic diseases occur in humans and pigs, with isolated cases reported in cats and dogs (Fox *et al*., 1985; Godfrey *et al*., 2005; Johnson *et al*., 1988; Whyte, 2002) and may result from a defect in vitamin D activation, end-organ unresponsiveness or renal phosphate wasting (Whyte, 2002).

Over two lambing seasons (2004-2005), a commercial sheep farm in Marlborough had up to 20 lambs per year showing skeletal abnormalities, the clinical signs and skeletal lesions strongly suggesting a diagnosis of rickets. Phosphorus deficiency was considered unlikely, as the sheep were grazing

improved pasture with high soil Olsen P levels and vitamin D deficiency was also improbable, as the Marlborough region has a high number of sunshine hours (National Institute of Water and Atmospheric Research, 2006). A hereditary form of rickets was therefore considered likely. The farmer had purchased rams from the same breeder for approximately 12 years, and so it was possible that a defective gene could be widespread throughout the flock (Thompson *et al*., 2007).

In order to confirm the hypothesis that the disease was inherited, two breeding trials were designed. The first trial used embryo transfer technology, while the second was a larger scale out-cross followed by a back-cross trial to determine the mode of inheritance. The aims of the breeding trials were to verify that this skeletal disease of Corriedale sheep was inherited, to determine the mode of inheritance, and to generate further animals for study.

2.2 Materials and Methods

2.2.1 Animals

The breeding trials were based on ten affected Corriedale sheep and one adult Corriedale ram that were transported from the flock of origin in Marlborough to a property in the Manawatu at 5 months of age. The nine recipient sheep for the embryo transfer trial were Romney cross mixed aged ewes from a commercial Manawatu sheep farm.

The adult ram was one of four rams that had been shown by DNA analysis to have sired affected lambs in the 2004 breeding season and was therefore a putative heterozygous animal. The out-cross trial was conducted on a commercial sheep and beef enterprise near Turakina. The ewes used in the first year of the large scale breeding trial (2006) were 250 mixed-aged Romney sheep. In 2007, after mating with the putative heterozygous ram, 77 female Romney-Corriedale hoggets mated by the ram in the back-cross trial were transported from Turakina to Keebles Farm, Massey University, Palmerston North. Approval was obtained from the Massey University Animal Ethics Committee for the trials (approval number 05/123).

2.2.2 Embryo Transfer

The embryo transfer program was carried out by Premier Breeding Services, Feilding. Three affected ewes were available for superovulation. Semen was successfully collected from an affected ram using an artificial vagina, and a ewe treated with oestrogen. The oestrus cycles of the three donor ewes, and nine recipient ewes were synchronised (Appendix 1), using controlled internal drug releasing (CIDR) intravaginal devices (CIDR-G, Pharmacia & Upjohn Ltd. Co., New Zealand), follicle stimulating hormone (Folltropin-V, Vetrepharm Inc., Canada) and pregnant mare serum gonadotrophin (Pregnecol, Vetrepharm Inc., Canada). The artificial insemination and embryo transfer was performed laparoscopically on anaesthetised sheep. In total, 16 embryos were collected from the three donor ewes and inserted into nine suitably prepared recipient ewes.

2.2.3 Out-Cross and Back-Cross Trial

In 2006, 250 phenotypically normal, unrelated Romney ewes were out-crossed to the phenotypically normal putative heterozygous ram, with the aim of producing daughters for back-crossing to their sire. The 101 daughters (F1 generation) were mated back to their sire in the following breeding season (2007).

For the out-cross, the heterozygous ram was harnessed with a coloured crayon and left with the ewes from 1 March 2006 to 28 March 2006. The female progeny from the out-cross mating were mated with the same heterozygous ram the following year from 2 May 2007 to 29 June 2007. The crayon was changed at the end of the first and second cycles. The ewes were vaccinated with Campylovexin (Schering-Plough Coopers Animal Health New Zealand Ltd., New Zealand) and Ultravac 5 in 1 (CSL New Zealand Ltd., New Zealand), and injected with iodised oil (Flexidine, Bomac Laboratories Ltd., New Zealand) prior to mating. Seven weeks after the end of mating, the ewes were ultrasound scanned for pregnancy.

2.2.4 Data Recording

2.2.4.1 Embryo Transfer Trial

Newly-born lambs were ear-tagged to their mother, and their birth date, rank and sex were noted. Blood was collected by jugular venipuncture into plain evacuated tubes weekly until 6 weeks of age, and then at 8, 10 and 12 weeks of age. The lambs were monitored for clinical signs consistent with rickets.

2.2.4.2 BACK-CROSS LAMBING

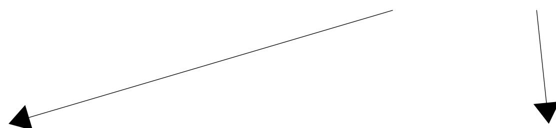
From 2 weeks prior to the calculated date of parturition until the end of lambing, the pregnant sheep were checked twice daily. Any newly-born lambs were caught and double ear-tagged to their mother. The tag number was also sprayed on their side to make identification in the paddock easier. The birth date, rank, and sex were noted. Blood was collected by jugular venipuncture into a plain evacuated tube. The blood collection process was repeated fortnightly for the first 8 weeks of life for each lamb, and then again at 12 weeks of age. Morphometric measurements as described in Chapter 3 were also performed at this time.

2.2.1 DATA ANALYSIS

The chi-squared test can be used as a goodness-of-fit test to determine whether the frequencies of some observed categorical variable in sample data complies with the hypothesised frequencies. However, when observed and expected numbers are less than five, an exact test should be used.

Based on an out-cross followed by a back-cross (Figure 2.1) the expected outcome if the disease has an autosomal recessive inheritance is that 12.5% of the lambs born in the back-cross will be affected.

Out-cross		Heterozygous Ram	
		R	**r**
Unrelated Ewes	**R**	RR	Rr
	R	RR	Rr



Back-cross		Heterozygous Ram	
		R	**r**
Homozygous F_1 ewes	**R**	RR	Rr
	R	RR	Rr

		Heterozygous Ram	
		R	**r**
Heterozygous F_1 ewes	**R**	RR	Rr
	r	Rr	rr

rr = affected animals
1/8 affected = 12.5%

Figure 2.1: Expected outcome from an out-cross with a heterozygous ram and unrelated ewes followed by a back-cross with the heterozygous ram and F_1 generation. From the back-cross 12.5% are expected to be affected with the disease if inheritance is autosomal recessive. R = dominant gene; r = recessive gene.

2.3 RESULTS (YEAR ONE)

2.3.1 EMBRYO TRANSFER

Of the 16 embryos transferred, 13 implanted successfully. One ewe (no. 28) died 2 weeks prior to the due date of parturition, but the fetus was retrieved for examination. The remaining eight ewes lambed over a 7 day period (18-25 October 2006) as shown in Table 2.1. Lamb 102 from dam 10, died 24 hours after birth due to hypoxia secondary to rib fractures sustained during birth.

Lamb 201 from dam 20 also sustained rib fractures during parturition and died at 6 days of age after developing dyspnoea and having difficulty suckling. Both lambs had required assisted delivery due to mild dystocia. Lambs 171, 241, 271, 272 and 291 were euthanased (see Table 2.1) once clinical signs indicated that they were suffering. All lambs were determined to be affected with rickets on the basis of clinical signs, serum calcium and phosphate concentrations and/or post-mortem examination and histology. The remaining lambs from the trial are being grazed on a commercial property in the Manawatu.

Table 2.1: Lambs born to recipient ewes in the embryo transfer breeding trial.

Dam ID	Sire ID	Recipient ID	Lamb ID	Date of birth	Sex	Comment
2363	2362	10	101	21/10/06	F	
			102	21/10/06	M	Died 22/10/06, assisted birth
		17	171	18/10/06	F	Euthanased 24/07/07
			172	18/10/06	F	
		20	201	23/10/06	M	Died 29/10/06, assisted birth
		21	211	20/10/06	F	
2353	2362	24	241	25/10/06	F	Euthanased 22/03/07
		26	261	20/10/06	M	
			262	20/10/06	F	
		27	271	19/10/06	M	Euthanased 28/03/08
			272	19/10/06	F	Euthanased 16/09/08
		28	281		M	Recipient ewe died 6/10/06
2358	2362	29	291	21/10/06	F	Euthanased 15/01/07

2.3.2 Out-Cross Trial - Year One

2.3.2.1 Mating and Parturition

During the 4-week mating period, the heterozygous ram marked 196 ewes. Of these, 25 ewes were re-marked by the commercial rams, and two were culled for other reasons, leaving 159 ewes considered pregnant to the heterozygous ram. At scanning on the 1 June 2006, 11 ewes were found to be empty, the rest scanned as in-lamb.

The 148 ewes pregnant to the heterozygous ram produced 212 lambs at docking (lambing percentage of 143% [212/148]), of which 105 were female and 107 were male. After weaning, 101 ewe lambs were available to back-cross in the second year of the breeding trial. The target mating weight for the ewe hoggets was 40 kg. By 1 May 2007, 83 of the ewe hoggets had reached this target with an average weight of 44.7 kg.

2.3.3 Back-Cross Trial - Year Two

2.3.3.1 Mating, Parturition And Observations

During the 8 week period of mating, 99 ewe hoggets were available for mating and the heterozygous ram marked 40 ewes to the first cycle, 37 to the second cycle, and none on the third cycle. In July, the 77 marked ewes were transported to Keebles Farm, Massey University, Palmerston North. Ultrasound scanning was performed on 10 August 2007 and showed that 58 ewes were empty, 13 were pregnant with singles, and five were carrying twins.

This gave an overall expected lambing percentage of 30% (23/77). The 18 hoggets gave birth to the expected 23 lambs. Of these, eight died either immediately after or within 2 days of birth. The first lamb was born on 1 October, and the last on 30 October 2007.

Of the eight lambs that died soon after birth, three died as a result of dystocia and three from suspected exposure. One lamb (5571) had a heart defect consisting of a dextraposed aorta and a ventricular septal defect, which would have been incompatible with life. The lamb that died at 2 days of age was autolysed and had been frozen, so the tissues were difficult to interpret and a cause of death was not determined. Histological examination of bones collected at post-mortem examination indicated that one of the eight lambs that died in the neonatal period (number 5557) had rickets.

One lamb was suspected at birth as being affected with rickets. Lamb 5573 walked gingerly and unsteadily with stiff, unbent legs, but gradually improved over the first week of life. This lamb also developed lordosis in the mid-thoracic region by 2 weeks of age. By 1 month of age, two other lambs had developed clinical signs consistent with rickets, including exaggerated lordosis in the mid-thoracic region, decreased growth rate and weight gain. Serial serum calcium and phosphate measurement and subsequent histological examination of bones collected at necropsy confirmed that all three lambs had lesions consistent with rickets.

2.3.3.2 STATISTICAL ANALYSIS

Rickets was diagnosed in four of the lambs born to the back-cross breeding trial, giving an incidence rate of 17.4% (Table 2.2). The expected incidence of affected animals if the disease has autosomal recessive inheritance is 12.5%.

Either chi-square or an exact test of the null hypothesis (that inheritance is autosomal recessive and the expected incidence of affected animals is 12.5%) gives a P-value of greater than 0.5. Therefore we can accept the hypothesis that the observed proportion of affected animals agrees with the expected proportion and any difference is likely to be due to chance (Table 2.2). There is no evidence to suggest that these results are not consistent with autosomal recessive inheritance.

Table 2.2: Number of offspring either affected or not affected with inherited rickets and chi-squared analysis of a cross between a putative heterozygous ram and F_1 generation ewes.

	Affected	**Not affected**	**Total**
Observed	4	19	23
Expected	2.875	20.125	23

2.4 DISCUSSION

The generation of lambs with rickets in these two breeding trials confirmed the inherited nature of the disease. All 13 lambs derived by embryo transfer from affected dams and an affected ram had the disease. The incidence of inherited rickets in the back-cross trial was 17.4% and together with the lack of evidence of disease in obligate heterozygotes, supports autosomal recessive inheritance.

In humans and pigs, vitamin D-dependent rickets type I caused by a mutation in the *1α-hydroxylase* gene, is inherited as an autosomal recessive trait (De Braekeleer and Larochelle, 1991; Fox *et al*., 1985). Similarly, hereditary vitamin D-resistant rickets of humans is due to autosomal recessive inheritance of a vitamin D receptor defect (Malloy and Feldman, 1999). In contrast, hypophosphataemic rickets of people may have either X-linked inheritance, autosomal dominant inheritance or autosomal recessive inheritance, depending on the mutation involved. X-linked hypophosphataemic rickets is the most common form of inherited rickets and is thought to be due to X-linked dominant inheritance of defects in the *PHEX* (Phosphate-regulating gene with Homologies to Endopeptidases on the X-chromosome) gene (Du *et al*., 1996). Autosomal dominant hypophosphataemic rickets is the result of the loss of a cleavage site on fibroblast growth factor 23 and, as the name suggests, is inherited in an autosomal dominant manner (Bai *et al*., 2003; The ADHR Consortium, 2000). Hereditary hypophosphataemic rickets with hypercalciuria and autosomal recessive hypophosphataemic rickets are rare forms of hypophosphataemic rachitic disease in humans with autosomal recessive inheritance (Feng *et al*., 2006; Tieder *et al*., 1985).

The emergence of an inherited form of rickets on a commercial sheep farm was perhaps unexpected, but the fact that purebred Corriedale sheep were being bred for wool production and the farmer had purchased rams from the same breeder for the past 12 years had created the opportunity. DNA analysis showed that four out of ten Corriedale rams used in the 2004 season had sired

affected lambs (Thompson *et al*., 2007). This suggested that the defective gene had become widespread in the ewe flock of the commercial farmer, and was common in the breeders flock.

All of the lambs derived from affected parents by embryo transfer had rickets, indicating that inheritance was most likely recessive. If the disease had dominant inheritance, then 50% of the offspring would have been expected to be clinically normal. By transferring the embryos to normal recipient ewes, any effect of dam hypocalcaemia, hypophosphataemia and vitamin D status on the developing fetus was removed.

Further support for autosomal recessive inheritance of the disease comes from the large scale two year breeding trial. In the first year of the breeding trial the phenotypically normal heterozygous ram was mated to unrelated ewes of a different breed in an out-cross. If the disease had dominant inheritance, then 50% of the progeny from this mating should have been affected, but all lambs born in the first year were clinically normal. In the second year of the breeding trial, the heterozgyous ram was mated back to the F1 generation (his daughters), and if the disease had autosomal recessive inheritance, 12.5% of the progeny should have been affected. In this case the percentage was slightly higher (17.4%, 4/23) than expected, but both chi-squared and an exact analysis indicated that this difference from the expected 12.5% was not significant.

While the results from the embryo transfer trial would be consistent with X-linked inheritance since all animals were affected, the heterozygous ram used in

the larger breeding trial was clinically normal, making X-linked inheritance highly unlikely. In addition, 50% of animals in the second year of the large scale breeding trial would have been expected to exhibit clinical signs of rickets, rather than the 17% obtained.

Of the 100 ewes available for mating in the second year of the breeding trial only 18 of the 77 mated by their sire became pregnant. The reasons for this are unclear, but it may be that the fertility of the ram had markedly declined between the first and second year. A reproductive examination of the ram at the beginning of the trial revealed adequate sperm numbers and quality, but the examination was not repeated in the second year. Another factor in the low pregnancy rate could be the age of the ewes, as lambing percentages from hogget matings can be disappointing (Kenyon *et al*., 2004). Because of the small number of lambs born the statistical power of the trial was reduced, but in spite of this the percentage of lambs with rickets was very close to what would be expected with autosomal recessive inheritance.

2.4.1 SUMMARY

The results of an embryo transfer programme using Corriedale ewes and ram with rickets and an out-cross/back-cross trial using a putative heterozygous ram have confirmed that the disease is inherited, and strongly supported autosomal recessive inheritance. It is likely that the defective trait is relatively common in the flock of at least one Corriedale breeder in New Zealand, and possibly within the breed itself.

Chapter 3

Clinical Signs and Imaging

3.1 Introduction

Rickets and osteomalacia are classic metabolic bone diseases of humans and animals caused by deficiency of either phosphorus or vitamin D. In humans the common clinical signs of rickets include skeletal deformities such as bowed legs, kyphosis and lordosis, prominent costochondral junctions (the so-called rachitic rosary), hypotonia and muscle weakness (Klein, 1999; Wharton and Bishop, 2003). Osteomalacia occurs in adults, and diffuse bone pain is the most common symptom (Klein, 1999). In animals, signs include stiffness and lameness, as well as either varus or valgus limb deformities and swollen joints (Ewer, 1951; Fitch, 1943; Theiler, 1934; Thompson, 2007). In severe cases, affected animals may develop pathological fractures (Theiler, 1931; Thompson, 2007).

A 10-point radiographic scoring method for rickets based on the severity of radiographic changes in the wrists and knees, to produce a score indicating the severity of nutritional rickets, has been developed for human patients (Thacher *et al*., 2000). The key features of this scoring system include widening of the growth plate, irregularity of the physeal-metaphyseal interface, and concavity or cupping (similar in appearance to a champagne glass) of the metaphysis (Pitt, 1995; Thacher *et al*., 2000). Other radiographic changes in humans with rickets may include, swollen costochondral junctions, stress fractures, delayed appearance of ossification centres, curving of long bones, metaphyseal sclerosis and generalised osteopenia (Klein, 1999; Pitt, 1995; Shore and Poznanski, 1999; Thacher *et al*., 2000; Wharton and Bishop, 2003). Stress fractures and decreased bone density are seen radiographically in osteomalacia (Klein, 1999).

Computed tomography (CT) is not used routinely to diagnose rickets or osteomalacia in human patients. In a study using CT to assess osteopenia in children, those with familial hypophosphataemic rickets had a marked decrease in cortical bone density and increased cortical thickness, but bone area was not altered (Kovanlikaya *et al*., 1996). The histogram also showed a broad distribution of cortical bone density values (Kovanlikaya *et al*., 1996). Similarly, CT examinations performed on vertebrae from historical skeletons with rickets revealed increased trabecular thickness but an overall decrease in trabecular bone density (Schamall *et al*., 2003). CT is often used in humans with osteomalacia to search for possible tumours that produce phosphatonins leading to renal phosphate wasting and tumour-induced osteomalacia, rather than to assess bone density (Bielesz *et al*., 2004; Jan de Beur, 2005).

CT is used as an imaging modality in small and large animal practice, but little has been published on CT alterations in animals with rickets. It has been used in sheep to evaluate models for new surgical techniques (Meakin *et al*., 2004; Sarkar *et al*., 2006; Schmidmaier *et al*., 2006) or to predict carcass composition for genetic selection (Lambe *et al*., 2006).

The aim of this study was to describe the clinical signs of inherited rickets in Corriedale sheep and examine in detail the changes present on radiography and peripheral quantitative computed tomography (pQCT). In addition, the study aimed to provide insight into disease progression, provide a reference for the diagnosis of rickets in the live animal, and to complement gross and microscopic pathology.

3.2 Materials and Methods

3.2.1 Animals Used and Samples Collected

In total, 28 animals were examined, 12 from the original Marlborough property, 13 from an embryo transfer breeding trial, and three from a back-cross breeding trial. Five animals, two from the embryo transfer trial, and three from the back-cross breeding trial, died naturally. The remaining animals were monitored regularly for clinical signs of disease, and were euthanased when clinical assessment indicated suffering.

At post-mortem examination, the right ribs 5-9, humerus, radius, metacarpus, femur and tibia, were cleaned of soft tissue, sectioned sagittally and stored in 10% neutral buffered formalin, until processed for histology. The left ribs 5-9, humerus, radius, metacarpus, femur and tibia were also cleaned of soft tissue and stored whole at -80°C prior to radiographic and/or pQCT examination. In nine animals, the mandible, proximal phalanx and either cervical or lumbar vertebrae were also collected and stored in either 10% neutral buffered formalin or at -80°C.

3.2.2 Morphometric Measurements and Analysis

Lambs born as a result of the back-cross breeding trial (Chapter 2), had their birth date, rank, and sex noted. Weight, crown-rump length, girth, forelimb and hindlimb length were measured. Birthweight was measured with a spring scale but from 2 weeks of age bodyweight was measured using a digital livestock scale (Tru-Test XR3000, Tru-Test Ltd., New Zealand). Crown-rump length was defined as the distance between the nuchal crest and the tuber ischii. Girth was measured immediately caudal to the scapula. Forelimb length was measured from the greater tubercle of the humerus to the tip of the hoof with the limb extended. Hindlimb length was measured from the greater trochanter of the femur to the tip of the hoof with the limb extended. Lengths were measured with the lamb in lateral recumbency using a flexible tape measure, with the metal end pressed onto the appropriate bony protuberance, and the length read to the nearest 0.5 cm. The measurements were repeated fortnightly for the first 8 weeks of life for each lamb, and then again at 12 weeks of age.

Statistical analysis was performed using Minitab 15 (Minitab Inc., 2008, USA) software. ANOVA (analysis of variance) general linear models were fitted to compare measurement and weight data between affected and non-affected animals. The basic model fitted was: $length_{(age)}$ ~ sex + birth rank + disease status. In addition, weight was added as a covariate. Growth rate was calculated by dividing the increase in measured length by the number of days. The same model was used for analysis as for measurement lengths.

3.2.3 Radiography and pQCT

Radiographic examination was performed on 12 animals, ranging in age from 1 day to 30 months, nine originating from the Marlborough property, and three obtained from the embryo transfer trial. The left ribs 4-7, left humerus, radius, metacarpus, femur, and tibia were radiographed in a dorso-palmar or dorso-plantar view. The radiographs were taken with the bones laid on a sheet of plastic on top of the radiograph plate using a Philips Optimus 65 X-ray machine (Philips Healthcare Ltd, Netherlands) and AD Mammo fine foil Fujifilm (AD-M Film, FujiFilm Europe GmbH, Germany).

CT examination using a XCT 2000 pQCT computed tomography instrument (Stratec Medizintechnik GmbH, Germany) was performed on the left tibia of 13 Corriedale sheep with inherited rickets aged from 11 weeks to 2 ¾ years, and six age-matched controls (one Corriedale cross, and five mixed breed). Of the affected animals, seven originated from the Marlborough property, three were obtained from the embryo transfer trial, and three from the back-cross trial. Due to the absence of soft tissue, the bone was immersed in a waterbath to

simulate soft tissue density (Figure 3.1A.). Three transverse scans were taken 8.5%, 20% and 50% along the length of the bone (Figure 3.1B.). These positions had been determined by examining radiographs of tibia, measuring the length of the bone and determining the position of the proximal cranial tibial metaphysis (8.5%), distal cranial tibial metaphysis (20%) and mid diaphysis (50%) of the bone as a percentage of bone length. When the reference line was placed after the scout view, the 8.5% scan of some affected sheep passed through the physis due to the thickened physis of sheep with rickets. As a result, another scan was manually placed in the upper metaphysis, immediately below the growth plate.

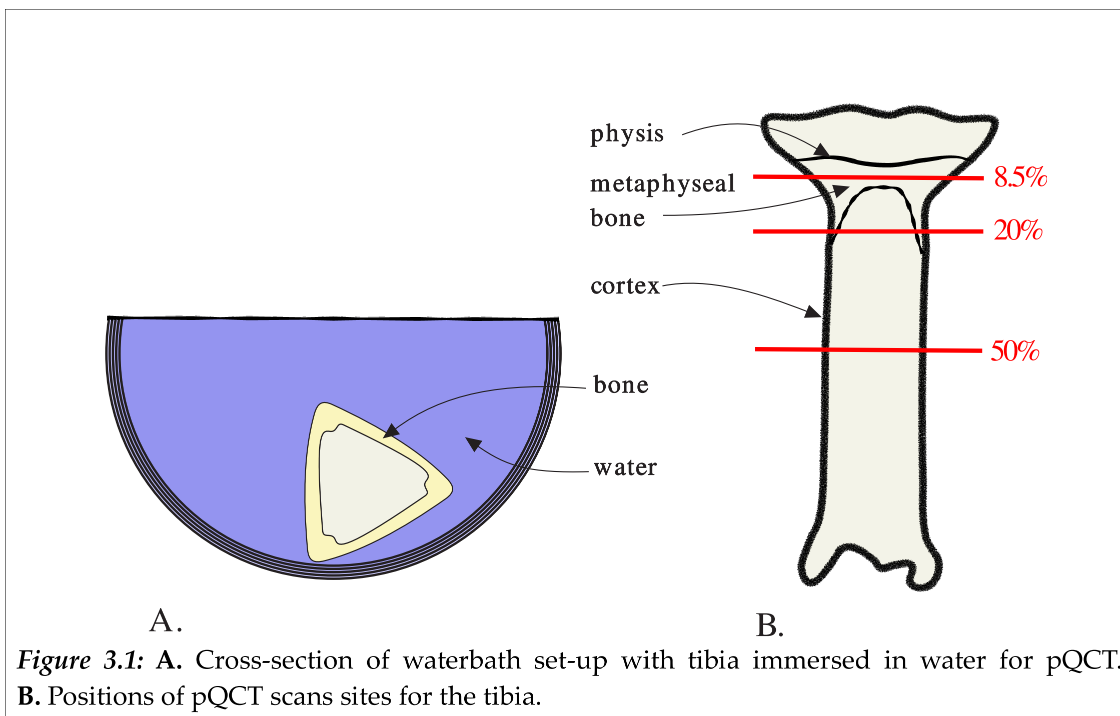


Figure 3.1: **A.** Cross-section of waterbath set-up with tibia immersed in water for pQCT. **B.** Positions of pQCT scans sites for the tibia.

Each tibia was measured to the nearest millimetre from the intercondylar eminence to the lateral malleolus with a plastic ruler prior to scanning, and the measurement inserted into the mask. The pQCT scan was performed under the following conditions: 2.0 mm slice thickness, 0.3 mm voxel size, 20 mm/s SV

(scout view) speed, and 25 mm/s CT speed. Analysis was performed using the manufacturer's XCT 550 software (v5.0). The cortical bone threshold was set at the standard 710 mg/cm^3, in addition, a cortical bone peel threshold of 280 mg/cm^3 was set in order to obtain results for immature and abnormal cortical bone (Firth, 2006). A regression model was fitted to the results, with affected/control (indicator variables, affected=1, control=0) and age (in months) as predictors, using Minitab 15 statistical software (Minitab Inc., 2008, USA).

The strength-strain index (SSI) is used for assessing bone strength. The formula for calculation of the SSI is: SSI=pCSMI x cortical $vBMD_i$ / d_{Mx} x $vBMD_{Mx}$; with pCSMI, polar moments of inertia of the cortical bone cross-sectional area; cortical $vBMD_i$, cortical volumetric bone mineral density; d_{Mx}, maximal distance from a voxel to the polar axis in the image; $vBMD_{Mx}$, maximal value the cortical volumetric bone mineral density could theoretically assume (Ferretti *et al*., 2001). The manufacturer's default threshold of 480 mg/cm^3 was used for the SSI (Schweizer *et al*., 2007).

3.3 RESULTS

3.3.1 CLINICAL SIGNS

Many of the affected lambs born during the 2006 and 2007 breeding trials appeared normal at birth, but three showed bone fragility consistent with rickets at birth. Two lambs, 102 and 201, born as a result of the embryo transfer trial in 2006, died as a consequence of bone fragility 1 and 6 days after birth. During difficult parturition, both lambs sustained fractures to the ribs, leading

to severe dyspnoea, and death within 6 days. For the first week of life one lamb in the 2007 breeding trial had a stilted, short stepping gait, with difficulty moving and minimal weight-bearing on the right forelimb. In the 2007 breeding trial, affected lambs were noticed within 3-4 weeks to be smaller in size and lagging behind the rest of the flock.

The growth rate and weight gain of affected lambs was significantly less ($P<0.05$) than unaffected lambs, and sheep with the disease attained a smaller adult size than unaffected sheep. If they survived to approximately 2 years of age, sheep with the disease appeared to stabilise, deteriorating only slowly from that age.

The severity of clinical signs varied between animals, but limb deformities were a prominent and consistent feature (Figure 3.2). These appeared from 6 weeks of age, were most severe in the forelimbs and became more pronounced with time. Some individuals had forelimb varus ("bow-legged") and others forelimb valgus ("knock-kneed") deformities, while others had a combination of varus and valgus deformities leading to a "wind-swept" appearance. The malalignment caused by limb deformities led to abnormally shaped hooves depending on where the most pressure from the limb deformity was exerted. The limb deformities also led to gait changes, and difficulty with rapid movement. In some animals with the valgus configuration, the limbs crossed and sometimes collided during locomotion.

Another common clinical sign was pronounced lordosis in the mid-thoracic region, caudal to the scapula (Figure 3.3). Some sheep were born with this abnormality and in others it developed from approximately 2 weeks of life. In severely affected animals, the curvature of the back, and shortened forelimbs meant that the head was often only slightly above the level of the pelvis.

Affected lambs appeared to be less active than their healthy counterparts, and moved tentatively, often with a short-stepping gait. In severe cases the lameness progressed to prolonged recumbency and difficulty in rising. Euthanasia was performed in sheep that progressed to this stage. Eighteen-month-old rams usually showed little inclination to mate and were unable to successfully mount ewes.

From approximately 2 years of age, hard swellings bridged the distal limb joints (distal to and including the tarsus) medially and laterally. The metatarsophalangeal and metacarpophalangeal joints were most severely affected, but the proximal phalangeal and hock joints were also involved. In many cases, the ability to flex and extend the joint was minimal. One animal developed a localised swelling in the left mandible and accumulation of grass within a gingival pocket adjacent to the lower molars. This animal had difficulty eating, lost weight and was euthanased.

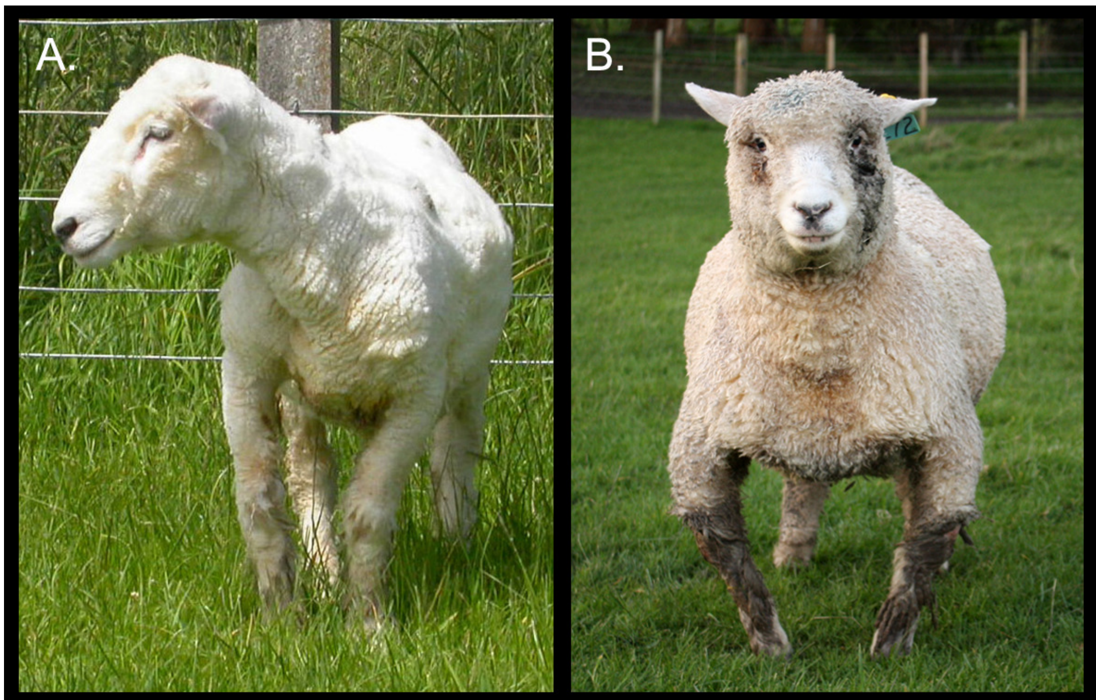


Figure 3.2: 2-year-old Corriedale sheep with inherited rickets showing angular limb deformities of the forelimbs.. **A.** Combined varus and valgus or "windswept" deformity; **B.** Valgus or "bow-legged" deformity.

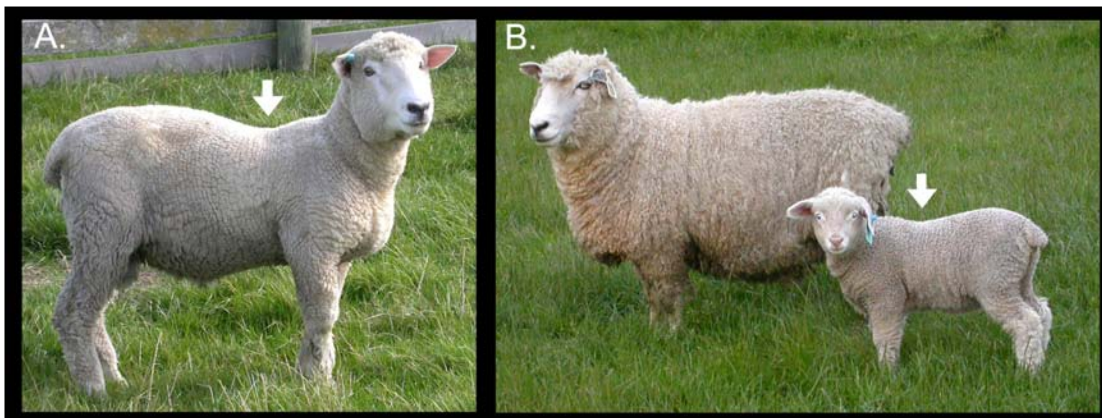


Figure 3.3: Corriedale sheep with inherited rickets showing lordosis of the spine in the mid-thoracic region (white arrows). **A.** 1½-year-old sheep; **B.** 3-week-old lamb with unrelated recipient ewe mother.

3.3.1.1 MORPHOMETRY

By 2 weeks of age, forelimb, hindlimb and crown-rump (C-R) length, weight and girth were all significantly less in affected lambs than in controls (P<0.05), when sex, rank and disease status were considered. Similarly, at 4, 6, 8, and 12 weeks of age, all length and weight measurements were less in affected lambs (P<0.1) than in controls. If results were corrected for weight however, then there were no significant differences between affected and control lambs. No difference was noted between affected and control lambs in the ratio of forelimb to hindlimb measurements, or in the ratio of growth rate of the forelimbs to growth rate of the hindlimbs. However, at 12 weeks of age, the C-R to hindlimb ratio was significantly less (P<0.05) in affected lambs than in controls.

The average daily weight gain over the 12 weeks was significantly less (P<0.05) in affected lambs, with control lambs gaining, on average, 100 grams more per day (Figure 3.4). The average growth rate of the forelimbs (Figure 3.4B) and C-

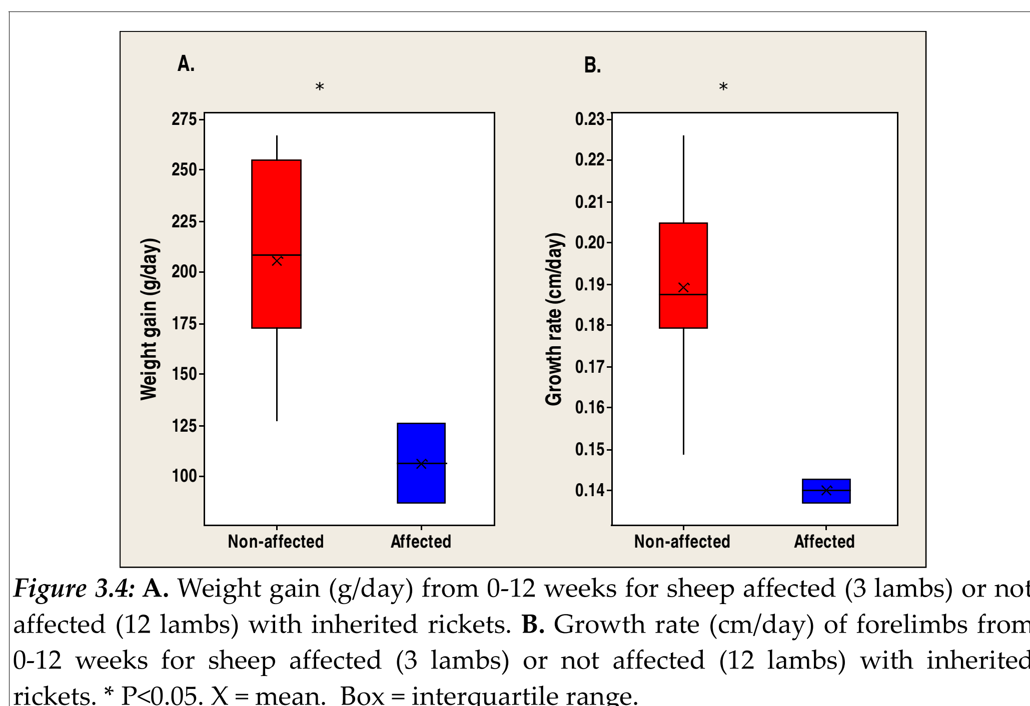


Figure 3.4: **A.** Weight gain (g/day) from 0-12 weeks for sheep affected (3 lambs) or not affected (12 lambs) with inherited rickets. **B.** Growth rate (cm/day) of forelimbs from 0-12 weeks for sheep affected (3 lambs) or not affected (12 lambs) with inherited rickets. * P<0.05. X = mean. Box = interquartile range.

R length over the 12 week measurement period for affected sheep was also significantly less (P<0.05) in affected than in control lambs.

3.3.2 Radiography

Growth plates were segmentally thickened in 3-12-month-old affected animals (Figure 3.5). In many animals, the metaphyseal/physeal interface was irregular with hazy margins. In 3-6-month-old animals, costochondral junctions were enlarged. The distal radius and the proximal tibial growth plate showed the most severe and consistent widening in 3-12-month-old animals. In a 3-month-old lamb, a 2 x 4 mm lucent area, consistent with retained cartilage, was present in the upper metaphyseal region of the distal radius. Radiographs of a 2-year-old control sheep showed closed growth plates, while growth plates in four affected animals 2-2 ½-years-old were still open.

Figure 3.5: Radiograph of bones from a 12-month-old Corriedale hogget with inherited rickets showing segmental lucency and thickening of the physes (white arrows) of **A.** distal radius and **B.** proximal tibia.

A consistent radiographic change in all animals was the presence of coarse metaphyseal trabeculae. These trabeculae had blurred margins, suggesting that they were lined by unmineralised osteoid. Metaphyseal sclerosis, suggestive of retention of the primary spongiosa, was present in all bones radiographed from the 1- and 6-day-old animals. In many animals, several radio-opaque lines parallel to the physis, consistent with growth arrest lines, were present in the metaphysis (Figure 3.6). Up to 15 such lines were sometimes present. The possibility that they represented healed infractions was considered, but their regularity and shape were more typical of what would be expected following periods of temporary physeal closure.

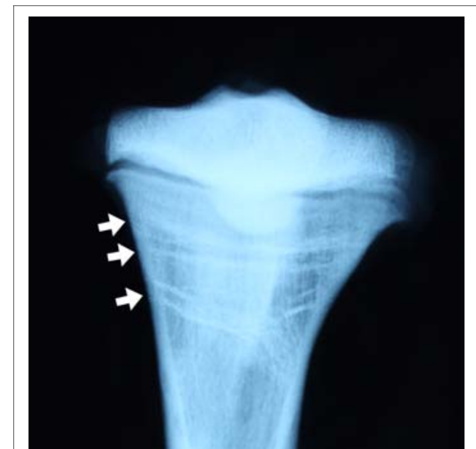


Figure 3.6: Tibia from 3-month-old Corriedale lamb with inherited rickets showing radio-opaque lines, consistent with growth arrest lines, parallel to the physis (white arrows).

The cortices of all long bones, at all ages, (with the exception of the femur of one 6-month-old sheep) were substantially thicker than in age-matched controls. The cortices had a porous appearance, with many fine, lucent linear foci. The medulla in the diaphysis of animals 6-months and older had patchy ill-defined lucent foci, with regular linear bars of sclerosis, consistent with bridging trabeculae, between cortices.

Many affected animals had either varus or valgus angular deformities, most commonly in the radius, tibia, and metacarpal bone. In bones with angular

deformities, the concave side of the cortex was considerable thicker than the convex side.

Fractures were present in lambs that died in the neonatal period. Complete non-displaced fractures of the ribs were seen in a 1-day-old lamb, while in a 6-day-old lamb the rib fractures were markedly displaced and overriding.

In animals 6-months and older, irregularity of the humeral head resulting in decreased epiphyseal bone thickness, was a consistent radiographic change (Figure 3.7). In one animal a piece of sclerotic bone surrounded by a lucent zone was present in the humeral head, resembling the radiographic appearance of a sequestrum.

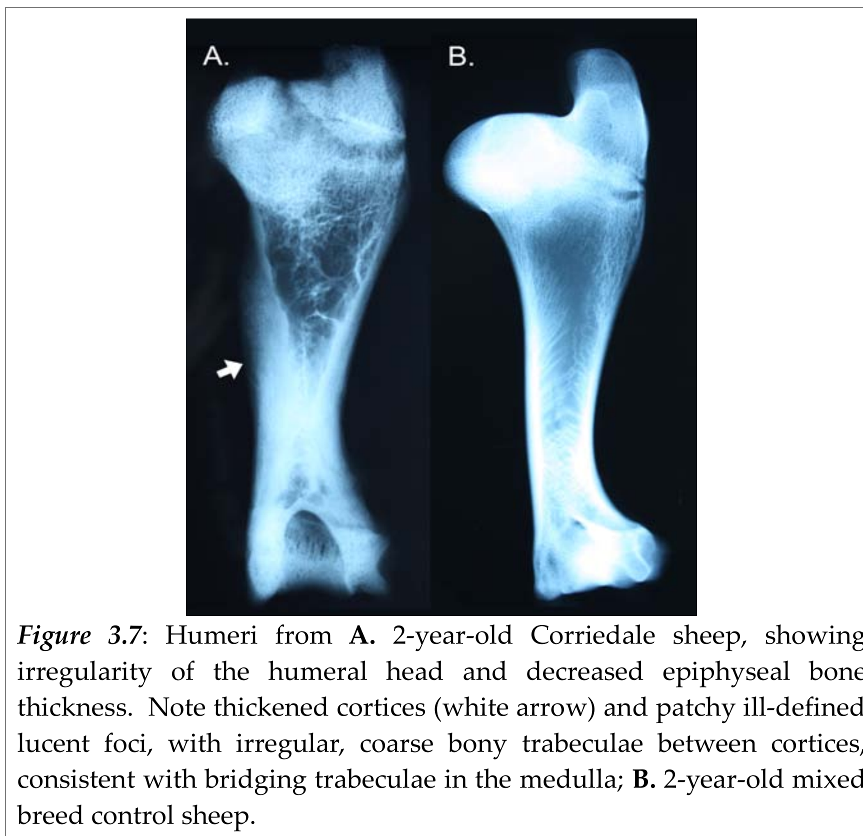


Figure 3.7: Humeri from **A.** 2-year-old Corriedale sheep, showing irregularity of the humeral head and decreased epiphyseal bone thickness. Note thickened cortices (white arrow) and patchy ill-defined lucent foci, with irregular, coarse bony trabeculae between cortices, consistent with bridging trabeculae in the medulla; **B.** 2-year-old mixed breed control sheep.

In affected sheep 2 years of age and older, considerable periosteal bone reaction was present spanning the tarsus, and metacarpophalangeal or metatarsophalangeal joints (Figure 3.8). The severity of this periosteal bone reaction varied between animals. The large bony proliferations often spanned both the metacarpophalangeal and metatarsophalangeal joints, with complete ankylosis of the metatarsophalangeal joints. Less severe periosteal bony proliferation was also present around the hock joint, and the proximal and distal interphalangeal joints. The joint spaces were not narrowed, and were uniform.

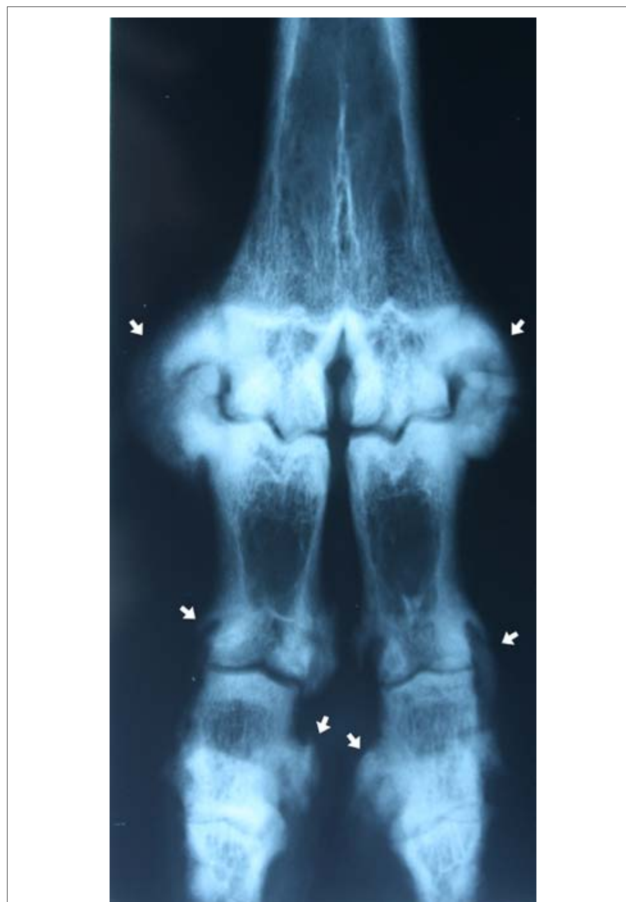


Figure 3.8: 25-month-old Corriedale sheep showing periosteal bone proliferation around the metacarpophalangeal, proximal and distal interphalangeal joints (white arrows).

3.3.3 pQCT

Peripheral quantitative computed tomography showed marked and consistent differences between affected and control sheep at all ages (Figure 3.9). The complete data are shown in Table 3.1

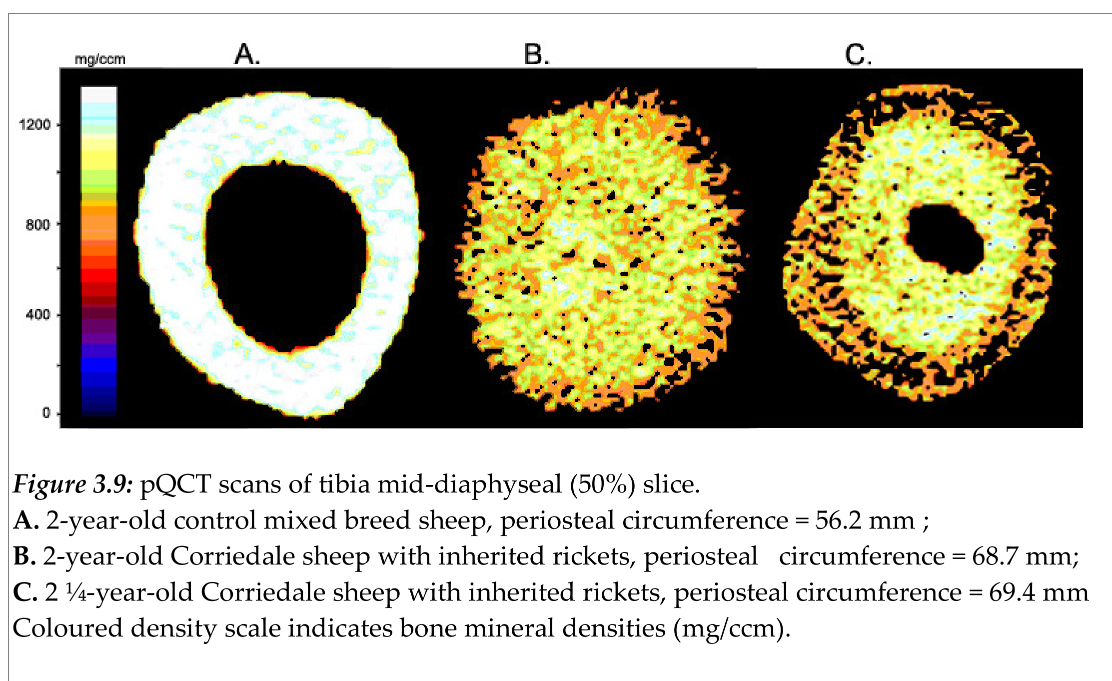


Figure 3.9: pQCT scans of tibia mid-diaphyseal (50%) slice.
A. 2-year-old control mixed breed sheep, periosteal circumference = 56.2 mm ;
B. 2-year-old Corriedale sheep with inherited rickets, periosteal circumference = 68.7 mm;
C. 2 ¼-year-old Corriedale sheep with inherited rickets, periosteal circumference = 69.4 mm
Coloured density scale indicates bone mineral densities (mg/ccm).

3.3.3.1 Mid-Diaphyseal pQCT Scan

The cortical bone density (cort BMD 710, using the standard 710 mg/mm^3 threshold) was 340 mg/mm^3 less ($P<0.001$) in sheep with inherited rickets than in control animals (Table 3.1). Similarly, cortical bone density when immature bone was included (cort BMD 280, using 280 mg/mm^3 threshold) was 332 mg/mm^3 less ($P<0.001$) in affected than in control sheep (Table 3.1). Cortical BMD 710 of sheep with inherited rickets ranged from 827.5 mg/mm^3 to 951.8 mg/mm^3, while cortical BMD 710 of control animals had a range of 1181.3 mg/mm^3 to 1321.5 mg/mm^3. While BMD of affected animals was less than that

Table 3.1: Differences in pQCT scan results of the left tibia, between 13 sheep with inherited rickets and 6 control sheep, analysed using a regression model.

	Mid diaphyseal				**Distal metaphyseal**				**Proximal metaphyseal**			
	Regr coeff	P-value	Age coeff	P-value	Regr coeff	P-value	Age coeff	P-value	Regr coeff	P-value	Age coeff	P-value
Trab BMC mg	N/A				24.21 ± 6.90	0.003	0.23 ± 0.29	0.451	7.21 ± 19.68	0.719	1.07 ± 0.86	0.229
Cort BMC 280 mg	59.06 ± 28.13	0.053	6.26 ± 1.20	<0.001	16.73 ± 16.71	0.333	5.49 ± 0.71	<0.001	N/A			
Cort BMC 710 mg	15.32 ± 25.59	0.558	3.71 ± 1.09	0.004	-47.01 ± 9.49	<0.001	4.67 ± 0.41	<0.001	N/A			
Trab BMD mg/mm^3	N/A				101.56 ± 29.84	0.004	-2.05 ± 1.28	0.129	36.05 ± 28.77	0.231	-0.01 ± 1.25	0.993
Cort BMD 280 mg/mm^3	-332.15 ± 38.13	<0.001	0.22 ± 1.63	0.895	N/A				N/A			
Cort BMD 710 mg/mm^3	-340.08 ± 24.50	<0.001	0.95 ± 1.05	0.380	-241.08 ± 16.63	<0.001	1.88 ± 0.71	0.018	N/A			
Cort Area 280 mm^2	135.21 ± 42.57	0.006	8.31 ± 1.82	<0.001	N/A				N/A			
Cort Area 710 mm^2	58.76 ± 26.08	0.040	3.78 ± 1.11	0.004	-22.29 ± 11.09	0.063	5.03 ± 0.47	<0.001	N/A			
Total Area mm^2	N/A				62.56 ± 61.32	0.324	10.77 ± 2.62	0.001	-36.6 ± 112.6	0.750	8.52 ± 4.89	0.104
Endo Circ mm	-12.31 ± 3.28	0.002	0.18 ± 0.14	0.229	-5.33 ± 4.27	0.231	0.60 ± 0.18	0.005	N/A	N/A	N/A	N/A
Peri Circ mm	30.39 ± 31.80	0.354	1.06 ± 1.36	0.447	4.18 ± 5.26	0.439	0.89 ± 0.22	0.001	-3.03 ± 7.241	0.682	0.55 ± 0.31	0.101
Peri C: Bone L mm	0.098 ± 0.019	<0.001	0.001 ± 0.001	0.350	-0.11 ± 0.08	0.181	0.006 ± 0.003	0.084	0.015 ± 0.263	0.565	-0.003 ± 0.001	0.013
SSI p mm^2	202.6 ± 144.3	0.181	35.78 ± 6.17	<0.001	57.0 ± 109.7	0.611	44.35 ± 4.69	<0.001	176.8 ± 138.6	0.221	48.25 ± 5.92	<0.001

Abbreviations: Trab = trabecular; Cort = cortical; BMC = bone mineral content; BMD = bone mineral density; 710 = BMC/BMD/Area of bone greater than 710 mg/mm^3; 280 = BMC/BMD/Area of bone greater than 280 mg/mm^3; Endo Circ = endosteal circumference; Peri Circ = Periosteal Circumference; Peri C: Bone L = Periosteal Circumference : Bone Length ratio; SSI p = polar stress strain index; Regr Coeff = regression coefficient ± standard error of coefficient; Age Coeff = regression coefficient for age (in months) ± standard error of coefficient for age. Regression model used age (in months) and disease status (indicator variables, affected = 1, control = 0) as predictors. For example; Cort BMD 710 is 340.08 mg/mm^3 less in affected animals compared with controls, and irrespective of disease status animals put on 6.26 mg of Cort BMC 280 mg every month.

of controls, bone mineral content tended to be greater in animals with inherited rickets than in controls. This difference was significant (P=0.053) when a threshold of 280 mg/mm^3 was used, and affected animals had 59.06 mg more mineral than did control animals (Table 3.1).

Cortical area (Cort area 710, using the standard 710 mg/mm^3 threshold) was 58.76 mm^2 greater (P=0.040) in sheep with inherited rickets than in control sheep, and the difference was even greater (135.21 mm^2, P=0.006) when a threshold of 280 mg/mm^3 ("Cort" Area 280) was used (Table 3.1). When the ratio of cort area 280 to cort area 710 was examined, cort area 280 was 1.61 ± 0.11 times greater than cort area 710 in affected animals, and only 1.19 ± 0.02 times greater in control animals (P=0.040). No significant difference was present in the polar stress strain index (SSI) between affected and control animals (Table 3.1). Endosteal circumference was significantly less in affected animals than in controls (P=0.002). Although no significant difference in periosteal circumference was present between affected and control sheep, when bone length was considered (Peri C:Bone length ratio) the periosteal circumference was significantly greater (P<0.001) for a given bone length in affected animals than in controls.

Age had a significant effect on a number of the variables examined in both affected and control sheep, including cort BMC 280, cort BMC 710, cort area 280, cort area 710 and SSI (Table 3.1). Cort BMC 280 and 710 increased by 6.26 mg per month and 3.71 mg per month respectively, as age increased. Similarly, cort area 280 and 710 increased by 8.31 mm^2 and 3.78 mm^2 per month

respectively as the animals aged. SSI increased by 35.78 mm^2 per month, as the animals aged. Age had no effect on cortical BMD 280 or 710 (Table 3.1).

The histograms of cort BMD 710 voxel spread for the mid-diaphyseal and distal metaphyseal scans showed consistent differences between affected and control sheep of all ages. The bone density voxels of affected sheep were positively skewed towards the lower densities, while in control animals, the bone densities voxels were negatively skewed towards the higher values. The skew in spread of bone densities is illustrated in Figure 3.10, which is representative of the tibias scanned from all sheep.

3.3.3.1 DISTAL METAPHYSEAL PQCT SCAN

Trabecular BMC and BMD were significantly greater in affected sheep than in controls (Table 3.1). Trabecular BMD was 101.56 mg/mm^2 greater (P=0.004) in animals with inherited rickets than in controls. In contrast, cort BMD 710 was

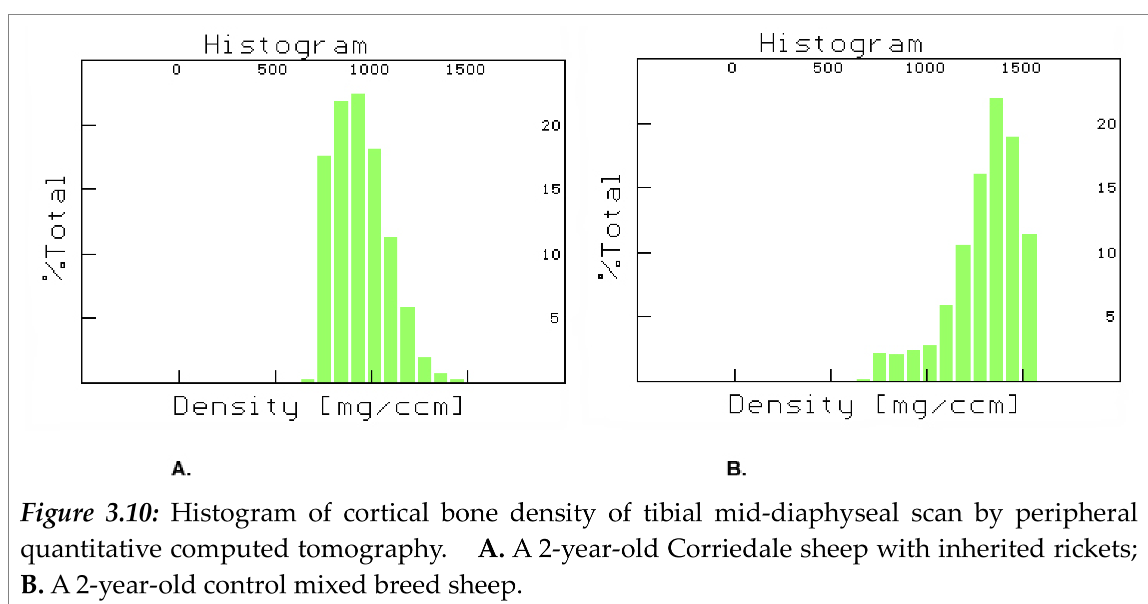


Figure 3.10: Histogram of cortical bone density of tibial mid-diaphyseal scan by peripheral quantitative computed tomography. **A.** A 2-year-old Corriedale sheep with inherited rickets; **B.** A 2-year-old control mixed breed sheep.

241.08 mg/mm^2 ($P<0.001$) less in sheep with inherited rickets than in control sheep (Table 3.1). Despite the differences in trabecular and cortical bone density, total bone density of the scan was not significantly different between affected and control sheep.

In contrast to the mid-diaphyseal scan, the cortical area 710 of the distal metaphyseal scan was 22.29 mm^2 less ($P=0.063$) in sheep with rickets than in control sheep (Table 3.1). However, the total area of the distal metaphyseal scan was not significantly different between the two groups (Table 3.1). Nor were there any differences between affected and control sheep, for endosteal circumference, endosteal circumference:bone length ratio (data not shown), periosteal circumference, periosteal circumference:bone length ratio and SSI (Table 3.1).

Age had no significant effect on trabecular BMC or BMD. However, cort BMC and BMD 710, cort and total area, endosteal circumference, periosteal circumference and SSI all increased with age in both affected and control animals.

3.3.3.2 Proximal Metaphyseal pQCT Scan

The 8.5% scan site was adequate for control bones. However, due to differences between affected and control sheep in the position and thickness of the proximal tibial growth plate, the proximal metaphyseal scan in affected animals was performed at an average position of 9.4 ± 0.3%.

No differences were present between affected and control animals for any of the variables examined (Table 3.1). Similarly, age did not have a significant effect on BMC, BMD or area. However, like the mid-diaphyseal and distal metaphyseal scans, SSI of the proximal metaphysis increased significantly with age (48.25 mm^2 per month, $P<0.001$) in both affected and control sheep.

3.4 DISCUSSION

The clinical signs of angular limb deformity, lordosis, fractures and an altered gait, together with the radiographic and computed tomography changes in these sheep are consistent with rickets (Klein, 1999). The cupping and fraying of the metaphysis in the 3-12-month-old animals is a classical radiographic change described in humans with rickets (Pitt, 1995; Thacher *et al*., 2000). While there are few published reports of CT scans from humans with this disease, the decreased bone mineral density and increase in bone area is comparable to changes seen in historical bones with rickets and in humans with familial hypophosphataemic rickets (Kovanlikaya *et al*., 1996; Schamall *et al*., 2003).

Pathological fractures, angular limb deformities, and lameness may be seen in other skeletal diseases of sheep, including osteogenesis imperfecta, osteopetrosis, and copper deficiency (Thompson, 2007), but all of these could be excluded in this investigation. Lambs with osteogenesis imperfecta seldom survive for more than a few hours after birth and, in addition to bone fragility, have other distinctive signs. These include skin fragility, blue sclera, marked joint laxity and poorly erupted pink teeth (Arthur *et al*., 1992), none of which were observed in affected Corriedale lambs. Osteopetrosis has recently been

diagnosed in neonatal lambs with excessive bone fragility and is characterised by persistence of the primary spongiosa (Leipold and Cook, 1977; K.G. Thompson, pers. comm.) similar to that seen in neonatal lambs with inherited rickets. However, osteopetrotic lambs are unlikely to survive beyond the first week of life and have different microscopic lesions (K.G. Thompson, pers. comm.). Copper deficiency was considered an unlikely differential diagnosis since the disease was confined to a small group of lambs on the original property and progressed in animals transferred to a different property where copper deficiency had never been diagnosed. The widened growth plates and angular limb deformities seen radiographically in sheep with rickets may also be seen in osteochondrosis, but this disease typically occurs in young fast growing animals, and bone fragility such as rib fractures and trabecular microfractures in long bones, is not a feature of the disease (Duff, 1986; Scott *et al*., 1996).

Many individuals with hereditary vitamin D-resistant rickets (HVDRR), in addition to the skeletal deformities of rickets, also have hypotrichosis or alopecia, but this was not a feature of the disease in Corriedale sheep. The presence of alopecia is thought to indicate more severe resistance to vitamin D (Marx *et al*., 1986; Whyte, 2002). Both the nutritional and hereditary rachitic diseases of humans show a range of dental abnormalities including delayed eruption, enamel hypoplasia and increased incidence of dental caries (Malloy and Feldman, 1999; Miller and Portale, 2003; Reid *et al*., 1989; Wharton and Bishop, 2003). No obvious abnormalities were detected in tooth eruption or enamel production in Corriedale sheep with inherited rickets. Muscle weakness is another symptom in people with nutritional rickets, vitamin D-

dependent rickets type I (VDDR) and HVDRR, but is not seen in X-linked hypophosphataemic rickets (XLH) (Drezner, 2003; Whyte and Thakker, 2005). Corriedale sheep with inherited rickets had difficulty rising and spent more time recumbent than their healthy counterparts, but this was probably related to skeletal lesions rather than to muscle weakness.

The morphometric analyses showed that by 2 weeks of age all skeletal measurements and body weights were less in affected lambs than in controls, and this remained consistent for the entire measurement period. The decrease in weight gain in affected sheep was particularly dramatic, with non-affected sheep gaining an average of 100 grams per day more than affected sheep over the 12 week period. By 12 weeks of age non-affected animals weighed on average 9.5 kg more. Reduced growth rate also occurs in humans with inherited rickets, where affected individuals also have decreased weight gain, and a general failure to thrive (Malloy and Feldman, 1999; Malloy *et al*., 2002a).

Once body weight was included in the analysis, there were generally no significant differences in skeletal measurements between affected and non-affected sheep. In particular, the forelimbs of affected lambs were not significantly shorter than those of controls. When the ratio of crown-rump length to hindlimb length was examined, this was significantly less in affected than control lambs, indicating that the hindlimbs were proportionately longer in comparison to body length in affected animals. This suggests the possibility that the lordosis seen in the shoulder region of affected animals may in fact be

due to proportionately longer hind limbs in comparison to body length, rather than to shorter forelimbs as originally thought.

While the radiographic changes seen in this study are consistent with a diagnosis of rickets, the growth plate widening and lucency was less obvious than that seen in animals and human patients with nutritional rickets (Malik *et al*., 1997; Shore and Poznanski, 1999). In humans with VDDR I and HVDRR, the radiographic changes of rickets are severe, with substantial widening and cupping of the physes (Pitt, 1995; Shore and Poznanski, 1999). However, in children with XLH, the radiographic changes may only be mild to moderate (Pitt, 1995; Shore and Poznanski, 1999). The thickened long bone cortices present radiographically in these sheep also occur in children with XLH, but are not seen in other forms of rickets (Shore and Poznanski, 1999).

The difference in growth rates in sheep with inherited rickets compared with animals or humans with nutritional rickets may also lead to differences in physeal width. The physeal widening in rickets is greatest in bones with the greatest growth, and minimal in slow-growing bones (Shore and Poznanski, 1999). The reduced growth rate of sheep with inherited rickets in this study, would have made severe growth plate widening less likely.

The varus and valgus deformities seen in the older sheep are common in all forms of rickets in humans (Pitt, 1995; Shore and Poznanski, 1999; Thacher *et al*., 2000). In humans, bowing and curving of the long bones may also be seen in forms of osteogenesis imperfecta and fibrous dysplasia (Shore and Poznanski,

1999), but growth plate widening is not a feature of these diseases and cortices are typically narrower than normal (Whyte, 1999a; Whyte, 1999b). During infancy and early childhood the bowing of limbs in rickets is thought be a result of movement of ossification centres due to asymmetric pulling of musculotendinous insertions, for example, the pull of the achilles tendon leading to anterior bowing of the tibia (Pitt, 1995). As an individual ages, angular limb deformities are thought to result from weight-bearing, leading to bending of the long bones (Pitt, 1995). The angular limb deformities present in Corriedale sheep with inherited rickets occurred most commonly and severely in the forelimbs, this is probably because the majority of body weight is on the forelimbs in sheep (Kim and Breur, 2008).

The bony bridging around the distal limb joints (from the carpus/tarsus distally) as the animals aged, could be due to either osteophytes or enthesophytes. Since osteophytes generally accompany degenerative joint disease (Milgram, 1990a), and no other radiographic evidence for degenerative joint disease was present in the Corriedale sheep with inherited rickets, the bony proliferations were considered to be enthesophytes. A similar age-related entity occurs in humans with XLH, where there is enthesopathic calcification and new bone formation at muscle and ligament attachments, and the capsules of appendicular joints (Burnstein *et al*., 1989; Pitt, 1995; Polisson *et al*., 1985).

Radiography is not a good indicator of bone density as the exposure factors are altered for each bone and animal in order to obtain a good quality radiograph (Shore and Poznanski, 1999). Instead, dual-energy x-ray absorptiometry

(DEXA) or CT are more reliable imaging modalities for bone density estimation, especially for serial measurements. (Shore and Poznanski, 1999). Nevertheless, radiographs demonstrated that the cortices of sheep with inherited rickets had increased porosity compared with controls, suggesting the presence of either unfilled resorption cavities and/or Haversian systems filled with poorly mineralised osteoid.

Corriedale sheep with inherited rickets had greater BMC on pQCT at the mid-diaphyseal scan than in control sheep. A similar finding was reported in humans with X-linked hypophosphataemic rickets, who also had bowing of the long bones, thickened cortices and a coarse trabecular pattern radiographically (Harrison *et al*., 1976), as seen in Corriedale sheep with inherited rickets. Histologically, affected human patients had greater than normal total bone tissue (mineralised bone and osteoid) and total bone calcium (Harrison *et al*., 1976). The authors considered that the structural weakness caused by osteomalacia led to the formation of new, poorly mineralised bone, which eventually became mineralised, leading to an increase in overall bone mineral content (Harrison *et al*., 1976).

Although BMC of the mid-diaphyseal scan was greater in affected sheep, BMD was substantially less, presumably a function of the increased bone area of the pQCT scan. When a lower cortical threshold (280 mg/mm^3) was used in the analysis, the cortical area of affected sheep was 2.3 times greater than using the standard threshold of 710 mg/mm^3. This indicated that a large proportion of the cortices of sheep with inherited rickets consisted of either immature or poorly

mineralised bone. When bone length was considered, the ratio of periosteal circumference to bone length of the mid-diaphyseal scan was significantly greater in affected sheep than in controls and endosteal circumference was decreased. The increase in bone area seen in Corriedale sheep with inherited rickets is therefore likely to be due to a combination of periosteal bone deposition and decreased endosteal bone resorption, as a result of increased strain on weakened bone (Ferretti *et al*., 2001).

Wolff's law states that bones grow and remodel during life in order to adapt to the mechanical environment (Pearson and Lieberman, 2004). Therefore, bone is deposited at sites of strain in order to decrease deformation associated with mechanical loading (Volz, 1995). Similarly, the mechanostat model states that bone tissue (presumably osteocytes) monitors the strain from mechanical forces and compares it with a pre-set target range (Rauch, 2006). It appears that the primary cilia of osteocytes may be the mechano-sensing mechanism (Whitfield, 2008). Bone loading leads to alterations in lacunocanalicular fluid flow which switches the cilia on and off, thereby altering gene expression (Whitfield, 2003; Whitfield, 2008). When strain leads to deformation that is outside the target range then bone architecture and mass is altered to compensate (Rauch, 2006). A fetus obtained in the breeding trial (see Chapter 4) had healed infraction lines in its long bones, neonatal lambs had infractions and rib fractures, and older animals had microfractures, indicating that strain had led to traumatic damage. This suggests that the cortical thickening at least in part, is due to the increased strain placed on under-mineralised bone. Further support for increased strain leading to cortical thickening, is the thickening of the concave side of bones with varus/valgus deformities. In addition, the thick bridging trabeculae seen

in the diaphysis on radiography suggests a buttressing effect or an attempt to strengthen the bone.

No differences in SSI were seen between Corriedale sheep with rickets and control sheep. This is in contrast to the findings of a study on osteomalacic rabbits (Anumula *et al*., 2008), where the ultimate strength was significantly less than in controls. The SSI is heavily influenced by the bone area, and distance of a particular voxel from the centre of the bone (neutral axis) (Pearson and Lieberman, 2004); it therefore may be falsely elevated as a result of the increased cortical area present in the diaphysis of affected sheep. Increasing the diameter of a bone substantially increases the bending strength, as the greater distance bone is away from the neutral axis, the better it is at resisting bending (Leonard *et al*., 2004; Rubin and Rubin, 1999). Given the extensive cortical thickening in affected sheep, it is not surprising therefore that the SSI was similar to that of controls. Rauch (2006) hypothesised that because of the weakened bone in humans with X-linked hypophosphataemic rickets, a given mechanical load will cause more strain than on normal bone. The osteocytes in XLH bone will therefore overestimate the amount of strain and the bone will adapt to higher loads than are actually present leading to bone with increased strength (Rauch, 2006).

Since the neutral axis changes as a sheep walks, estimates from cross-sections like SSI may be in error by 23-38% (Pearson and Lieberman, 2004). Also the SSI does not take into account other factors important to the material properties of bone, including fatigue damage (Ferretti *et al*., 2001). While the SSI may be

normal, it is only a measure of bending strength, bone also undergoes axial compression, twisting and shear forces in addition to bending (Pearson and Lieberman, 2004) and it may be that the under-mineralised bone has produced new bone in response to these loading forces. Infraction lines and microfractures present in many of the bones examined, suggest that bone strength is not normal in Corriedale sheep with inherited rickets, and that SSI may not be a reliable indicator of bone strength in this disease.

Mineralisation of bone is only one part of the material properties of bone, and other factors like collagen fibres, cement lines, osteons, and the amount of microdamage present should also be considered (Ferretti *et al*., 2001). The type 1 collagen gene promoter contains a vitamin D response element (Lichtler *et al*., 1989) and vitamin D is thought to regulate type-α1 collagen expression in proliferating osteoblasts (Owen *et al*., 1990). The effect of vitamin D deficiency on collagen structure and organisation however, does not appear to have been examined. Bone collagen markers such as carboxyterminal propeptide of type I procollagen (PICP – marker of bone formation), cross-linked carboxyterminal telopeptide of type I collagen (ICTP – marker of bone resorption) and cross-linked N-telopeptides of type I collagen (NTX – marker of bone resorption) are elevated in children with rickets (Baroncelli *et al*., 2000; Sharp *et al*., 1997). It seems likely that the bone resorption markers (ICTP and NTX) are elevated as a result increased bone resorption due to increased serum parathyroid hormone (Sharp *et al*., 1997). Sharp *et al.* (1997) found no correlation between serum PICP and either serum parathyroid hormone or vitamin D metabolites, and suggested PICP may be elevated as a result of increased strain on weakened bone leading to increased collagen production.

Cortical bone density of the mid-diaphyseal scan from the control animal at 2 years of age was 1.4-1.6 times that of affected animals (2-3 years of age). A study of rickets in historical skeletons using QCT found that overall bone density was decreased in rachitic vertebrae compared to controls (Schamall *et al*., 2003). Another study assessing osteopenia using CT considered that this imaging modality was able to accurately quantitate deficiencies in bone mineralisation, aid in the diagnosis of rickets, and allow a better assessment of the severity of the disease (Kovanlikaya *et al*., 1996). As observed in Corriedale sheep with inherited rickets, cortical bone density of children with hypophosphataemic rickets was significantly reduced suggesting that the use of CT in addition to radiography may be useful in the clinical diagnosis of rickets (Kovanlikaya *et al*., 1996).

Trabecular BMC and BMD in the distal metaphyseal pQCT site was significantly greater in Corriedale sheep with inherited rickets than in control sheep. This is mostly likely due to metaphyseal trabeculae of affected sheep extending further into the diaphyseal region than in control sheep. Since, trabecular bone has a greater turnover, and is thought to be a better indicator of bone remodeling than cortical bone (Lee *et al*., 2007), these results suggest that either decreased osteoclastic remodelling of metaphyseal trabeculae or increased peritrabecular bone apposition (Ferretti *et al*., 2001) is occurring in Corriedale sheep with inherited rickets. Given the extensive osteoclastic resorption present microscopically (see Chapter 4), it seems unlikely that the

increase in trabecular BMC and BMD in the distal metaphyseal pQCT site is the result of inadequate osteoclastic remodelling.

This study provides an insight into the effects of life-long rickets and osteomalacia. Most animals were euthanased by 15 months of age, as a result of increased recumbency and weight loss. After this age, probably as a result of declining growth rate, their condition appeared to stabilise, and some animals were used in a successful treatment trial (see Chapter 7). The angular limb deformities became more prominent with time, as did the lordosis in the shoulder region. Cortical area, as measured by CT, increased with age, while bone density remained low and showed little increase.

3.4.1 SUMMARY

The clinical signs, radiographic and computed tomography changes observed in Corriedale sheep with an inherited skeletal disease are consistent with rickets. The clinical signs include angular limb deformities, prominent lordosis of the mid-thoracic spine and decreased weight gain. Physeal widening, cortical thickening, coarse metaphyseal trabeculae and bridging trabeculae were prominent radiographic changes. This is the first study reporting computed tomography changes in animals with rickets, and Corriedale sheep with inherited rickets had significantly greater BMC, and bone area, but significantly less BMD than controls.

Neonatal lambs sustained rib fractures during difficult parturition as a consequence of bone fragility and died within 6 days of birth. This skeletal

disease is likely therefore, to be more common in the Corriedale breed than currently detected due to undiagnosed cases in neonatal lambs. The combination of clinical signs, radiographic and CT changes present in Corriedale sheep with inherited rickets most closely resembles XLH of humans.

Chapter 4

Pathology

4.1 Introduction

Rickets is a metabolic bone disease caused by a deficiency in either vitamin D or phosphorus and characterised by failure of mineralisation of physeal cartilage and newly formed osteoid. In adults, vitamin D or phosphorus deficiency is manifested as osteomalacia, where there is impaired mineralisation of newly-formed osteoid during bone remodelling. Classical lesions of rickets seen in all species include, enlarged costochondral junctions, irregular thickening of the physeal cartilage and spontaneous fractures (Fitch, 1943; Milgram, 1990b). Histologically, there is persistence of the hypertrophic zone of the physes, disorganisation of the primary spongiosa, and thick seams of unmineralised osteoid lining trabeculae (Fitch, 1943; Milgram, 1990b; Theiler, 1934).

The purpose of this study was to describe in detail the macroscopic and microscopic findings in Corriedale sheep with an inherited skeletal disease that had features of rickets. Although preliminary investigations suggested a diagnosis of rickets, inherited forms of this disease are rare in animals and had not been reported previously in sheep. In order to characterise the disease and confirm the diagnosis it was important to examine affected animals at different ages to gain insight into disease progression. This study also enabled comparison of this disease with inherited forms of rickets in humans and provided a basis for studies aimed at treatment.

4.2 Materials and Methods

4.2.1 Animals

In total, 22 affected animals were examined, 11 from the original Marlborough property, seven from an embryo transfer breeding trial, and four from a back-cross breeding trial (Chapter 2). Five animals, two from the embryo transfer trial, and three from the back-cross breeding trial, died naturally. An affected fetus was also collected from an embryo transfer recipient ewe that died of unknown causes prior to parturition. The remaining animals were monitored regularly and were euthanased when clinical assessment indicated suffering. The ages of animals examined ranged from a fetus of approximately 133 days gestation to young adults 32 months of age (Table 4.1). Age-matched controls included ten Corriedale cross, and seven mixed breed sheep.

Table 4.1: Animal number, age at post-mortem examination, sex and source of affected sheep from which samples were available for examination.

Animal Number	Sex	Source	Age at post-mortem
fetus	Male	Embryo transfer	133 days gestation
5557	Female	Back-cross	newborn
102	Male	Embryo transfer	1 day
201	Male	Embryo transfer	6 days
5585	Female	Back-cross	11 weeks
291	Female	Embryo transfer	3 months
5573	Male	Back-cross	4 months
5583	Male	Back-cross	4.5 months
241	Female	Embryo transfer	5 months
64	Male	Marlborough flock	6 months
60	Male	Marlborough flock	6 months
55	Male	Marlborough flock	9 months
59	Male	Marlborough flock	9 months
171	Female	Embryo transfer	9 months
52	Male	Marlborough flock	12 months
57	Male	Marlborough flock	13 months
54	Male	Marlborough flock	15 months
271	Male	Embryo transfer	17 months
53	Female	Marlborough flock	25 months
63	Female	Marlborough flock	28 months
61	Male	Marlborough flock	30 months
62	Male	Marlborough flock	32 months

4.2.2 Tissue Processing and Staining

At necropsy, the right ribs 5-9, humerus, radius, metacarpus, femur and tibia, were cleaned of soft tissue, sectioned sagittally and stored in 10% neutral buffered formalin until processed for histology. In nine animals, the mandible, proximal phalanx and either cervical or lumbar vertebrae were also collected and stored in 10% neutral buffered formalin until required. Bone slabs 5-8 mm thick were trimmed using a band-saw, and decalcified overnight, or longer if necessary, with Osteomoll® (Merck KGaA, Germany). Bone slabs (with the exception of rib sections) from affected sheep 3 months and older required prolonged decalcification times of 1-8 weeks. Once decalcified, the bone slabs were trimmed to the size of a cassette, dehydrated in graded alcohol and embedded in paraffin wax. Sections 3 µm thick were cut and stained with haematoxylin and eosin (H & E). Thyroid glands from 13 affected sheep were stored in formalin until processed for histology and serial sectioning to locate the internal parathyroid glands.

Selected bones were prepared for silver staining by a method described by Tripp and McKay (Tripp and MacKay, 1972) designed to demonstrate osteoid seams. Bones were fixed in 10% neutral buffered formalin and cut into 1-2 mm thick slabs using a band-saw. The bone slabs were washed in distilled water to remove all traces of formalin, placed in 2% aqueous silver nitrate for 48 h in complete darkness, washed for 4 h, and then placed in reducer (5 g sodium hypophosphite, 0.2 mL 0.1 M sodium hydroxide, 100 mL distilled water) for 48 h. Bone slabs were then washed in tap water for 1 h, and placed in 5% aqueous sodium thiosulphate (anhydrous) for 24 h. This was followed by a wash in water for 1 h and finally decalcification in 10% aqueous formic acid. The bone

slabs were then paraffin-embedded, sectioned at 3 μm, and stained by the van Gieson's picro-fuchsin method. The stain relies on the replacement of calcium ions with silver ions, forming a silver phosphate complex that is reduced to black metallic silver (Tripp and MacKay, 1972). Previously mineralised osteoid remains black, while collagen in unmineralised osteoid stains red (Tripp and MacKay, 1972).

Undecalcified sections were prepared from rib metaphyses from two sheep with rickets (1 and 2 years of age) and from a 2-year-old control sheep. These sections were prepared at a specialist medical histology laboratory (Histology Laboratory, Middlemore Hospital, Auckland), following embedding in methacrylate and were stained by Goldner's trichrome and Von Kossa's methods.

4.2.2.1 HISTOMORPHOMETRY

Histomorphometry was performed on sections of the distal femoral metaphysis stained by the Tripp & MacKay silver method. Red-stained collagen was regarded as representing unmineralised osteoid. Osteoid surface as a percentage of trabecular surface was measured using the linear intercept method at 50 random sites in the metaphysis (Revell, 1983). The osteoid area was measured on the 10x objective using AnalySIS docu software (Olympus Corp., Germany) at 10 randomly chosen sites in the secondary spongiosa. The results from each site were summed to give osteoid area, trabecular area, and osteoid area as a percentage of trabecular area. Osteoid seam width, from the trabecular edge to the black edge of mineralised bone, was measured using the

AnalySIS docu software at 50 randomly chosen sites, and the mean width calculated. The Mann-Whitney test for two samples was employed, using Minitab 15 statistical software (Minitab Inc., 2008, USA), to determine differences between control sheep and Corriedale sheep with inherited rickets.

4.3 RESULTS

4.3.1 GROSS PATHOLOGY

The most consistent gross lesion was focal to segmental thickening of the physes of long bones, in particular those of the distal radius, distal metacarpus and proximal tibia (Figure 4.1). In some sheep, the distal femoral and proximal humeral growth plates were also affected. Costochondral junctions were enlarged in animals 3-17 months of age (Figure 4.2A). Tongues of physeal cartilage often extended into the metaphyses (Figure 4.2B). In addition, separate islands of cartilage persisted in metaphyseal regions, especially in the proximal humerus, distal femur and ribs. Haemorrhage and disruption of trabeculae was commonly seen in the metaphyses. In some affected sheep, long bones had multiple horizontal sclerotic lines in the metaphysis parallel to the growth plate (also seen radiographically (Figure 3.6)). These were consistent with growth arrest lines (Figure 4.1D), reflecting periods where bone growth had been temporarily interrupted. In the fetus and lambs that died in the neonatal period, sagittal sectioning of long bones revealed a wedge-shaped core of persistent primary spongiosa, filling metaphyseal regions.

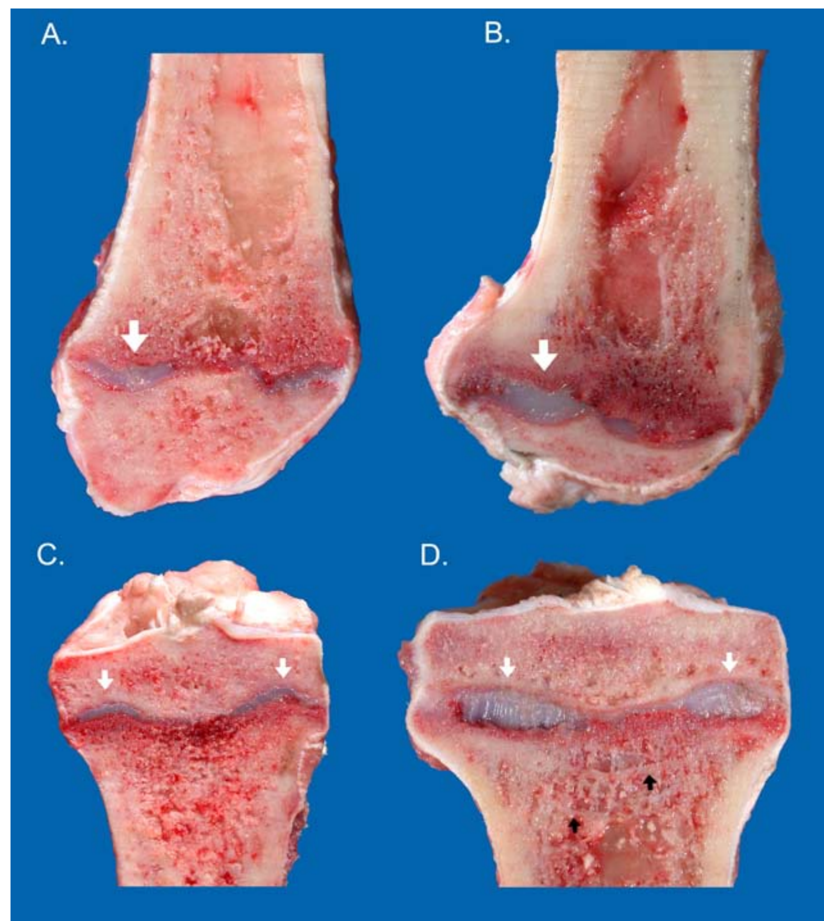


Figure 4.1: Sagittal sections of bones from Corriedale sheep with rickets. Note the variable degree of physeal thickening (white arrows) in the distal radius of a 12-month-old sheep (**A**), distal radius of a 6-month-old sheep (**B**), proximal tibia of a 12-month-old sheep (**C**) and proximal tibia of a 13-month-old sheep (**D**). Also note in (**D**) horizontal sclerotic lines parallel to the growth plate consistent with growth arrest lines (black arrows).

Figure 4.2: Ribs from Corriedale sheep with rickets. **A.** Markedly enlarged costochondral junctions from a 6-month-old sheep; **B.** Tongues and islands of cartilage (white arrows) in metaphysis of rib from 13-month-old sheep. Note the bulging metaphysis, and irregular outline of the physis.

Another lesion present in all affected animals from approximately 6 months of age, was bilateral collapse of the subchondral bone of the humeral head, leading to flattening and irregularity of the articular surface (Figure 4.3). On sagittal section, the humeral lesion varied from early separation of the articular cartilage from the collapsed subchondral bone to extensive under-running and formation of a cartilage flap (Figure 4.3). In some animals, the collapsed subchondral bone had foci of yellow grey discolouration, interpreted as areas of necrosis. In animals older than 2 years, the humeral head was sclerotic as a result of subchondral bone collapse, but two out of the four animals examined at this age still had separation of the articular cartilage from the underlying bone. The humeral head changed shape as the animals aged, becoming increasingly flattened with a ventrally protruding lip, resulting in decreased epiphyseal bone thickness (Figure 4.3B).

In most affected sheep the cortices of the long bones were greatly thickened and coarse trabeculae frequently bridged the diaphyseal medulla (Figure 4.4). The humerus was often most severely affected. Thickened cortical bone in affected animals was pale pink and often contained many fine red streaks (Figure 4.4B). In spite of the thickened cortices, long bones cut more easily than normal with a band saw. Serous atrophy of medullary fat was frequently present on sagittal sectioning of long bones. Metaphyseal trabeculae were at times resorbed to the level of the physis, especially in the distal radius, distal femur, and proximal tibia.

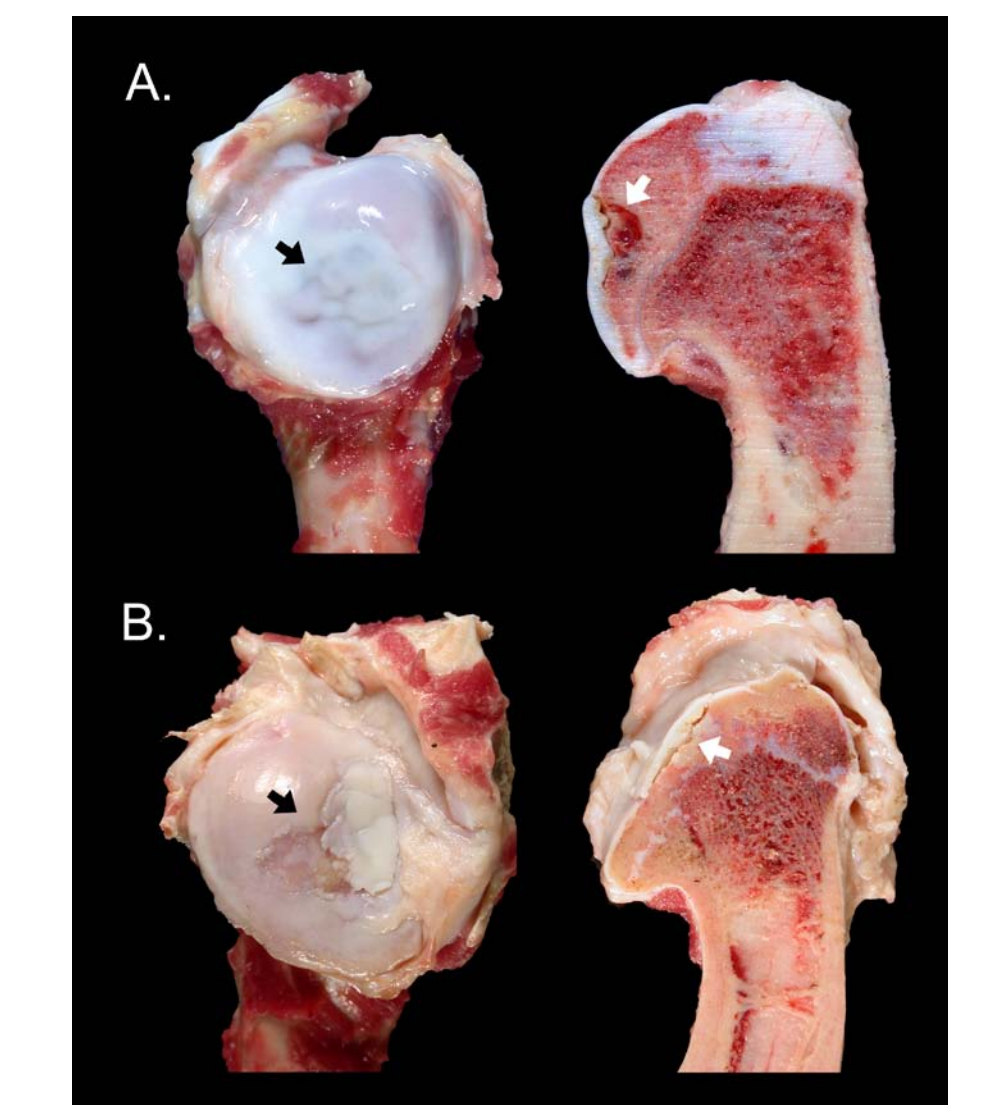


Figure 4.3: Humeri from sheep with rickets, showing articular cartilage defect (black arrows), and on sagittal section separation of articular cartilage from collapsed subchondral bone (white arrows). **A.** 9-month-old Corriedale with thickening and under-running of articular cartilage; **B.** 32-month-old Corriedale, with flattening of humeral head, reduced thickness of epiphyseal bone and a ventrally protruding lip.

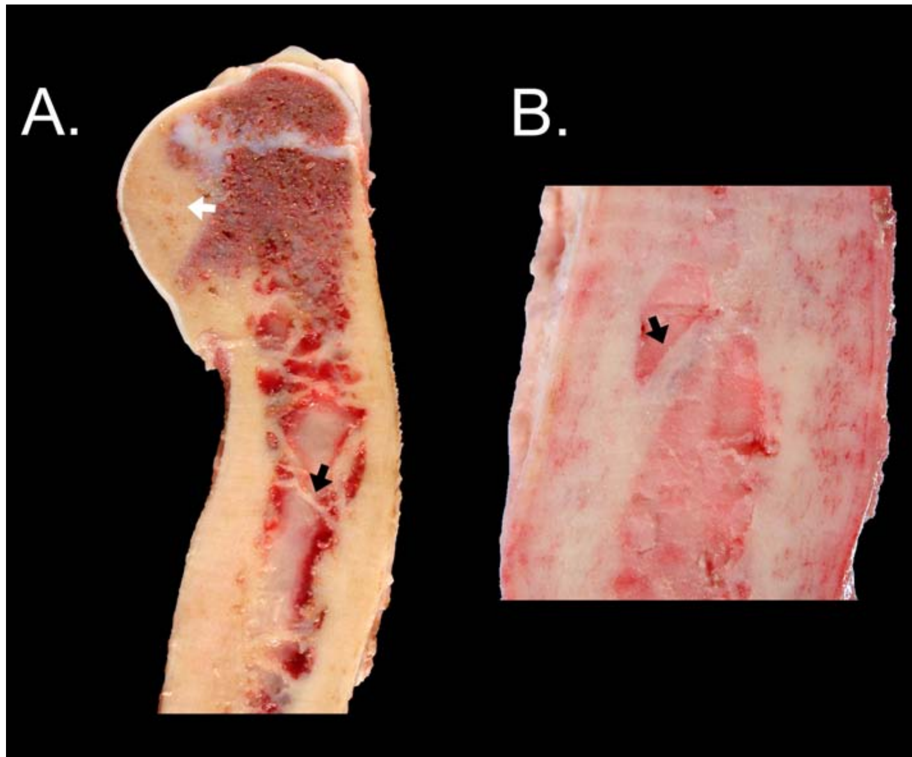


Figure 4.4: Sagittal sections of humeri from Corriedale sheep with rickets showing thickened cortices and thick bridging trabeculae (black arrows). **A.** Humerus from 30-month-old sheep, note also sclerosis of humeral head (white arrow); **B.** Humerus from 12-month-old sheep, note fine red longitudinal streaks in cortex.

In some animals, angular limb deformities were detected in various long bones (Figure 4.5). The radius, metacarpus and tibia were most severely affected, showing varus, valgus and forward curvatures. The angular deformities appeared to be due to asymmetric physeal growth as a result of growth plate lesions. The cortices on the concave surface of the curvature were often thicker than those on the convex side (Figure 4.5B).

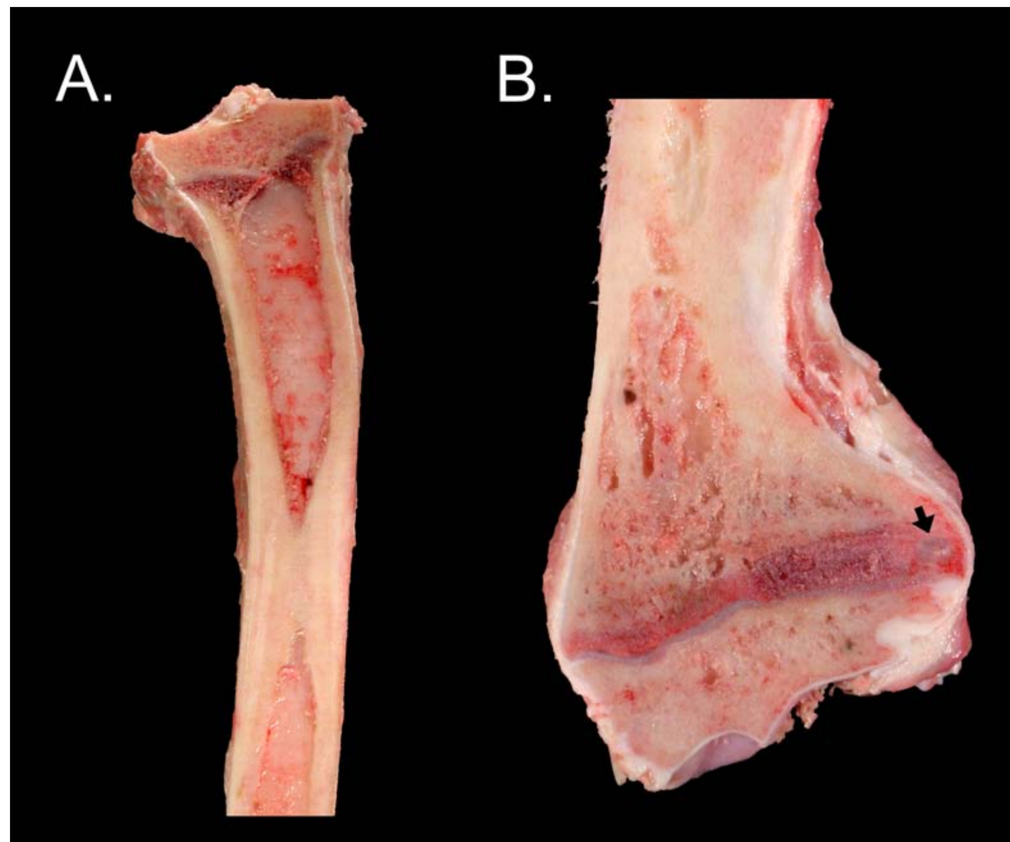


Figure 4.5: Sagittal sections of bones from Corriedale sheep with rickets and angular deformities. **A.** Tibia from 28-month-old sheep showing varus curvature and thickened cortices; **B.** Radius from 13-month-old sheep. Note the abnormal alignment of the distal radial growth plate and thickening of the convex side of the curvature. Also, a small island of persistent physeal cartilage is present in the metaphysis (black arrow).

Two neonatal lambs that died at 1 and 6 days of age following assisted birth had acute, bilateral fractures of several ribs. The fractures were displaced in the 6 day old lamb, and bulged into the thoracic cavity (Figure 4.6). Haemorrhage was associated with the fractures in both animals and there was no obvious callus formation, suggesting that they occurred during parturition. In animals less than 6 months of age the ribs were easy to fracture, and sometimes bent like cardboard. Likewise, the vertical ramus of the mandible was easily fractured in most animals.

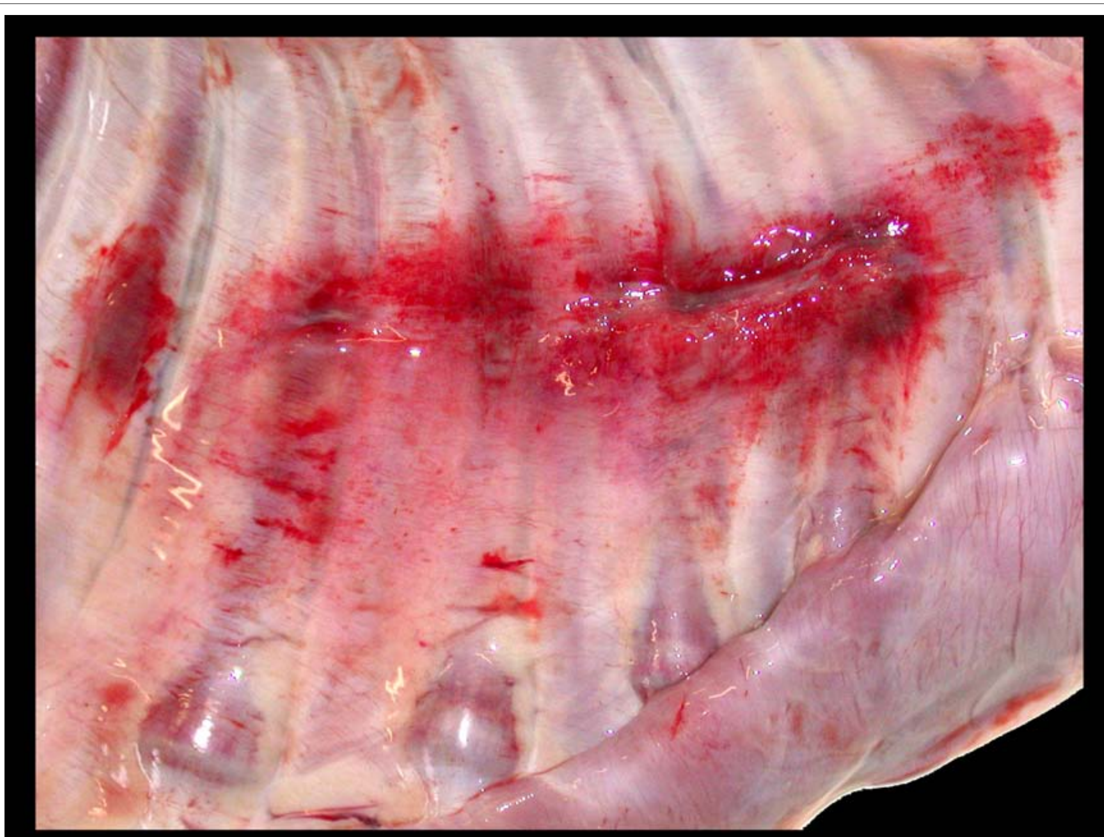


Figure 4.6: Ribs from 6-day-old Corriedale lamb with rickets, showing multiple acute rib fractures. Note the line of haemorrhage and no obvious callus formation.

In animals 2 years of age and older, exostoses of variable size projected from points of muscle and tendon insertion, including the supraglenoid tubercle of the scapula, greater trochanter of the femur, lateral malleolus of the tibia and the patella. The tarsus, metacarpophalangeal, metatarsophalangeal joints, proximal and distal interphalangeal joints had bony outgrowths and ligament ossification, which in some animals led to complete bridging of the joint (Figure 4.7).

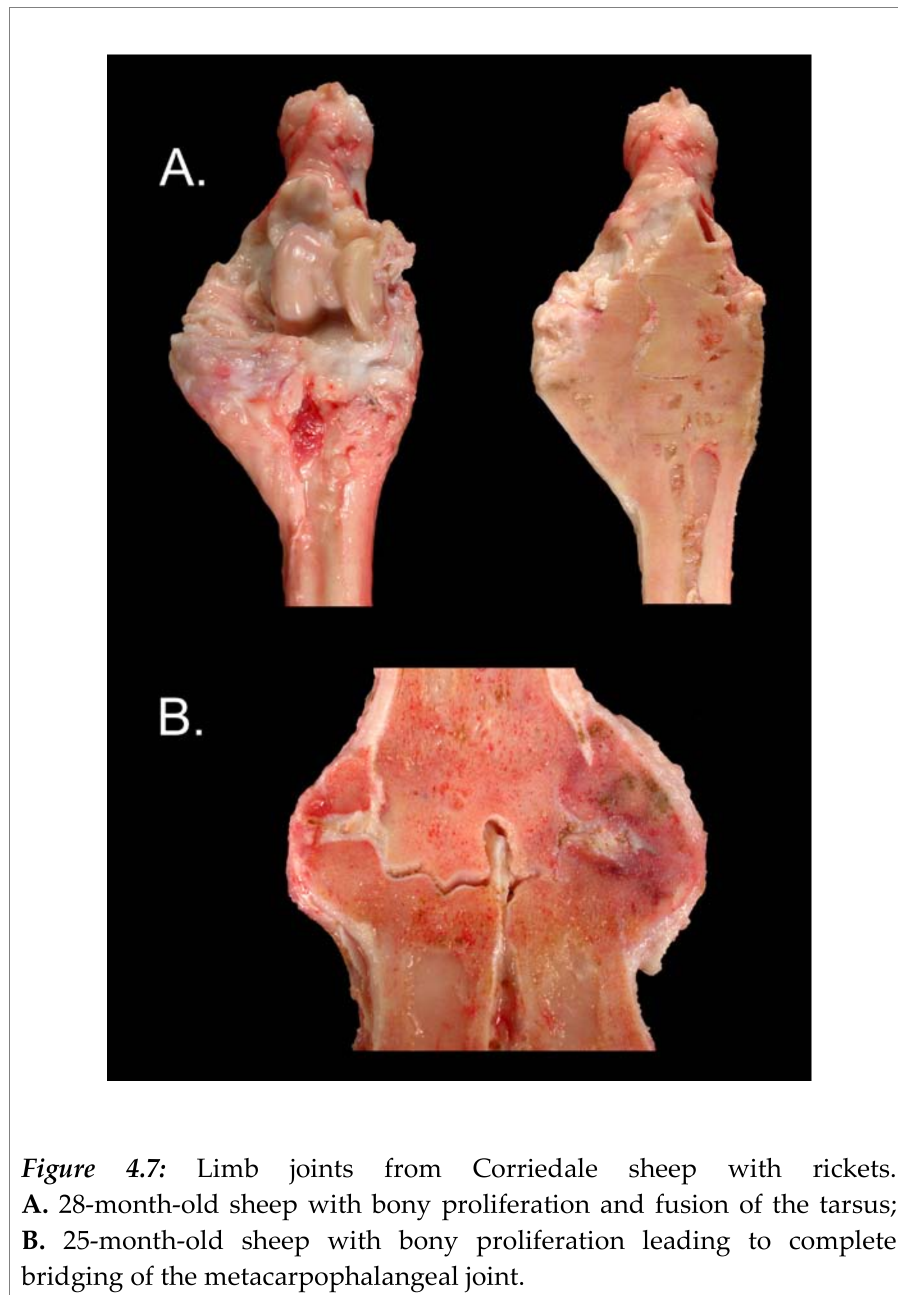


Figure 4.7: Limb joints from Corriedale sheep with rickets. **A.** 28-month-old sheep with bony proliferation and fusion of the tarsus; **B.** 25-month-old sheep with bony proliferation leading to complete bridging of the metacarpophalangeal joint.

Significant gross lesions were not present in extraskeletal organs except for multiple small yellow brown scabs with matted wool, most commonly on skin of the ears but also the skin over the withers and other parts of the body of many animals. These lesions were consistent with *Dermatophilus congolensis* infection and confirmed histologically.

4.3.2 HISTOPATHOLOGY

The severity and distribution of lesions varied with age and growth rate. The physeal lesions were characterised by irregular thickening of the hypertrophic zone and disorganisation of chondrocyte columns. Such lesions varied from focal to segmental thickening, the latter sometimes involving up to half the width of the growth plate. Tongues of persistent hypertrophic chondrocytes extended from the physis into the metaphysis (Figure 4.8). These varied in width from one to greater than 100 cells, but were generally 2-20 chondrocytes wide. The cartilage matrix in these tongues of persistent cartilage was eosinophilic, and at times fibrin was interspersed between clumps of chondrocytes (Figure 4.8C).

The physes of the costochondral junctions were consistently and severely affected in all animals. In the appendicular skeleton, the proximal tibial, distal radial and distal metacarpal growth plates had the greatest segmental thickening of the hypertrophic zone of chondrocytes. The proximal humeral, and proximal and distal femoral growth plates were less frequently affected. Lesions were uncommon in other long bone physes.

Growth plate lesions were present in one or more physes from all animals up to 2 years of age, and were present in the proximal femoral and costochondral physes of the fetus and newborn animals. The most severe physeal lesions were in animals 6-15 months of age, but by 2 years of age most physes were narrow and capped by a thin layer of osteoid, indicating the start of growth plate closure.

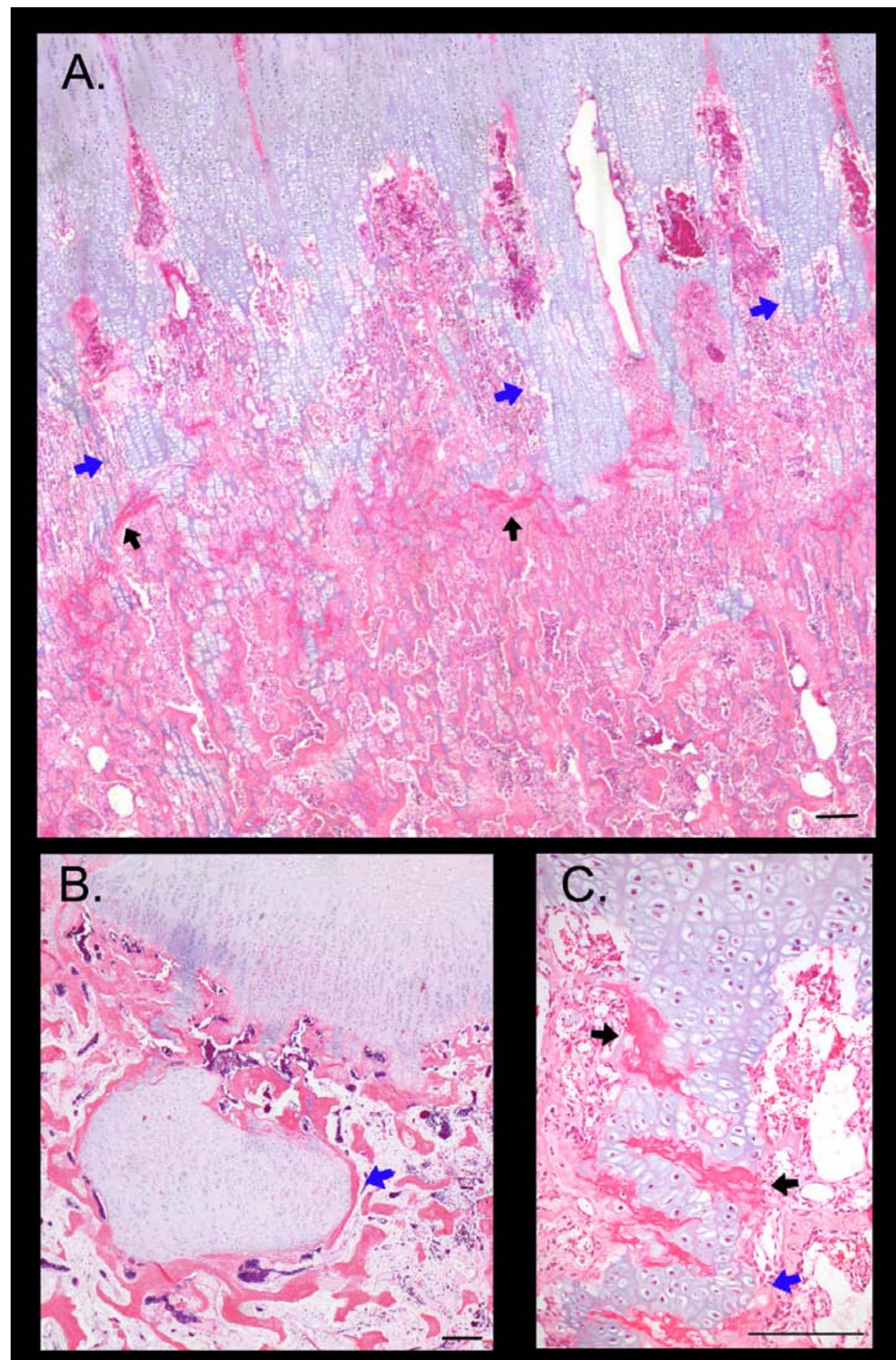


Figure 4.8: Physeal lesions in Corriedale sheep with inherited rickets. **A.** Proximal femoral physis from a 133-day Corriedale fetus, note thickening of physeal hypertrophic zone with tongues of hypertrophic chondrocytes extending into the metaphysis (blue arrows), and the infraction line (black arrows); **B.** Costochondral junction from 6-month-old lamb. Note island of cartilage in metaphysis (blue arrow); **C.** Costochondral junction from 6-month-old lamb showing tongue of hypertrophic chondrocytes (blue arrow) extending into metaphysis. Note fibrin indicating traumatic damage (black arrows). Bar = 200 μm.

Metaphyses usually consisted of thickened, disorganised trabeculae of woven bone, devoid of cartilage cores and separated by fibrous connective tissue (Figure 4.9A). In the metaphysis of some older animals, only short, wide, stubby trabeculae were present, the remainder having been resorbed almost to the level of the growth plate (Figure 4.9B). In many animals, thick transverse trabeculae, consistent with growth arrest lines, extended across the metaphysis parallel to the growth plate. In addition, there was serous atrophy of fat in the marrow cavity of many bones.

Islands of necrotic hypertrophic chondrocytes surrounded by eosinophilic cartilage matrix were present in the metaphysis of many bones. The cartilage islands were often partly surrounded by fibrin and haemorrhage, indicating traumatic damage. Trabecular microfractures were common in the metaphysis,

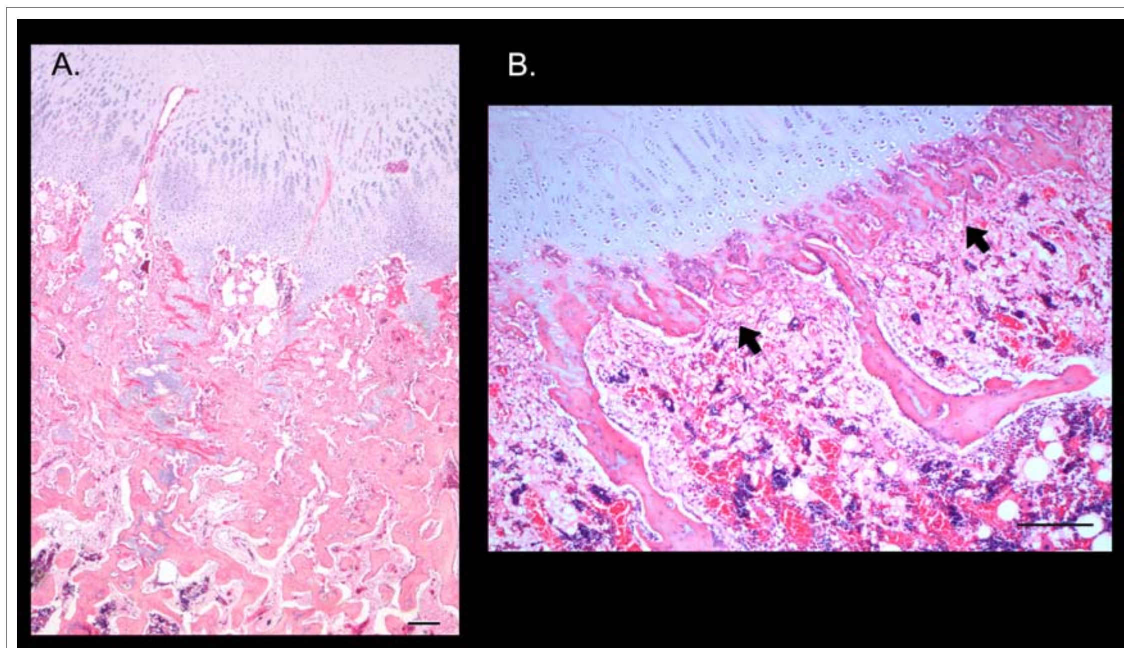


Figure 4.9: Metaphysis of Corriedale sheep with inherited rickets. **A.** Rib from 6-month-old lamb showing tongues of hypertrophic chondrocytes, microfractures, and disorganisation of trabeculae in the metaphysis; **B.** Proximal humerus from 9-month-old sheep showing shortened metaphyseal trabeculae. Bar = 200 µm.

particularly underneath thickened physes (Figure 4.8A, 4.8C, 4.9A). In some animals, the rib cortex was fractured, and showed fibrous callus formation.

In the fetus and neonatal lambs, there was diffuse persistence of the primary spongiosa, the metaphysis consisting of densely packed, thin, calcified cartilage spicules covered by a thin layer of osteoid, often joined laterally creating a lattice appearance (Figure 4.10A). In the long bones of these animals, there were multiple irregularly-spaced transverse lines of thickened trabeculae surrounding disorganised cartilage spicules, representing healed infraction lines (Figure 4.10B, C). The infractions were generally encased in osteoid, but otherwise there was little reaction, except in some cases where increased osteoclastic activity was noted above and below these areas. The fetus also had recent infraction lines with haemorrhage, fibrin and fibrous connective tissue, extending beneath the physes of multiple bones.

Excessive and inappropriate osteoclastic bone resorption was a feature in all animals. Thick metaphyseal trabeculae often contained resorption cavities lined by osteoclasts and Howship's lacunae, sometimes referred to as “dissecting osteitis” (Milgram, 1990c)(Figure 4.11B). Large resorption cavities were also present in the cortices (Figure 4.11A), even in young animals, and secondary osteons with scalloped margins were partly filled with pale staining osteoid. Thick, pale eosinophilic matrix consistent with poorly mineralised osteoid lined trabeculae and filled cortical Haversian systems in the long bones and mandible of all animals, including the fetus (Figure 4.12A, B). The unmineralised nature of this matrix was supported by red staining using the

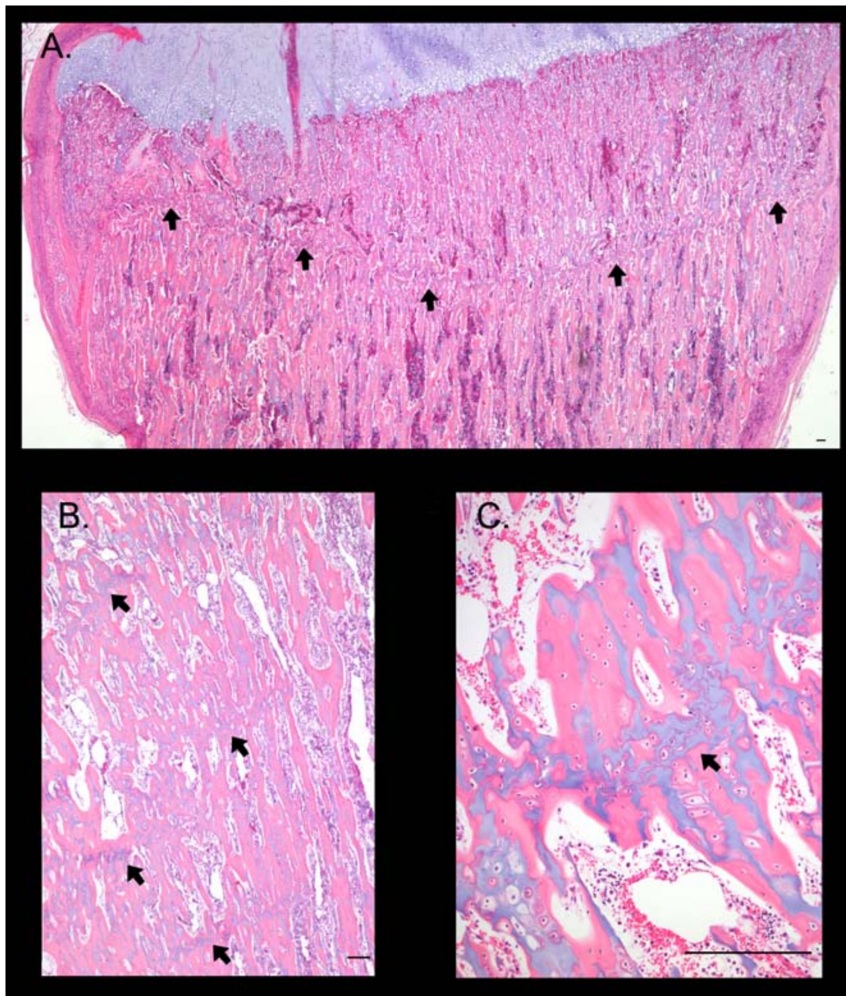


Figure 4.10: **A.** Rib from 5-day-old Corriedale lamb with inherited rickets showing infraction line (black arrows) and persistence of primary spongiosa; **B.** and **C.** Proximal femur from 1-day-old Corriedale lamb with inherited rickets showing healed infraction lines (black arrows). Bar = 200 μm.

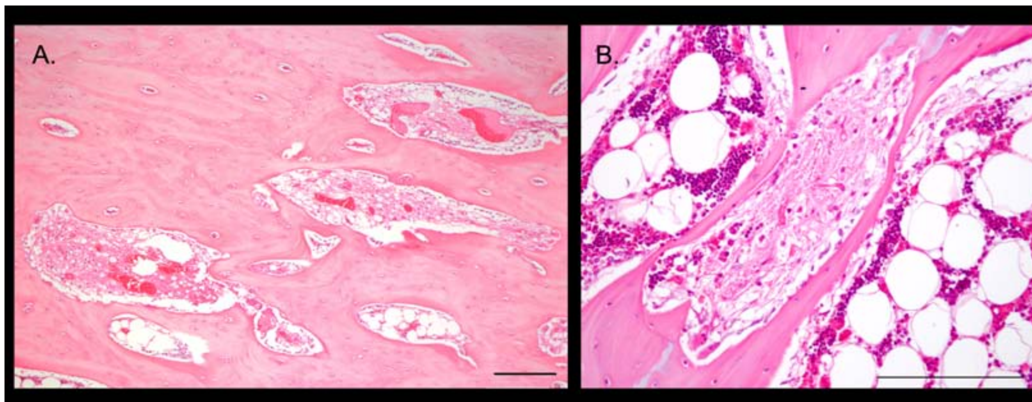


Figure 4.11: Excessive osteoclastic resorption in bones from Corriedale sheep with inherited rickets. **A.** Proximal humerus of 9-month-old lamb showing resorption cavities in cortical bone; **B.** Proximal femur from 9-month-old lamb with trabecular resorption cavity. Bar = 200 μm.

Tripp & MacKay silver method, and by Goldner's trichrome method in undemineralised sections (Figure 4.12 C, D, E, F).

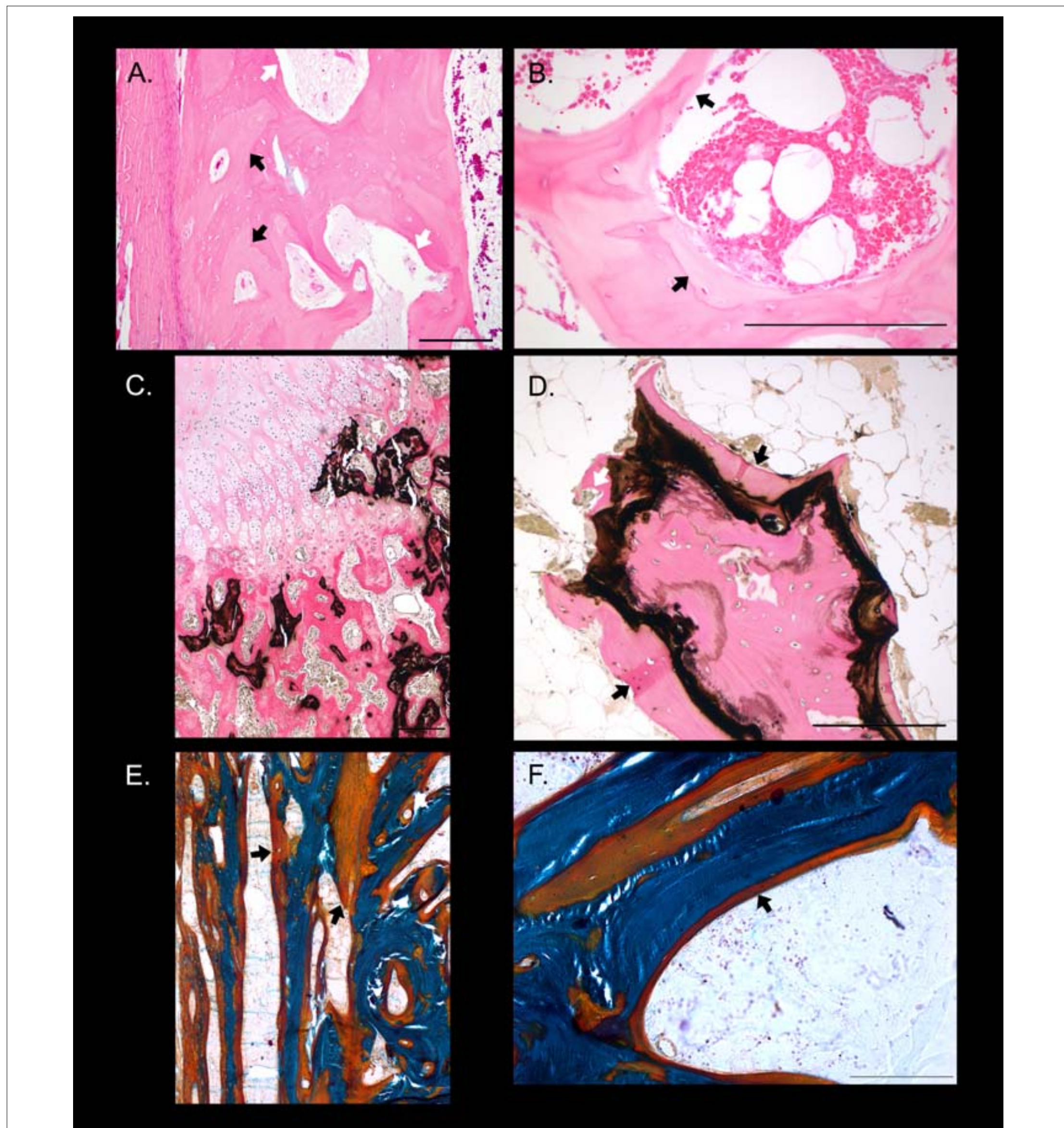


Figure 4.12: Unmineralised osteoid seams lining trabeculae and filling secondary osteons in the cortex of Corriedale sheep with inherited rickets. **A.** Rib cortex of 9-month-old lamb, showing resorption cavities (white arrows) and secondary osteons with scalloped margins partly filled with pale staining osteoid (black arrows); **B.** Rib of 25-month-old hogget showing osteoid seams lining trabeculae (black arrows); **C.** Rib of 12-month-old hogget, showing unmineralised nature of metaphyseal trabeculae (Tripp & MacKay method) **D.** Distal femur of 12-month-old hogget showing unmineralised osteoid seams lining trabeculae (black arrows), note osteoclastic resorption and Howship's lacunae (white arrow) (Tripp & MacKay method); **E.** Rib cortex of 12-month-old hogget, showing thick unmineralised osteoid seams (black arrows)(Goldner's trichrome method); **F.** Rib from 25-month-old hogget showing thick osteoid seams lining trabeculae (black arrow)(Goldner's trichrome method). Bar = 200 μm.

A consistent lesion in animals 6 months and older (except for two sheep older than 2 years) was collapse of the subchondral bone of the humeral head. The area of subchondral bone collapse consisted variably of necrotic bone with empty lacunae, chondrocytes with pyknotic nuclei, eosinophilic cartilage matrix, fibrin, and granulation tissue (Figure 4.13). Osteoclastic resorption occurred around the edges of these lesions. Intact trabeculae in the surrounding area were thickened and lined by wide osteoid seams. The articular cartilage overlying the affected area tended to be thicker than in non-affected areas and in several sheep, the articular cartilage was separated from the collapsed subchondral bone. In the 28- and 30-month-old sheep, no obvious subchondral bone collapse was present, but there was sclerosis of the epiphysis

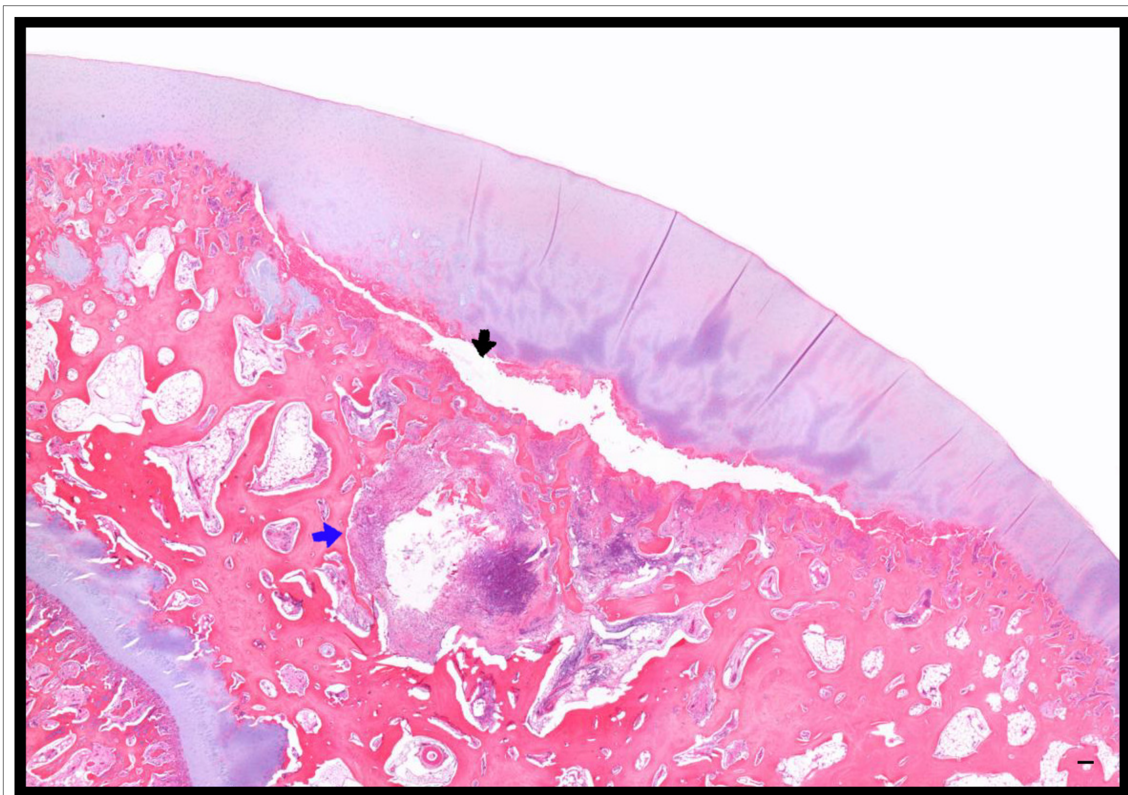


Figure 4.13: Subchondral bone collapse of the humeral head from a 9-month-old Corriedale sheep with inherited rickets. Note, separation of thickened articular cartilage from underlying subchondral bone (black arrow), degenerate articular cartilage, necrotic bone, fibrin and granulation tissue (blue arrow). Bar = 200 µm.

of the humeral head. In the oldest animal examined (32 months), the articular cartilage of both the humerus and scapula was eroded, and extensively replaced by fibrocartilage.

Animals 2 years and older developed bony proliferations around the distal limb joints. The bony proliferations consisted of dense lamellar bone, with a few areas of cartilage and reactive woven bone. The new bone formation appeared to be centred on sites of tendon and ligament insertion, consistent with these being enthesophytes.

In spite of a careful search, external parathyroid glands were not detected in any sheep. The internal parathyroid glands consisted of nests of closely packed vacuolated epithelial cells and loss of pericapillary spaces (Figure 4.14), consistent with parathyroid hyperplasia.

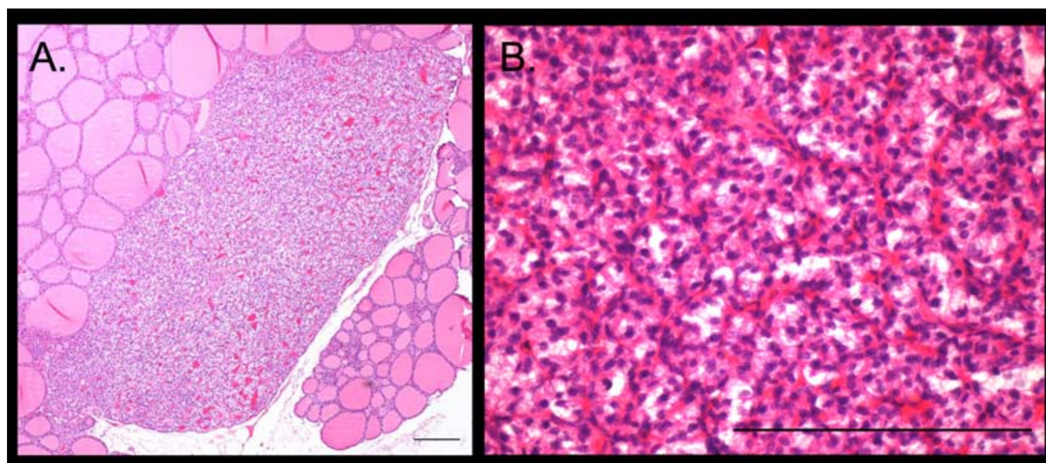


Figure 4.14: Hyperplasia of the internal parathyroid gland in Corriedale sheep with inherited rickets. **A.** Low power view of parathyroid gland partly surrounded by thyroid tissue in a 25-month-old hogget; **B.** Higher power view of hyperplastic chief cells with vacuolated cytoplasm and loss of pericapillary spaces in a 12-month-old hogget. Bar = 200 μm.

4.3.2.1 HISTOMORPHOMETRY

As presented in Table 4.2, affected sheep had a mean osteoid surface, as a percentage of trabecular surface, of 59.9 ± 5.3% and a mean osteoid seam thickness of 30.7 ± 1.2 µm. These values were significantly greater than mean osteoid surface percentage (8.2 ± 1.2%) and mean osteoid seam thickness (4.9 ± 0.8 µm) of control sheep. Mean osteoid area as a percentage of trabecular area was significantly greater in affected sheep (19.7 ± 1.2%) than in control sheep (1.05 ± 0.05%).

Table 4.2: Histomorphometry results of osteoid seam measurements in the distal femoral metaphysis of control sheep, and sheep with inherited rickets.

Status	***Osteoid Surface % (Mean ± SEM)**	**Mean osteoid seam thickness (µm) (Mean ± SEM)**	****Osteoid area % (Mean ± SEM)**
Control	9.4	4.05	1.0
Control	7.0	5.69	1.1
	(8.2 ± 1.2)	(4.9 ± 0.8)	(1.1 ± 0.1)
Affected	66.7	27.75	23.0
Affected	76.4	29.28	19.4
Affected	42.1	27.64	14.7
Affected	58.8	31.17	20.2
Affected	55.4	35.84	18.8
Affected	44.0	34.36	23.7
Affected	76.0	28.53	17.9
	(59.9 ± 5.3)*	(30.7 ± 1.2)*	(19.7 ± 1.2)*

*Osteoid surface as a percentage of trabecular surface. ** Osteoid area as a percentage of trabecular area. SEM = standard error of mean *P=0.03. P-value obtained from Mann-Whitney two sample test.

4.4 DISCUSSION

The gross and microscopic lesions described in these Corriedale sheep are consistent with rickets and osteomalacia, with concurrent hyperparathyroidism (Milgram, 1990b; Milgram, 1990c). Thickening of the physes due to persistence of hypertrophic chondrocytes in the metaphysis, in particular costochondral junctions, are classic lesions of rickets in humans and animals (Fitch, 1943; Milgram, 1990b; Theiler, 1934), and were present in all affected Corriedale sheep less than 2½ years of age. The wide osteoid seams seen in affected Corriedale sheep are characteristic of osteomalacia (Milgram, 1990b).

Increased osteoclastic activity was a feature in the majority of Corriedale sheep with this inherited skeletal disease. Resorption cavities within trabeculae ("dissecting osteitis"), together with parathyroid gland hyperplasia, is suggestive of hyperparathyroidism (Milgram, 1990c). This is expected in an animal with vitamin D deficiency, as reduced intestinal calcium absorption will lead to hypocalcaemia, resulting in secondary hyperparathyroidism and skeletal lesions of fibrous osteodystrophy, together with rickets (Milgram, 1990c; Whyte, 2002).

An unusual finding, was persistence of the primary spongiosa in neonatal animals. In a mouse model with inactivation of the vitamin D receptor (VDR) in chondrocytes, juvenile mice showed a transient increase in metaphyseal trabecular density as a result of decreased vascular invasion and osteoclastogenesis (Masuyama *et al*., 2006). The authors suggested this was due

to decreased VDR-mediated signaling in chondrocytes leading to decreased receptor activator of NF-κB ligand (RANKL) and vascular endothelial growth factor (VEGF) expression required for osteoclast differentiation and vascular invasion (Masuyama *et al*., 2006). A similar mechanism may have led to persistence of the primary spongiosa in neonatal Corriedale lambs with rickets, with hyperparathyroidism leading to excessive osteoclastic resorption postnatally.

Thickening of the hypertrophic zone in physes of domestic animals has also been reported in association with copper deficiency, fluoride toxicosis and osteochondrosis (OCD). However, none of these would be expected to cause the spectrum of skeletal lesions seen in these Corriedale sheep. OCD is characterised by both physeal thickening and separation of articular cartilage from subchondral bone, but bone fragility and thick osteoid seams, as described here in affected Corriedale sheep, are not features of OCD (Scott *et al*., 1996). In OCD the articular defect originates in the cartilage rather than in subchondral bone. The pathogenesis of OCD is poorly understood, however it is thought to be due to ischaemic damage to the articular-epiphyseal cartilage complex leading to focal cartilage necrosis and localised failure of endochondral ossification (Carlson *et al*., 1989; Carlson *et al.*, 1995; Ytrehus *et al*., 2007). The eventual lesion is seen as underrunning of articular cartilage and subchondral cystic lesions (Thompson, 2007; Ytrehus *et al*., 2007). In sheep with rickets, collapse of the subchondral bone leads to formation of the cartilage flap. Although copper deficiency is reported to cause physeal thickening in cattle, lambs with copper deficiency typically develop osteoporosis and pathological fractures (Suttle *et al*., 1972). Fluoride toxicity is also unlikely due to the

absence of the classic brown staining and excessive wearing of incisor teeth (Thompson, 2007). Furthermore, history, relocation to a new farm, and the genetic nature of the disease make copper deficiency and fluorosis unprobable causes of the bone lesions in affected Corriedale sheep.

The increased physeal thickness in rickets is due to persistence of hypertrophic chondrocytes, as a result of a failure of provisional calcification. The reason for this persistence is poorly understood, but rather than an increase in proliferation of these cells it is most likely due to a failure of the hypertrophic chondrocytes to undergo apoptosis (Donohue and Demay, 2002). Circulating (rather than local) phosphate concentration is thought to influence hypertrophic chondrocyte apoptosis via phosphate-mediated activation of the caspase-9 mitochondrial pathway (Sabbagh *et al.*, 2005). Hypophosphataemia could therefore decrease apoptosis of hypertrophic chondrocytes by this mechanism, and lead to their persistence in animals with rickets.

Thick osteoid seams were a striking feature in Corriedale sheep with inherited rickets. Because of the technical difficulties involved with undecalcified bone section preparation, and the expense of having them performed elsewhere, a stain developed by Tripp & MacKay (Tripp and MacKay, 1972) was used to better visualise the osteoid seams, especially for histomorphometry. Results from the undecalcified sections were used to validate the interpretation of the Tripp & MacKay method, which was a cheaper and more practical alternative. Milgram (1990b), states that in humans osteoid seams greater than 20 μm are diagnostic for osteomalacia. Other authors suggest that osteoid seams of 12.5

µm or thicker indicate this disease (Rauch, 2003). A diagnosis of osteomalacia is supported by a demonstration of increased mineralisation lag time, which was not measured in this study. However, the presence of osteoid seams lining trabeculae and in osteons of bones at all ages examined, suggests that mineralisation lag time was delayed in affected sheep.

Despite a high incidence of vitamin D deficiency rickets in many parts of the world, histomorphometric data is lacking, as the diagnosis can usually be made radiographically and treatment is straightforward (Rauch, 2003). However, a study of 15 children with rickets from South Africa, showed that trabecular osteoid surface was increased in 11 children, and trabecular osteoid volume and mean osteoid surface width were increased in 14 children (Raubenheimer *et al*., 1997) Data collected from XLH rickets patients showed that the surface area of trabeculae covered by osteoid was increased, but that the osteoid seam thickness was usually normal (Reid *et al*., 1989).

Collapse of subchondral bone in the humeral head was present with varying severity in all animals 6 months and older. In outbreaks of rickets in growing pigs in the United Kingdom and New Zealand, many affected animals also had humeral head cartilage ulceration and loss of subchondral bone (Pepper *et al*., 1978; Thompson and Robinson, 1989). This lesion has also been reported in rachitic calves (Craig and Davies, 1943). In sheep, the forelimbs bear 59% of body weight (Kim and Breur, 2008). In comparison to the other joints of the forelimb, which are hinge joints, the shoulder joint has a greater capacity for movement in all directions (Dyce *et al*., 1996). The increased severity of lesions

in the shoulder joint of Corriedale sheep with inherited rickets may therefore be related to the increased weight-bearing of the forelimb in quadrapeds and the greater range of movement in the shoulder joint, making it more susceptible to weight-bearing stress.

The bridging around the tarsus, metacarpophalangeal, metatarsophalangeal, proximal and distal interphalangeal joints was consistent with the formation of enthesophytes. In a study of XLH rickets in people, 33% of patients younger than 30 years of age, and all those older had enthesopathies (Reid *et al*., 1989). The pathogenesis of the enthesophytes in hypophosphataemic rickets is unclear, but may be the end-result of a "long-standing mineralization defect" (Burnstein *et al*., 1989, p787). In the case of the enthesophytes in sheep with inherited rickets, the most likely pathogenesis involves strain on ligament/tendon attachments to weakened, poorly mineralised bone, resulting in the new bone formation that is seen clinically and radiographically. Such lesions are not seen in human patients with vitamin D-dependent rickets type I (VDDR I) or hereditary vitamin D-resistant rickets (HVDRR) (Pitt, 1995).

The severity of physeal lesions in the long bones was most likely associated with the rate of growth just prior to post-mortem examination, as growth plate thickening in rickets is greatest in bones with the fastest growth (Shore and Poznanski, 1999). After birth, the relative growth of the limb bones is greater proximally than distally (Davies *et al*., 1984). However, this does not fit well, with the timing of growth plate changes in the animals examined in this study. The proximal femoral growth plate was thickened in the fetus and neonatal

animals, while the distal radius, proximal tibia and distal metacarpal growth plates were most severely thickened in animals 6-15 months of age. This is probably because these growth plates are responsible for the majority of growth for those bones. The distal radial growth plate for example, contributes 75% of radial growth (Farnum, 1994). Disorganisation of the primary spongiosa and a decrease in vascular invasion of the metaphysis in association with weight-bearing (Rauch, 2003), may also contribute to the increase in physeal width.

Although it was reported from the farm of origin that the lambs were normal at birth (Thompson *et al*., 2007), the fractures suffered by dystocic lambs and infraction lines seen in the fetus provide strong evidence for intrauterine bone fragility. The physeal thickening and wide osteoid seams in an affected fetus, despite the normocalcaemic status of the recipient ewe, add further support to the intrauterine development of rickets in this inherited disease of Corriedale sheep. In humans with inherited rickets, affected individuals do not develop clinical signs of skeletal disease until 3-4 months of age. During pregnancy, intestinal absorption of calcium is doubled, but not by vitamin D-dependent mechanisms (Kovacs, 2008). Pregnant vitamin D-deficient rats and *vitamin D receptor* (*VDR*) null mice have up-regulation of intestinal calcium absorption at the same rate as normal pregnant rats and mice (Kovacs and Kronenberg, 1997). Other potential calcium-regulating hormones are increased during pregnancy, and include prolactin, placental lactogen, and most importantly parathyroid hormone-related peptide (PTHrP) (Kovacs, 2008). The placenta in rats, mice and humans is thought to provide calcium without relying on vitamin D (Kovacs, 2008). Vitamin D-deficient and *VDR* null mice placentas express

normal concentrations of vitamin D-dependent calbindin-D_{9k} and Ca^{2+}-ATPase (Kovacs, 2008; Kovacs *et al*., 2005).

The development of rickets in intrauterine Corriedale sheep fetuses, could reflect differences between sheep and other species in placental calcium transport. In monkeys and rats, passive movement of calcium across the placenta accounts for the majority of fetal calcium (Lester, 1986). In sheep, passive diffusion is only a minor part of placental calcium transport, with active transport being more important (Lester, 1986). Nephrectomy of the ovine fetus leads to a drop in $1,25(OH)_2D_3$ and serum calcium concentration (Lester, 1986; Kovacs and Kronenberg, 1997). The decreased serum calcium concentration was partially normalised by administration of $1,25(OH)_2D_3$ to the fetus (Kovacs and Kronenberg, 1997). A lack of $1,25(OH)_2D_3$ in the ovine fetus may therefore cause inadequate mineralisation of bones and a failure of endochondral ossification due to an inability of the fetus to transport calcium across the placenta. Further studies on this inherited form of rickets in Corriedale sheep may therefore generate useful information on ovine placental calcium transport.

The ruminant external parathyroid gland is considered to be located cranial to the thyroid but caudal to the carotid bifurcation, possibly embedded in the thymus or mandibular gland and are 8-10 mm in size (Dyce *et al*., 1996). Despite a careful search, and evidence of hyperplasia of internal parathyroid glands, the external parathyroid glands were not detected in any affected sheep. This highlights the difficulties in locating the ovine external parathyroid gland,

especially as it would have been expected to be more prominent than normal in these sheep.

Microscopically, the hereditary forms of rickets (with the exception of XLH rickets) are indistinguishable from nutritional rickets (Pitt, 1995). VDDR I and HVDRR produce severe rickets and secondary hyperparathyroidism similar to nutritional rickets (Pitt, 1995). XLH rickets is generally accompanied by only mild to moderate lesions of rickets, and secondary hyperparathyroidism is absent (Rauch, 2003). Microscopically, in patients with this disease periosteocytic hypomineralised lesions are present, and are considered pathognomonic (Rauch, 2003; Reid *et al*., 1989). Due to variation in the depth of silver penetration of the Tripp and MacKay method, the demonstration of periosteocytic hypomineralised lesions in sheep with inherited rickets was not possible.

The animal models developed for studying hereditary rachitic diseases all show similar microscopic features. The *VDR* null mouse, *1α-hydroxylase* null mouse, and *Hyp* mouse all exhibit increased expansion and disorganisation of the growth plate and accumulation of osteoid in trabecular and cortical bone (Carmeliet *et al*., 2003). Microscopically, both the *VDR* null mouse and *1α-hydroxylase* null mouse show features of secondary hyperparathyroidism, but the *Hyp* mouse does not (Carmeliet *et al*., 2003). Unlike humans with HVDRR, the corresponding mouse phenotype (*VDR* null) can be "rescued" by supplying a diet containing 2% calcium, 20% lactose and 1.25% phosphorus (Amling *et al*., 1999; Li *et al*., 1998).

In conclusion, although this inherited skeletal disease in Corriedale sheep has lesions typical of rickets, it does not appear to be analogous to any of the forms reported to date in either human patients or in animal models. Nor has a similar disease been reported in domestic animals. It is possible therefore that this disease represents a new form of inherited rickets with a different mechanism than those diseases described to date.

4.4.1 Summary

The macroscopic and microscopic changes described in Corriedale sheep in this study are consistent with rickets. Although the most severe lesions were seen in growing lambs 6-15 months of age, there was convincing evidence of intrauterine bone fragility, sometimes resulting in rib fractures during parturition and death in the perinatal period. The inappropriate and excessive osteoclastic resorption present in most bones was likely a result of secondary hyperparathyroidism due to hypovitaminosis D and concurrent hypocalcaemia. This study confirms that the skeletal disease in Corriedale sheep is a newly discovered form of inherited rickets and suggests that the genetic defect may be different from inherited forms of rickets described to date in humans and animals.

Chapter 5

Serum Chemistry

5.1 Introduction

Rickets is a metabolic bone disease caused by either vitamin D deficiency or phosphate deficiency. Vitamin D is an essential hormone for calcium and phosphate homeostasis (DeLuca, 2004). A lack of vitamin D leads to decreased intestinal calcium and phosphate absorption and a decrease in serum ionized calcium concentration (Holick, 2006). This leads to an increase in the secretion of parathyroid hormone (PTH), which mobilises calcium and phosphate from bone, increases renal tubular reabsorption of calcium, and decreases renal tubular phosphate reabsorption (Holick, 2006). Typically, in humans with rickets caused by vitamin D deficiency, serum calcium concentrations are normal to low-normal, while serum phosphate concentrations are low to low-normal, reflecting the greater homeostatic control of serum ionised calcium

(Holick, 2006; Wharton and Bishop, 2003). Once skeletal calcium stores are depleted, hypocalcaemia occurs (Holick, 2006).

Vitamin D, whether it is obtained from the diet or formed by ultraviolet induced thermal isomerisation of 7-dehydrocholesterol, undergoes two hydroxylations, the first in the liver and the second in the kidney, to become 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active form of vitamin D (Holick, 1999). The first hydroxylation in the liver produces 25-hydroxyvitamin D (25(OH)D), the major circulating form of vitamin D (Holick, 2006). This form is used as an estimate of vitamin D status, and is low (less than 25 nmol/L) in the circulation of children with nutritional rickets (Holick, 2006; Wharton and Bishop, 2003). Since PTH stimulates increased production of 1,25$(OH)_2D_3$ in the kidney, serum 1,25$(OH)_2D_3$ concentration tends to be normal or high in most cases of nutritional rickets (Holick, 2006; Wharton and Bishop, 2003).

Measurement of the different forms of vitamin D in serum may be useful in distinguishing between the various hereditary forms of rickets in humans. Patients with X-linked hypophosphataemic rickets (XLH) have inappropriately normal to low normal serum 1,25$(OH)_2D_3$ concentrations given the degree of hypophosphataemia, similar to humans with autosomal dominant hypophosphataemic rickets where serum 1,25$(OH)_2D_3$ concentrations are also inappropriately normal (Whyte, 2002). Patients with vitamin D-dependent rickets type I (VDDR I) have normal serum 25(OH)D, but low serum 1,25$(OH)_2D_3$ concentrations (Whyte, 2002). In contrast, patients with hereditary vitamin D-dependent rickets (HVDRR) have elevated serum 1,25$(OH)_2D_3$

concentrations, sometimes as much as ten times normal (Malloy and Feldman, 1999; Whyte, 2002).

The serum concentration of the non-collagenous bone protein osteocalcin may be measured as a biomarker of bone formation (Hauschka *et al*., 1989; Lian *et al*., 1999b). The concentration of osteocalcin in serum reflects that which is not incorporated into mineralised osteoid, and in adults this comprises approximately one-third of osteocalcin production (Hauschka *et al*., 1989). Although the *osteocalcin* gene is vitamin D-dependent, and contains vitamin D-response elements in its promoter (Lian *et al*., 1989; Lian *et al*., 1999b) and treatment with vitamin D increases serum osteocalcin, no correlation has been found between serum $1,25(OH)_2D_3$ concentrations and serum osteocalcin concentrations in humans with rickets, as other factors such as PTH and glucocorticoids can also alter osteocalcin synthesis (Hauschka *et al*., 1989; Lian *et al*., 1999b). Cultured fibroblasts from humans with HVDRR, have minimal to no induction of osteocalcin in response to treatment with $1,25(OH)_2D_3$ (Yagi *et al*., 1993).

Serum calcium and phosphate concentrations provided support for the initial diagnosis of rickets in Corriedale sheep with inherited rickets. The aim of this study was to obtain information on vitamin D, calcium and phosphate metabolism in affected sheep, in an effort to gain further insight into disease progression and the disease mechanism.

5.2 Materials and Methods

5.2.1 Sample Collection and Animals Used

Three groups of animals had blood taken for measurement of serum calcium and phosphate concentrations, as shown in Table 5.1. The groups of affected animals included the Corriedale sheep obtained from Marlborough at 5 months of age, lambs derived from the embryo transfer breeding trial from 0-12 weeks of age (Chapter 2), and lambs born to the heterozygous ram back-cross breeding trial from 0-12 weeks of age (Chapter 2). The control animals for the Marlborough group were obtained from the same farm. The age-matched control lambs for the embryo transfer breeding trial were collected from another Manawatu farm, 6 weeks prior to the time of serum collection from affected animals. This was necessary because of the anticipated unavailability of controls at the time the embryo transfer lambs were born. The controls for the heterozygous ram breeding trial were the normal lambs born to the breeding trial.

Table 5.1: Groups of animals from whom serum calcium and phosphate concentration were measured, and the ages at which the samples were taken.

Group	Number Affected	Number Control	Age
Original Marlborough	14	8	5 months
Derived from embryo transfer	12 2 died by 1 week of age	16 2 died before 2 weeks of age	Weekly from 0-6 weeks of age, then fortnightly until 12 weeks.
Heterozygous ram back-cross breeding trial	3	12	Fortnightly from 0-8 weeks of age, then again at 12 weeks.

The results were analysed using unpaired Student's t-test by either Minitab 15 (Minitab Inc., 2008) or GraphPad Prism 3.0 (GraphPad Software, USA).

5.2.1.1 SERUM CALCIUM AND PHOSPHATE

Blood samples were taken by jugular venipuncture into plain evacuated tubes. The serum was separated by centrifugation at 2000 *g* for 10 min and frozen at -80°C until processed. Total serum calcium and phosphate were measured using a Roche Hitachi 911 Chemistry Analyser (Roche Diagnostics, USA) at a commercial veterinary diagnostic laboratory (New Zealand Veterinary Pathology, Palmerston North, NZ). The laboratory was unable to measure serum ionised calcium concentrations.

5.2.1.2 URINE

Urine was collected by the free-catch method from eight animals with inherited rickets. The urine was centrifuged to remove any gross contamination, and then creatinine, calcium and phosphate were measured by a commercial veterinary diagnostic laboratory (New Zealand Veterinary Pathology Limited, Palmerston North) using a Roche Hitachi 911 Chemistry Analyser (Roche Diagnostics, USA). Blood was collected by jugular venipuncture into plain evacuated tubes at the same time, to measure serum creatinine, calcium and phosphate concentrations. Fractional excretion of calcium and phosphate was calculated using the following equation (Lefebvre *et al*., 2008):

$$(\text{urine}_{\text{electrolyte}} \text{ x } \text{serum}_{\text{creatinine}}) / (\text{serum}_{\text{electrolyte}} \text{ x } \text{urine}_{\text{creatinine}}) \text{ x } 100.$$

5.2.2 VITAMIN D

Blood for vitamin D estimation from the affected 5-month-old Marlborough sheep was collected and processed as described in Section 5.2.1.1. The vitamin D metabolites – $25(OH)D_3$ and $1{,}25(OH)_2D_3$ were measured by radioimmunoassay at Sullivan Nicolaides Pathology Laboratory (Brisbane, Australia). The results were analysed with an unpaired Student's t-test using GraphPad Prism 3.0 statistical software.

5.2.3 OSTEOCALCIN

Blood for osteocalcin estimation was collected, as described in Section 5.2.1.1, from 10 lambs with inherited rickets (derived from embryo transfer) and 10 age-matched control lambs at 1 week and 12 weeks of age. Samples were collected at the same time in the morning, and placed in a chilled polystyrene container. The serum was separated by centrifugation at 2000 *g* for 10 min and frozen at -80°C within 2 h of collection, as osteocalcin has been reported to be sensitive to proteolysis. A commercial enzyme immunoassay for the quantitation of intact osteocalcin in serum (Metra® Osteocalcin, Quidel Corporation Specialty Products, USA) was used as per the manufacturer's instructions. The kit had 100% cross-reactivity with bovine osteocalcin. The optical density was measured at 405 nm using a Molecular Devices V_{max} kinetic microplate reader (Molecular Devices Corp., USA). A 4-parameter calibration curve fitting equation, $y=(A-D)/1=(x/C)^B)+D$, was used to analyze the assay results with GraphPad Prism 3.0 statistical software. The concentration of samples from affected and control sheep was determined from the standard curve. A two-sample t-test was used to analyse differences between the two groups with Minitab 15 statistical software.

5.3 Results

5.3.1 Calcium and Phosphate

5.3.1.1 Serum

The 5-month-old affected sheep from Marlborough had a mean serum phosphate concentration of 1.242 mmol/L and a mean serum calcium concentration of 2.008 mmol/L (Figure 5.1). This was significantly different (P<0.001) than control animals from the same farm, which had mean serum phosphate and calcium concentrations of 2.233 and 2.718 mmol/L respectively (Figure 5.1). This data was also presented in the intial report of this disease (Thompson *et al*., 2007).

Lambs with inherited rickets derived from the embryo transfer breeding trial had significantly lower serum calcium and phosphate concentrations (P<0.05) at all ages from 0-12 weeks, than control sheep of the same age (Table 5.2). The difference between affected and control sheep was particularly obvious in the serum phosphate concentrations (Figure 5.2).

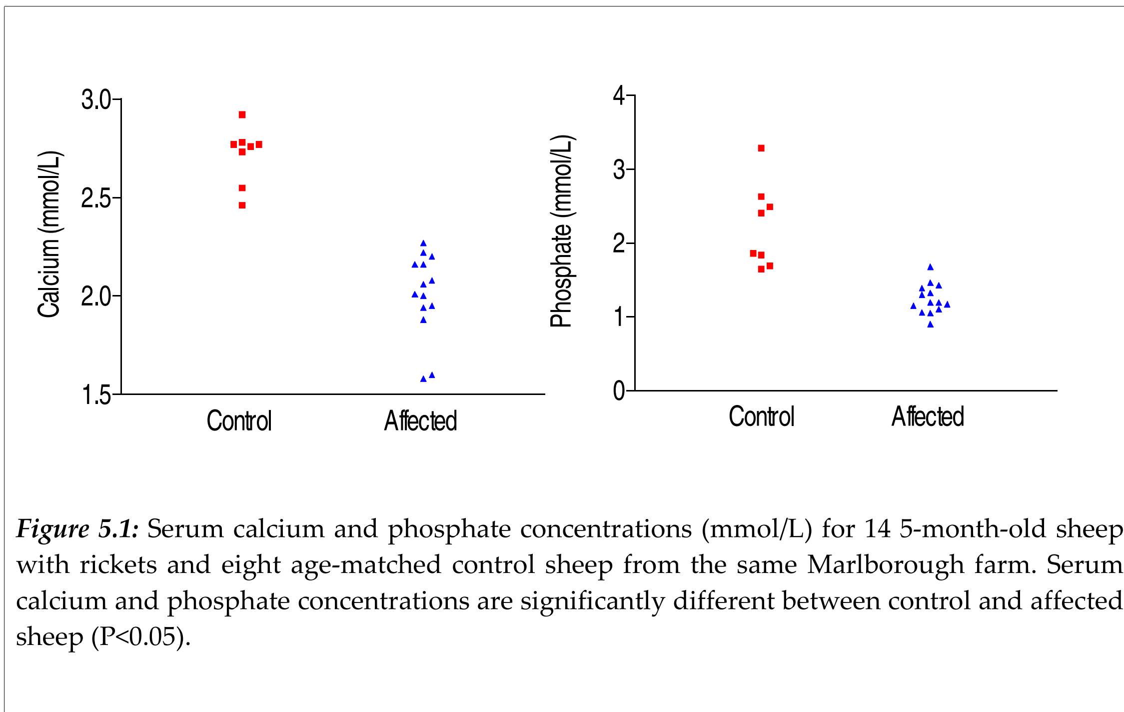


Figure 5.1: Serum calcium and phosphate concentrations (mmol/L) for 14 5-month-old sheep with rickets and eight age-matched control sheep from the same Marlborough farm. Serum calcium and phosphate concentrations are significantly different between control and affected sheep (P<0.05).

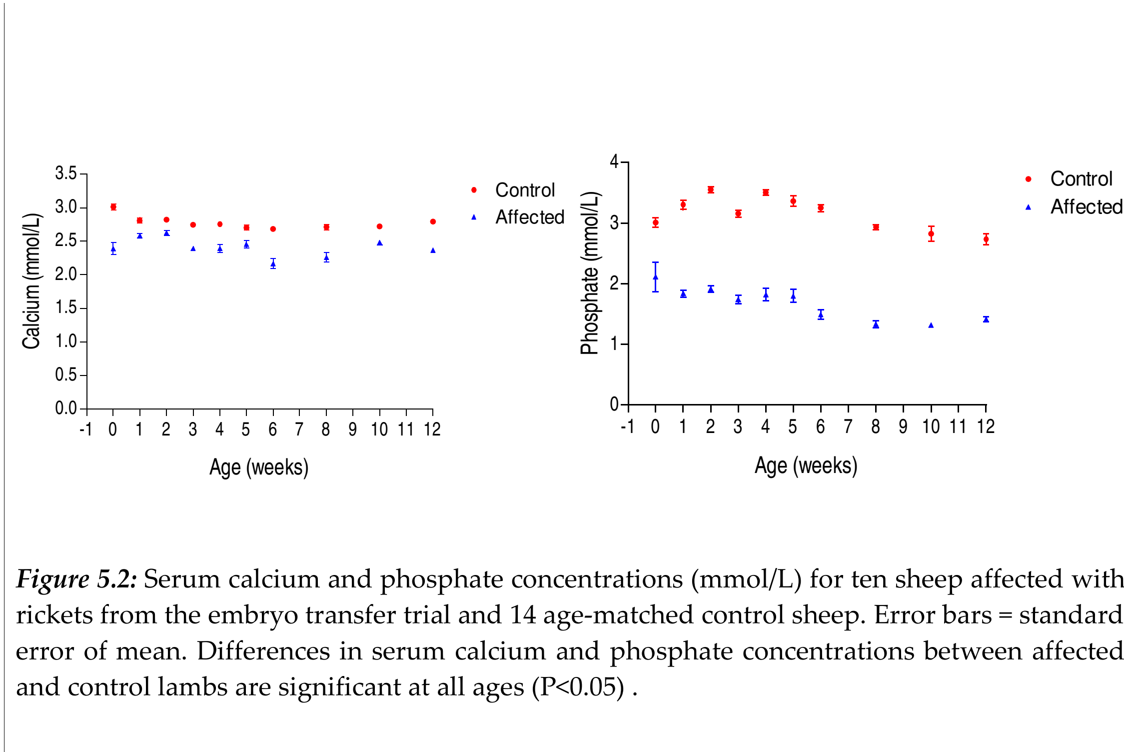


Figure 5.2: Serum calcium and phosphate concentrations (mmol/L) for ten sheep affected with rickets from the embryo transfer trial and 14 age-matched control sheep. Error bars = standard error of mean. Differences in serum calcium and phosphate concentrations between affected and control lambs are significant at all ages (P<0.05) .

Table 5.2: Comparison of mean serum calcium and phosphate concentrations (mmol/L) in lambs with rickets derived from either embryo transfer or the back-cross breeding trial, and their respective age-matched controls. ET = embryo transfer breeding trial; BT = heterozygous ram back-cross breeding trial. P-value determined using students two sample t-test.

Age	Group	Affected/Control	Mean serum calcium	P-value	Mean serum phosphate	P-value
0 weeks	ET	Affected	2.391	<0.001	2.117	0.004
		Control	3.013		3.013	
1 week	ET	Affected	2.581	<0.001	1.842	<0.001
		Control	2.809		3.307	
2 weeks	ET	Affected	2.625	<0.001	1.919	<0.001
		Control	2.821		3.554	
	BT	Affected	2.613	0.075	2.067	<0.001
		Control	2.717		3.373	
3 weeks	ET	Affected	2.392	<0.001	1.742	<0.001
		Control	2.746		3.158	
4 weeks	ET	Affected	2.394	<0.001	1.822	<0.001
		Control	2.751		3.509	
	BT	Affected	2.330	0.397	1.573	<0.001
		Control	2.504		3.048	
5 weeks	ET	Affected	2.456	0.002	1.805	<0.001
		Control	2.702		3.366	
6 weeks	ET	Affected	2.167	<0.001	1.496	<0.001
		Control	2.678		3.250	
	BT	Affected	2.337	0.132	1.407	0.001
		Control	2.605		3.042	
8 week	ET	Affected	2.259	<0.001	1.334	<0.001
		Control	2.711		2.935	
	BT	Affected	2.230	0.018	1.423	<0.001
		Control	2.653		2.668	
10 week	ET	Affected	2.480	<0.001	1.322	<0.001
		Control	2.719		2.825	
12 week	ET	Affected	2.365	<0.001	1.418	<0.001
		Control	2.791		2.739	
	BT	Affected	1.923	0.169	1.073	<0.001
		Control	2.511		2.218	

In the large scale breeding trial, lambs with inherited rickets had lower serum calcium and phosphate concentrations (Table 5.2), but while serum phosphate concentrations were significantly less in affected sheep, calcium concentrations were not significantly different (except at 8 weeks of age, P=0.018). The three lambs determined clinically to have inherited rickets, consistently had the lowest phosphate concentrations (Figure 5.3).

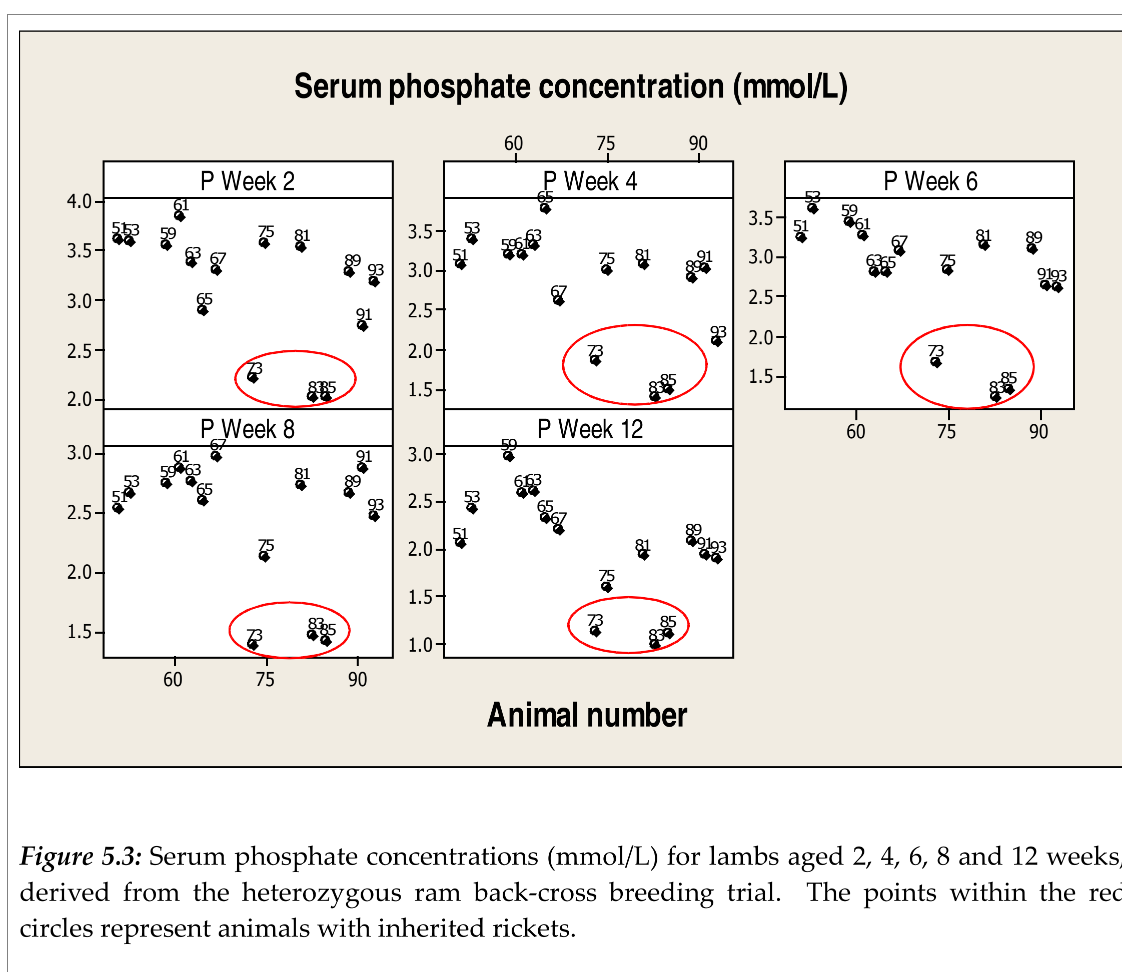


Figure 5.3: Serum phosphate concentrations (mmol/L) for lambs aged 2, 4, 6, 8 and 12 weeks, derived from the heterozygous ram back-cross breeding trial. The points within the red circles represent animals with inherited rickets.

5.3.1.2 URINE

The mean urine calcium and phosphate concentrations of affected sheep were 0.160 ± 0.033 mmol/L and 0.419 ± 0.252 mmol/L. Mean serum calcium and

phosphate concentrations at the corresponding time were 1.715 ± 0.095 mmol/L and 1.180 ± 0.081 mmol/L respectively. The mean fractional excretion rates were 0.131 ± 0.018% for calcium and 0.362 ± 0.175% for phosphate. According to published reference ranges for cattle and sheep, these results appear to be within normal limits (Lefebvre *et al.*, 2008).

5.3.2 VITAMIN D

Mean serum $25(OH)D_3$ concentrations were similar in affected and control sheep, with means of 32.93 and 31.75 nmol/L respectively (P=0.7313, Figure 5.4). However, the mean serum $1,25(OH)_2D_3$ concentration of affected sheep was 235.3 pmol/L, which was significantly greater (P<0.001) than 123.4 pmol/L the mean serum $1,25(OH)_2D_3$ concentration of control sheep (Figure 5.4). This data was also presented in the intial report of this disease (Thompson *et al.*, 2007).

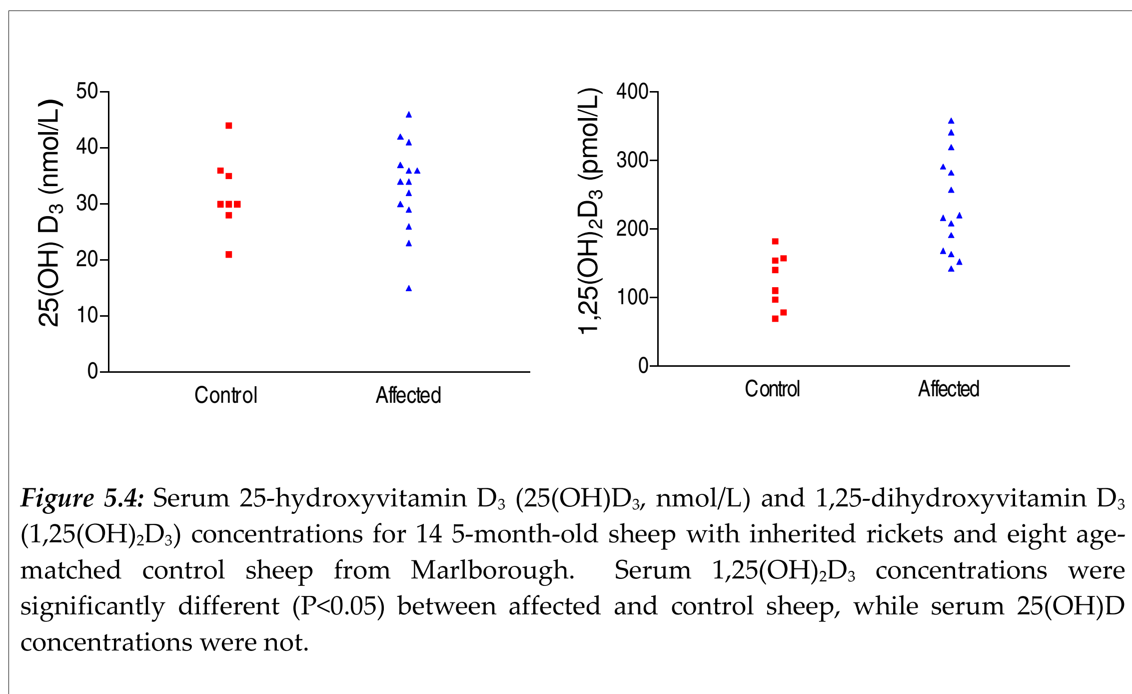


Figure 5.4: Serum 25-hydroxyvitamin D_3 ($25(OH)D_3$, nmol/L) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) concentrations for 14 5-month-old sheep with inherited rickets and eight age-matched control sheep from Marlborough. Serum $1,25(OH)_2D_3$ concentrations were significantly different (P<0.05) between affected and control sheep, while serum 25(OH)D concentrations were not.

5.3.3 OSTEOCALCIN

The mean serum intact osteocalcin concentration at 1 and 12 weeks of age for lambs affected with rickets was greater than that of control lambs (Figure 5.5). At 1 week of age serum osteocalcin was close to being significantly higher in affected lambs (P=0.06). The difference was significant at 12 weeks, where the mean serum intact osteocalcin concentration in lambs with rickets was 1.5 times greater (P=0.001) than that of control lambs.

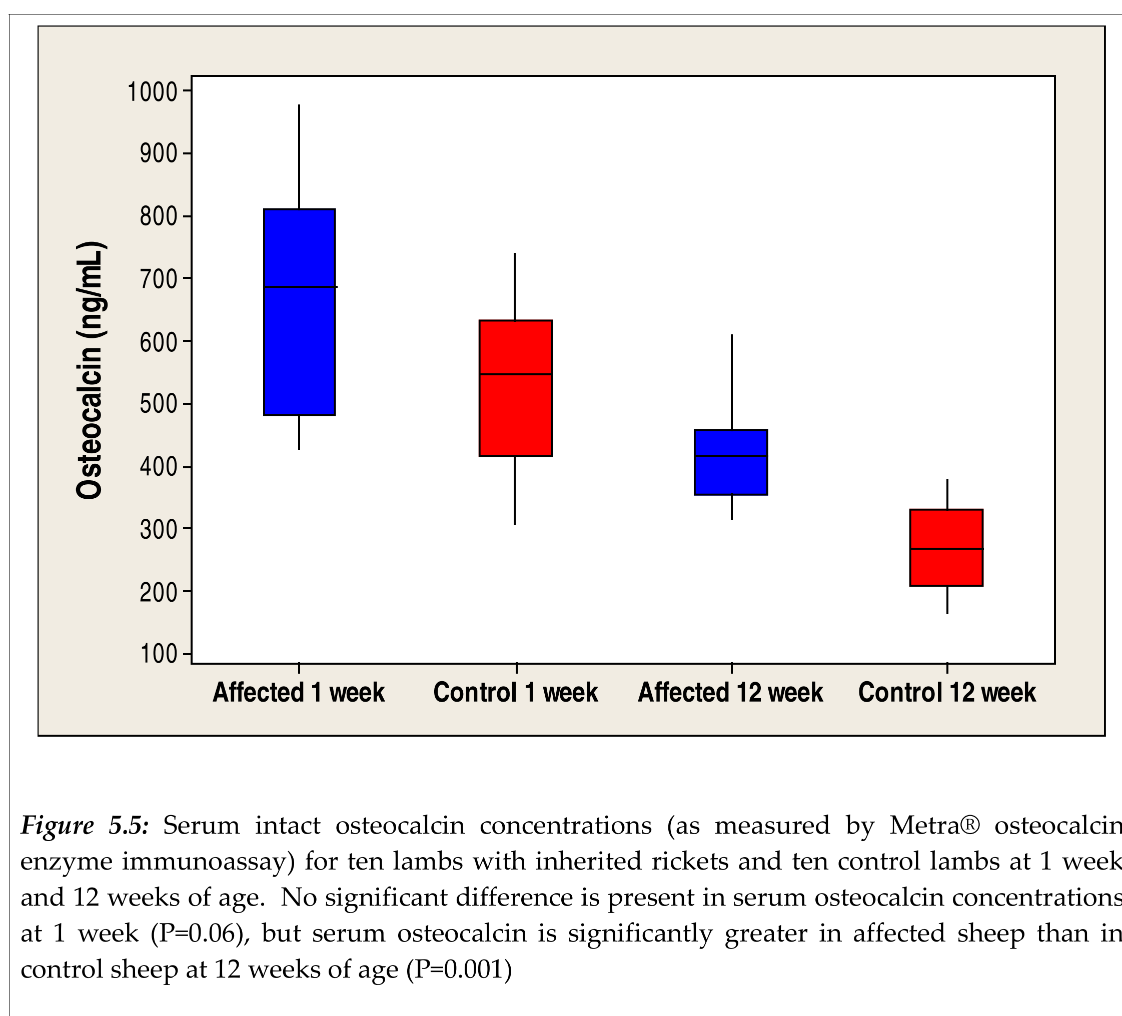


Figure 5.5: Serum intact osteocalcin concentrations (as measured by Metra® osteocalcin enzyme immunoassay) for ten lambs with inherited rickets and ten control lambs at 1 week and 12 weeks of age. No significant difference is present in serum osteocalcin concentrations at 1 week (P=0.06), but serum osteocalcin is significantly greater in affected sheep than in control sheep at 12 weeks of age (P=0.001)

5.4 DISCUSSION

Persistent hypocalcaemia and hypophosphataemia, present from birth, was a consistent finding in Corriedale lambs with inherited rickets. In fact, hypophosphataemia was a useful marker of affected lambs in the back-cross breeding trial. Affected and control lambs had similar serum 25(OH)D_3 concentrations, but serum 1,25$(OH)_2D_3$ concentrations were significantly greater in affected lambs, suggesting end-organ unresponsiveness. Affected and control lambs had similar urinary calcium and phosphate fractional excretion rates, making renal phosphate wasting an unlikely mechanism for the disease.

Similar serum chemical alterations occur in some forms of inherited rickets in human patients. The degree of hypophosphataemia in Corriedale sheep with inherited rickets was more severe than that typically seen in children with nutritional rickets due to vitamin D or phosphorus deficiency, where serum phosphate concentration is low to low-normal (Holick, 2006; Wharton and Bishop, 2003). Marked hypophosphataemia is however a feature of human patients with inherited forms of rickets (Holick, 2006; Malloy *et al*., 1999). Serum calcium concentrations in patients with hypophosphataemic rickets are typically within normal limits, but are reduced in patients with VDDR I and HVDRR (Holick, 2006; Malloy *et al*., 1999; Miller and Portale, 2003). Affected lambs derived from the embryo transfer trial had significantly lower serum calcium concentrations than control lambs. The differences in serum calcium concentrations between affected and control lambs were not significant in the back-cross breeding trial, probably because of the small number (3) of affected lambs in this trial, and the low statistical power. The presence of hypocalcaemia and hypophosphataemia at birth in affected lambs is consistent with the bone

fragility and microscopic lesions observed in neonatal lambs (Chapters 3 & 4) and provide further evidence of the requirement for $1,25(OH)_2D_3$ in ovine placental calcium and phosphate transport.

Normal serum $25(OH)D_3$ and elevated serum $1,25(OH)_2D_3$ concentrations obtained from affected sheep in this study suggest that the underlying defect in Corriedale sheep with inherited rickets is end-organ unresponsiveness. Human patients with VDDR I have decreased serum $1,25(OH)_2D_3$ concentrations, while patients with HVDRR have serum $1,25(OH)_2D_3$ concentrations greater than normal (Malloy *et al*., 1999). HVDRR is caused by a defect in end-organ responsiveness, most commonly a fault with the vitamin D receptor (Malloy and Feldman, 1999). However, only a two-fold increase in mean serum $1,25(OH)_2D_3$ was present in Corriedale sheep with rickets, while the increase seen in humans with HVDRR is typically 5-20 times greater than normal (Malloy *et al*., 1999).

The serum osteocalcin concentrations obtained from the control lambs in this study are similar to those previously reported in sheep of the same age (Pastoureau *et al*., 1991). At 12 weeks of age, Corriedale lambs with inherited rickets had significantly greater mean serum osteocalcin concentrations than those of control lambs. Increased serum osteocalcin is reported in human patients with healing rickets, VDDR I, XLH, and hyperparathyroidism, but not in patients with HVDRR, and nutritional rickets (Hauschka *et al*., 1989; Malloy *et al*., 1997). The increased serum osteocalcin concentration seen in affected lambs could reflect either increased bone formation or reduced availability of

mineral in affected sheep to bind newly formed osteocalcin (Hauschka *et al*., 1989). Alternatively, elevated 1,25$(OH)_2D_3$ concentrations may have led to increased genetic expression of osteocalcin in affected sheep (Carpenter and Gundberg, 1996). Experiments performed with *Hyp* mice (a model for XLH) have shown that increased serum osteocalcin concentrations in these mice result from increased osteocalcin mRNA production and altered gene expression (Carpenter and Gundberg, 1996). No difference was seen in the clearance of osteocalcin, or the affinity of osteocalcin for mineral in affected mice (Carpenter and Gundberg, 1996).

5.4.1 SUMMARY

Corriedale sheep with inherited rickets are persistently hypocalcaemic and hypophosphataemic from birth. Normal serum 25(OH)D and elevated 1,25$(OH)_2D_3$ concentrations are consistent with a defect in end-organ responsiveness. However, the increase in 1,25$(OH)_2D_3$ is not as great as patients with HVDRR. The overall combination of serum chemical changes differs from that reported in human hereditary rachitic diseases.

CHAPTER 6

DISEASE MECHANISM

6.1 INTRODUCTION

Vitamin D is essential for normal calcium and phosphate homeostasis (DeLuca, 2004). This steroid hormone is obtained from the diet or formed in the skin after thermal isomerisation of 7-dehydrocholesterol (Holick, 1981). This is followed by the addition of hydroxyl groups in the liver and kidney to form 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active form of vitamin D (Prosser and Jones, 2004). In target tissues, 1,25$(OH)_2D_3$ is the activating ligand for the heterodimeric nuclear receptor, which comprises the vitamin D receptor (VDR) and the retinoid X receptor (RXR) (Dusso *et al*., 2005). This complex binds to vitamin D-response elements to effect changes in transcription of 1,25$(OH)_2D_3$-regulated genes (Figure 1.3), such as osteocalcin, 25-hydroxyvitamin D_3-24-hydroxylase (CYP24 or 24-hydroxylase) and parathyroid hormone (PTH) (Haussler *et al*., 1998). One of the pathways responsible for the breakdown of

$1,25(OH)_2D_3$, involves C-24 oxidation to calcitroic acid (Reddy and Tserng, 1989). CYP24 (25-hydroxyvitamin D_3-24-hydroxylase), a cytochrome P450 protein, is the enzyme responsible for 24-hydroxylation and is capable of catalyzing multiple steps in the catabolic pathway (Beckman *et al*., 1996).

Defects at various stages of vitamin D activation have been reported in humans and animals. These include vitamin D-dependent rickets type I (VDDR I), caused by defects in the 1α-hydroxylase enzyme in the kidney, and hereditary vitamin D-resistant rickets (HVDRR), caused by defects in the VDR (Fraser *et al*., 1973; Malloy *et al*., 1999). Hypophosphataemic forms of rickets due to renal phosphate wasting also occur. For example, X-linked hypophosphataemic (XLH) rickets is associated with mutations in *PHEX* (Phosphate regulating gene with Homologies to Endopeptidases on the X-chromosome)(Bielesz *et al*., 2004).

In humans, $1,25(OH)_2D_3$ induction of 24-hydroxylase and vitamin D-binding analyses are commonly performed on cultures of skin fibroblasts for confirming a diagnosis of HVDRR (Chen *et al*., 1984; Feldman *et al*., 1982; Malloy *et al*., 2002a). Studies on the induction of 24-hydroxylase by $1,25(OH)_2D_3$ have been performed using cell cultures of pig, rat, mouse, monkey and human tissues (Akeno *et al*., 1997; Chandler *et al*., 1984; Feldman *et al*., 1982; Tashiro *et al*., 2004; Zierold *et al*., 2000). The VDR is present in a number of tissues and induction of 24-hydroxylase has been observed in fibroblasts, renal tubular cells, keratinocytes, intestinal cells, and peripheral blood mononuclear cells (Chen *et al*., 1994; Furuichi *et al*., 1998; Koeffler *et al*., 1990; Tashiro *et al*., 2004; Zierold *et*

al., 2000). Typically, humans with HVDRR show no, or minimal induction of 24-hydroxylase, in response to 1,25$(OH)_2D_3$ (Malloy *et al*., 1999).

A vitamin D-binding analysis on fibroblast cultures from humans with HVDRR is sometimes used to further clarify the nature of the VDR defect (Malloy *et al*., 1999). This enables determination of the affinity (K_d, equilibrium dissociation constant) and the capacity (B_{max}, maximal number of receptor binding sites) of the VDR for 1,25$(OH)_2D_3$. By determining these values, the location of a mutation in the VDR may be detected. For example, a low-affinity, high-capacity result may indicate a defect in the ligand-binding domain of the VDR, whereas normal affinity may be due to a mutation in the DNA-binding domain (Malloy *et al*., 1999).

Studies described in Chapter 2 confirmed the inheritance of a new skeletal disease with features of rickets in Corriedale sheep. Serum chemistry results discussed in Chapter 5 suggested a defect in end-organ responsiveness as a likely mechanism. The aim of this study was to determine if the disease was due to a defect in the VDR.

6.2 Materials and Methods

6.2.1 Vitamin D-Binding Analysis

6.2.1.1 Animals Used and Skin Biopsy Technique

The subjects were two clinically normal, female cross-bred Perendale sheep and three Corriedale sheep with inherited rickets (one male, two female). All sheep were 2 years of age. The sheep were sedated with 0.05-0.1 mg/kg intravenous acepromazine maleate (Ethical Agents Ltd., New Zealand). Local anaesthetic (Ethical Agents Ltd., New Zealand) was injected intradermally and subcutaneously at the site of biopsy. Skin biopsies taken from the nape of the neck were approximately 1-2 cm in size. The biopsies were transported in Dulbecco's phosphate-buffered saline (Dulbecco's PBS, Invitrogen Corp., New Zealand) with 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen Corp., New Zealand). Animal ethics approval for this investigation was obtained from the Massey University Animal Ethics Committee (Approval number 05/123). Genetic technology approval for this investigation was obtained from the Massey University Genetic Technology Committee (Organism Approval Code GMD004382).

6.2.1.2 Cell Cultures

The protocol for explant fibroblast culture from skin biopsies was adapted from a method previously described for primary human skin fibroblast culture (Rittie and Fisher, 2005). After collection, the skin was washed twice in PBS with 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen Corp., New Zealand)

to remove gross contamination. Subcutaneous tissue was removed, and the skin sliced into pieces approximately 5 mm square. The pieces of skin were incubated overnight in 0.3% trypsin (0.3 g trypsin (1:250) Invitrogen Corp., Canada) in PBS (100 mL) at 4°C. Subsequently, the epidermis was scraped off with a scalpel, and the dermis cut into approximately 1-2 mm squares and placed in 24-well plates. The explants were allowed to dry for 30 min to increase attachment to the bottom of the dish prior to the addition of 1.5 mL of complete media - 1:1 Dulbecco's Modified Eagle's Medium/Hams F-12 (Invitrogen Corp., New Zealand), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen Corp., New Zealand), 2.5 μg/mL amphotericin B (Invitrogen Corp., United Kingdom), and 10% fetal bovine serum (Invitrogen Corp., New Zealand). The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Once confluent, the fibroblasts were subcultured into 75 cm^2 flasks with 20 mL of complete media.

6.2.1.3 CYTOSOL PREPARATION

Media was removed from the flasks and 10 mL of ice cold PBS added. Cells were removed using a cell scraper, without prior trypsinisation and washed twice in ice cold PBS, centrifuging at 2100 *g* for 5 min. The cells were then resuspended in KTEDM buffer (300 mM KCl, 10 mM sodium molybdate, 1.5 mM EDTA, 10 mM Tris, 1 mM dithiothreitol) with protease inhibitors (Roche Complete mini, protease inhibitor cocktail tables, Roche Diagnostics Corp., Germany) (Griffin and Zerwekh, 1983; Hirst *et al*., 1985; Skowronski *et al*., 1993). The cells were disrupted by sonication for 10 s using a VCX-500 Ultrasonic processor with a 13 mm probe (Sonic and Materials Inc., USA), and then cooled on ice for 1 min. This process was repeated three times. The sonicate was

centrifuged at 16 000 *g* for 60 min at 4°C. The protein concentration of the supernatant was measured using a ND-1000 Spectrophotometer at a wavelength of 280 nm (Nanodrop Technologies, USA).

6.2.1.1 VITAMIN D-BINDING ANALYSIS

The initial experimental conditions were based on those used for human vitamin D-binding analyses (Chandler *et al*., 1984; Malloy *et al*., 1989; Skowronski *et al*., 1993). Cytosol (200 μL at 1 mg/mL protein concentration) was incubated for 16 h with increasing concentrations (0-1 nM) of $[^3H]1,25(OH)_2D_3$ (1α,25-dihydroxy[26,27-*methyl*-^{3}H]cholecalciferol, 158 Ci/mmol, GE Healthcare Co., UK) at 4°C, with or without 250-fold excess of unlabelled $1,25(OH)_2D_3$ (Sigma-Aldrich Co., USA).

Glass fibre filters (Whatman glass microfibre filters grade GF/C, 1.2 μm, Whatman plc., UK) were soaked in 10 mM Tris/HCl, pH 7.4, placed in a Millipore manifold, and a vacuum applied to dry (Juntunen *et al*., 1999). Cytosol and radioligand were applied to the filters and a gentle vacuum employed to trap receptor and bound radioligand onto the filter, and remove unbound radioligand. The filters were washed three times with 10 mL portions of buffer (50 mM Tris/HCl, 150 mM NaCl, 0.01% Triton X-100, pH 7.4) to minimize non-specific binding (Juntunen *et al*., 1999). Suction was continued to remove excess moisture. The damp filters were placed in liquid scintillation cocktail (4 g 2,5-diphenyloxazole, 100 mg 1,4-bis(5-phenyloxazol-2-yl)benzene, 667 mL toluene, 333 mL Triton-X 100) for 24 h. The filters were counted for 5 min in a Wallac 1450 Microbeta Trilux liquid scintillation and luminescence

counter (Perkin-Elmer Inc., USA). Non-linear regression analysis using GraphPad Prism™ 3.0 (GraphPad Software Inc., USA) was used to calculate B_{max} and K_d. Mann-Whitney two-sample test was performed using Minitab 15 (Minitab Inc., 2008, USA).

Subsequent experimental conditions examined in an effort to optimise the method included incubation for 12 h, protein concentrations of 0.1, 0.25 and 0.5 mg/mL, and 0-25 nM $[^3H]1,25(OH)_2D_3$ concentrations. The optimal conditions for vitamin D-binding analysis using sheep cytosol were determined to be: 200 μL of cytosol (0.1 mg/mL protein concentration) incubated for 12 h with increasing concentrations of $[^3H]1,25(OH)_2D_3$ (0-10 nM) at 4°C, with or without 250-fold excess of unlabelled $1,25(OH)_2D_3$.

6.2.2 Induction of 24-Hydroxylase mRNA by $1,25(OH)_2D_3$

6.2.2.1 Primer Development

Reverse transcriptase polymerase chain reaction (RT-PCR) primers for sheep *Cyp24* were designed by comparing rat and human CYP24 mRNA and genomic DNA to find consensus sequences, and then selected using Lightcycler Probe Design Software 2 Version 1.0 (Idaho Technology Inc., USA) (Table 6.1). The primers were designed to span an intron, eliminating amplification of any genomic DNA present in the RNA preparation. The expected product was 218 base pairs long and consisted of exons 4 and 5.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as a house-keeping gene as it had previously been used in this role for examining collagen expression in ovine dermal and tendon fibroblasts by RT-PCR (Brink *et al*., 2006; Budhia *et al*., 2006). Primer sequences for *GAPDH* had previously been reported (Budhia *et al*., 2006) and are listed in Table 6.1.

The forward sequencing primer was the same consensus primer used for RT-PCR (*Cyp24* Forward) and the reverse sequencing primer was selected from the rat CYP24 mRNA sequence. The sequencing primers were also used to sequence rat *cyp24* cDNA.

Table 6.1: **List of primers used in RT-PCR or for sequencing.**

Primer Name	**Sequence (5'-3')**	**T_m (1 M Na^+)**	**Product Size**
Cyp24 RT-PCR			
Cyp24 Forward	AAT CAA TGA GGT CTT GGC	62	218
Cyp24 Reverse	TCC CAA ACG TGC TCA TCA	65	
House-keeping gene			
GAPDH Forward	GGC GTG AAC CAC GAG AAG TAT AA	72	120
GAPDH Reverse	CCC TCC ACG ATG CCA AAG T	70	
Sequencing and rat primers			
Cyp24 Forward	AAT CAA TGA GGT CTT GGC	62	219
Cyp24 Rev Seq	CGT AAT GAA GGT CAG C	60	

Cyp24 = 25-hydroxyvitamin D_3-24-hydroxylase; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

6.2.2.2 1,25$(OH)_2D_3$ Treatment

Cells were obtained and cultured as described in Sections 6.2.1.1 and 6.2.1.2. Fibroblasts, no older than passage 10, were trypsinised (0.3% trypsin in PBS) and counted. Approximately 8 x 10^5 cells were then added to each well of a 6-well plate with 5 mL of complete media and grown to at least 80% confluence. The 1α,25$(OH)_2D_3$ (Sigma-Aldrich Co., USA) was solubilised in 0.1% ethanol, and added to the medium decanted from the cultures at the desired concentration. The 1α,25$(OH)_2D_3$ concentrations examined included: 10^{-10} M, 10^{-8} M, 5 x 10^{-8} M, 10^{-7} M, 5 x 10^{-7} M, 10^{-6} M. The old medium plus sterol or ethanol alone (control), a total volume of 2 mL, was then replaced and the cultures incubated for 6, 12, 14 or 16 h. Treatments were performed in duplicate, and the experiment repeated at least twice. The optimal incubation time was determined to be 12 h by RT-PCR, and this was used for subsequent experimental conditions.

6.2.2.3 RNA Extraction

Total RNA was extracted using Tri Reagent as per the manufacturer's instructions (Sigma-Aldrich Co., USA). The medium was removed from the wells, and the cells lysed directly by adding 1 mL of Tri Reagent to each well. The RNA pellet was resuspended in 100 µL of DEPC (diethylpyrocarbonate)-treated water and either used immediately or stored at -80°C until required. RNA concentration was measured with a ND-1000 Spectrophotometer at a wavelength of 260 nm (Nanodrop Technologies, USA).

6.2.2.1 Reverse Transcriptase Polymerase Chain Reaction

Synthesis of cDNA was performed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen Corp., USA) and oligo(dT) primers as per manufacturer's instructions. To each 0.2 mL thin-walled PCR tube on ice, 5 μM oligo(dT) , 1 mM dNTP mix, 0.3-0.6 μg RNA were added and made up to a total volume of 10 μL with DEPC-treated water. A control lacking RNA, to determine if any contamination occurred during this step, was also included. The tubes were incubated at 65°C for 5 min, and then held at 4°C for at least 1 min. The cDNA reaction mix (2 X RT buffer, 10 mM $MgCl_2$, 20 mM dithiothreitol, 40 units RNaseOUT, 200 units Superscript III Reverse Transcriptase per sample, total volume 10 μL) was prepared on ice and added to each incubated RNA mixture. The samples were incubated at 50°C for 50 min, 85°C for 5 min, and then chilled at 4°C. RNase H (2 units) was added to each tube, and incubated at 37°C for 20 min. The cDNA product (5 μL) was used to amplify the *CYP24* and *GAPDH* genes as follows: the 50 μL PCR reaction mix contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 mM each primer and 1 unit Platinum *Taq* DNA polymerase (Invitrogen Corp., USA). A separate reaction mix for each concentration was set up for *CYP24* and *GAPDH*. Rat *Cyp24* cDNA[1] was used as a positive control. The PCR conditions were: 95°C for 5 min, 35 cycles of 30 s at 95°C, 30 s at 54°C and 1 min at 72°C, and finally a 10 min elongation step at 72°C. In all reactions, negative controls were performed to check for the presence of contaminants. All PCR products were analysed on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen Corp., USA) containing ethidium bromide and visualized under UV light on a transilluminator.

1 Kindly donated by Dr Ohyama and Dr Noshiro of Hiroshima University, Japan.

6.2.2.1 SEQUENCING REACTION

Amplicons to be used for sequencing were purified using the PureLink PCR purification kit (Invitrogen Corp., USA) and with the sequencing primers listed in Table 6.1, subjected to automatic dye-terminator cycle sequencing with BigDye^{TM} Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc., USA).

6.3 RESULTS

6.3.1 VITAMIN D-BINDING ANALYSIS

The vitamin D-binding analysis performed using human cells utilises a protein concentration of 1 mg/mL. To determine optimal conditions for the sheep vitamin D-binding analysis, protein concentrations of 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL were examined. As can be seen in Figure 6.1, 0.1 mg/mL was the only protein concentration at which saturation binding of $[^3H]1,25(OH)_2D_3$ occurred. Non-specific binding was minimal, and was subtracted along with background radioactivity from the total counts.

Once optimal conditions were established, the average B_{max} and K_d of the VDR of fibroblasts from control sheep were estimated to be 307.4 pmol/mg protein (322.1 and 292.6 pmol/mg protein) and 6.482 nM (8.832 and 4.132 nM) respectively. In comparison, the average B_{max} for the VDR of fibroblasts from Corriedale sheep with inherited rickets was 249.2 pmol/mg protein (177.0, 200.1, 370.4 pmol/mg protein), and the average K_d was 5.852 nM (3.350, 6.994, 7.213).

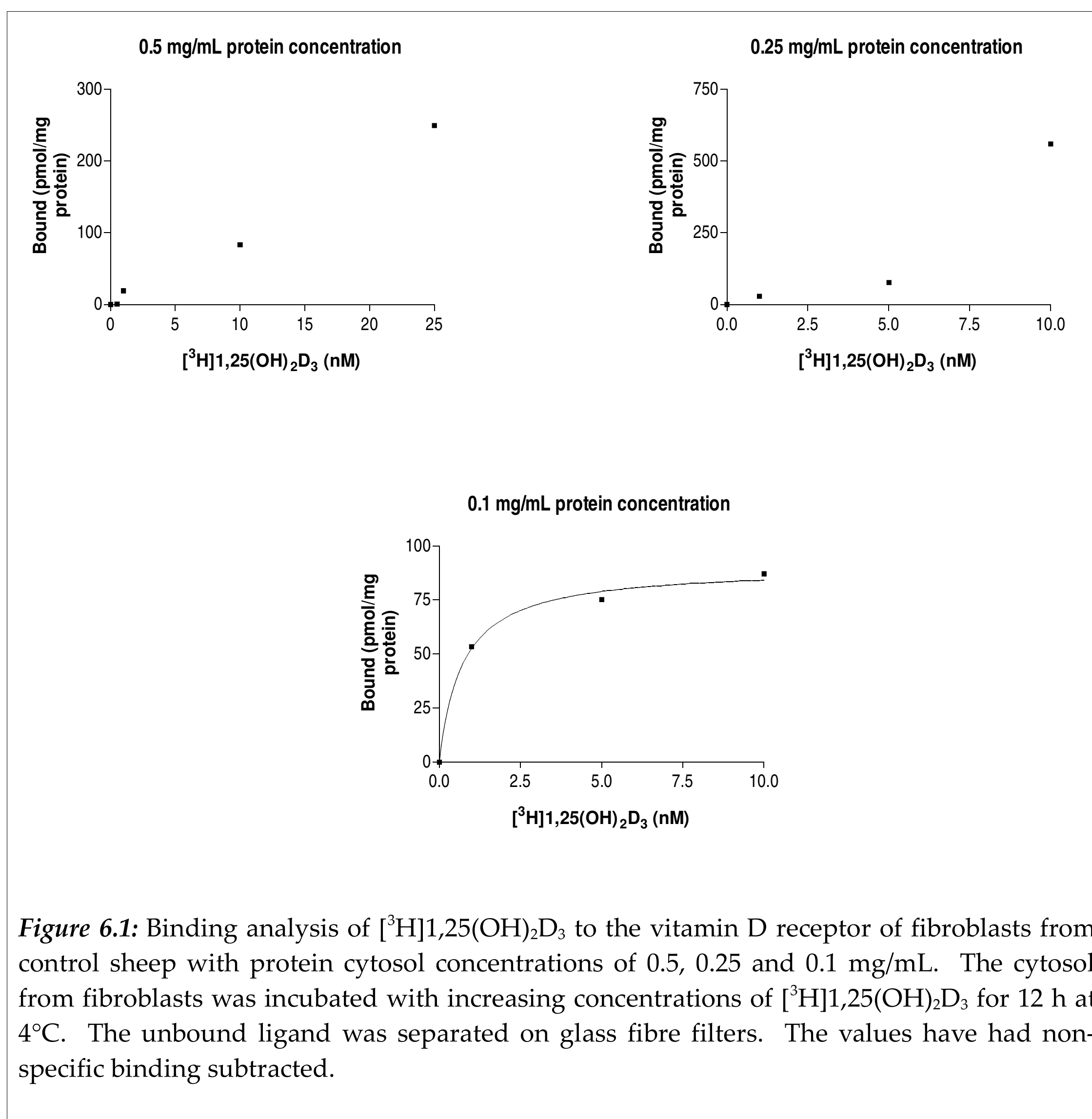


Figure 6.1: Binding analysis of $[^3H]1,25(OH)_2D_3$ to the vitamin D receptor of fibroblasts from control sheep with protein cytosol concentrations of 0.5, 0.25 and 0.1 mg/mL. The cytosol from fibroblasts was incubated with increasing concentrations of $[^3H]1,25(OH)_2D_3$ for 12 h at 4°C. The unbound ligand was separated on glass fibre filters. The values have had non-specific binding subtracted.

nM). Using Mann-Whitney two-sample test, no signficant differences in B_{max} and K_d between affected and control sheep were detected. The full results are shown in Figure 6.2 .

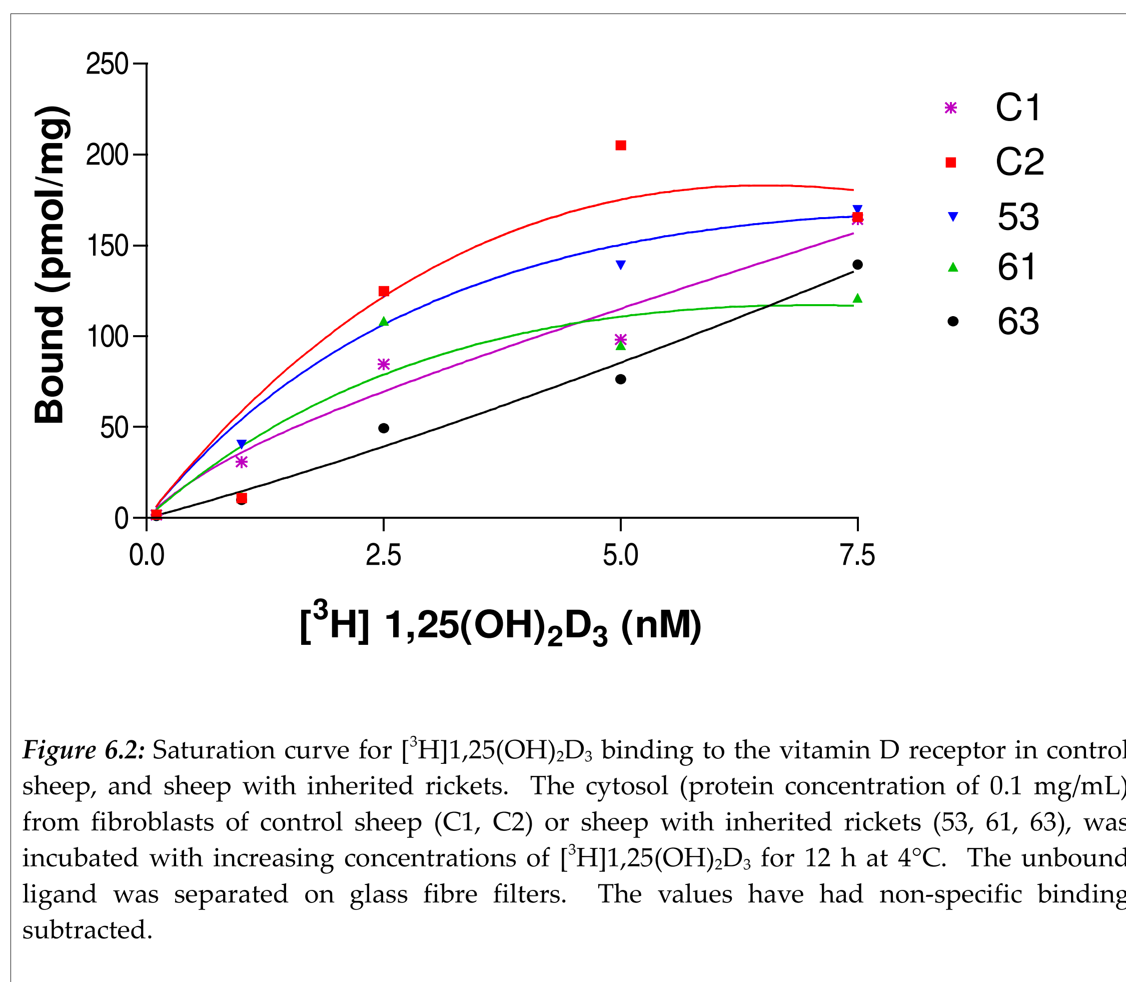


Figure 6.2: Saturation curve for [^{3}H]1,25(OH)$_2$D$_3$ binding to the vitamin D receptor in control sheep, and sheep with inherited rickets. The cytosol (protein concentration of 0.1 mg/mL) from fibroblasts of control sheep (C1, C2) or sheep with inherited rickets (53, 61, 63), was incubated with increasing concentrations of [^{3}H]1,25(OH)$_2$D$_3$ for 12 h at 4°C. The unbound ligand was separated on glass fibre filters. The values have had non-specific binding subtracted.

6.3.2 Induction of 24-Hydroxylase mRNA By 1,25(OH)$_2$D$_3$

6.3.2.1 Partial Sequence of Ovine 25-Hydroxyvitamin D$_3$ 24-Hydroxylase

The amplicons obtained from RT-PCR after treatment with 10^{-6} M 1,25(OH)$_2$D$_3$, representing ovine *24-hydroxylase*, amplified at 218 base pairs as expected and were sequenced, as was the positive control, rat *24-hydroxylase*. The rat *24-hydroxylase* sequence showed 100% identity to *Rattus norvegicus* cytochrome P450 CYP24a1 mRNA (**NM_201635.1**) and RNA for 25-hydroxyvitamin D$_3$ 24-hydroxylase (**X59506.1**). A partial sequence for ovine *25-hydroxyvitamin D$_3$ 24-*

hydroxylase was obtained, as shown in Figure 6.3A. This sequence is similar to *Bos taurus* (96%, **XM591370**), *Sus scrofa* (93%, **NM_214075**), *Homo sapiens* (91%, **BC109084**) and *Mus musculus* (86%, **NM_009996**) genes. The deduced amino acid sequence (Figure 6.3B) showed identity to the corresponding *Bos taurus* (96%), *Sus scrofa* (93%) and *Homo sapiens* (92%) amino acid sequences for 24-hydroxylase. The DNA and amino acid sequences were submitted to Genbank with the accession number **EF635857**.

6.3.2.2 TIME EFFECT OF $1,25(OH)_2D_3$ ON CYP24 MRNA LEVELS

Ovine fibroblasts from control sheep were treated with 10^{-10} M, 10^{-8} M, 10^{-7} M and 10^{-6} M of $1,25(OH)_2D_3$ for 6, 12, 14 and 16 h, since protocols for studies in humans, rats and pigs suggested these times as appropriate lengths of treatment (Feldman *et al*., 1982; Furuichi *et al*., 1998; Malloy *et al*., 2004; Zierold *et al*., 2000). After 6 h of treatment, a faint PCR product representing CYP24 mRNA was seen only at the highest dose of 10^{-6} M (Figure 6.4A). After 12 and 14 h of $1,25(OH)_2D_3$ treatment, bands indicated a CYP24 mRNA response to 10^{-6} and 10^{-7} M of $1,25(OH)_2D_3$ (Figures 6.4B and 6.4C). Densitometry indicated that band strength at 12 h for 10^{-6} and 10^{-7} M was 1.06 and 1.18 times stronger respectively than at 14 h (data not shown). After 16 h, a band was observed only at 10^{-6} M $1,25(OH)_2D_3$ (Figure 6.4D). At all times during the experiments, cells cultured in the absence of $1,25(OH)_2D_3$ were included and no CYP24 mRNA bands were seen at any time in these cells. The housekeeping gene, GAPDH, showed consistent amplification in treated and untreated cells.

A)

534

5' A A T C A A T G A G G T C T T G G C T G A T T T T A T G G G
T A G A A T A G A C G A G C T C T G T G A T G A G A G A G G
C C G C A T T G A A G A C T T A T A C A C G G A G C T G A A
C A A A T G G T C T T T T G A A A G T A T C T G T C T T G T
G C T G T A T G A G A A G A G A T T T G G G C T C C T T C A
G A A G A A G G C A G G G G A G G A A G C T T T G A A C T T
C A T C A T G G C C A T C A A A A C A A T G A T G A G C A C
G T T T G G G A A 3'

752

B)

179

Ovis aries	I N E V L A D F M G	R I D E L C D E R G	R I E D L Y T E L N
Bos taurus	. . . .	. . . .	. . . .
Sus scrofa	. . . . S	. . . .	C . . . . . S . . .
Homo sapiens	. . . .	. . . .	H V . . . . S . . .
Ovis aries	K W S F E S I C L V	L Y E K R F G L L Q	K K A G E E A L N F
Bos taurus	. . . .	. . . .	. N . . . .
Sus scrofa	. . . .	. . . .	. N . . . .
Homo sapiens	. . . .	. . . .	. N . . D . . V . .
Ovis aries	I M A I K T M M S T	F G	
Bos taurus	. . . V . . . . . M	. .	
Sus scrofa	. T	. .	
Homo sapiens	. . . .	. .	

250

Figure 6.3: **Partial sequences of ovine 25-hydroxyvitamin D_3-24-hydroxylase.** **A)** Partial DNA sequence of ovine *25-hydroxyvitamin D_3-24-hydroxylase* (**EF635857**). DNA was obtained from ovine fibroblasts after $1,25(OH)_2D_3$ treatment, RNA extraction, RT-PCR, and then sequenced on a Capillary ABI3730 Genetic Analyzer from Applied Biosystems using specifically designed primers. **B)** Deduced partial amino acid sequence of ovine 25-hydroxyvitamin D_3-24-hydroxylase compared to the corresponding amino acid sequences of *Bos taurus*, *Sus scrofa* and *Homo sapiens*.

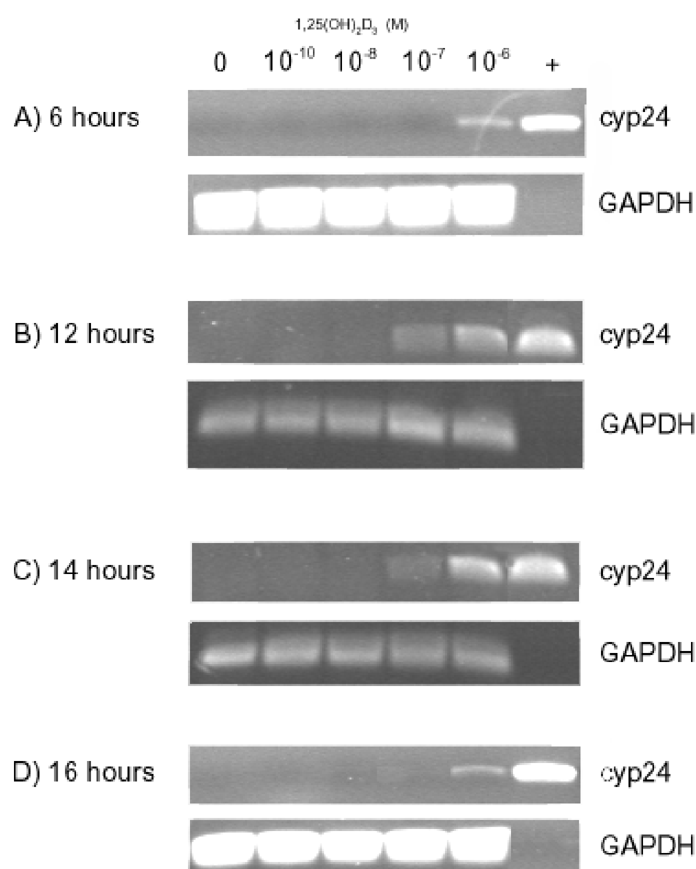


Figure 6.4: Effect of time of 1,25 $(OH)_2D_3$ treatment on cyp24 induction in ovine fibroblasts from control sheep. DNA was obtained from fibroblasts after 1,25$(OH)_2D_3$ treatment, RNA extraction, RT-PCR, and analysed on 1.5% (w/v) agarose gel containing ethidium bromide and visualised under UV light on a transilluminator. Treatment concentrations included, no treatment (vehicle only = 0), 10^{-10} M, 10^{-8} M, 10^{-7} M and 10^{-6} M of 1,25$(OH)_2D_3$. A positive control (+), rat cyp24 cDNA was included in the PCR. *GAPDH* was used as a housekeeping gene for RNA extraction and RT-PCR. **(A)** 6 h 1,25$(OH)_2D_3$ treatment. **(B)** 12 h 1,25$(OH)_2D_3$ treatment. **(C)** 14 h 1,25$(OH)_2D_3$ treatment. **(D)** 16 h 1,25$(OH)_2D_3$ treatment.

6.3.2.3 DOSAGE EFFECT OF 1,25$(OH)_2D_3$ ON CYP24 MRNA LEVELS

Further graduation of the 1,25$(OH)_2D_3$ dose at 12 h was required to demonstrate a dose specific response. A range of doses was used, including 10^{-8} M, 5 x 10^{-8} M, 10^{-7} M, 5 x 10^{-7} M and 10^{-6} M. A gradual increase in the strength of the CYP24 band was seen as the dose increased from 5 x 10^{-8} M to 10^{-6} M of 1,25$(OH)_2D_3$

(Figure 6.5). Densitometry showed that the band strength of the 5 x 10^{-7} and 10^{-6} M treatments was similar and approximately 3.7 and 11.5 times stronger than the bands at 10^{-7} M and 5 x 10^{-8} M respectively (data not shown). No response was observed at 10^{-8} M $1,25(OH)_2D_3$. GAPDH bands were present in all samples.

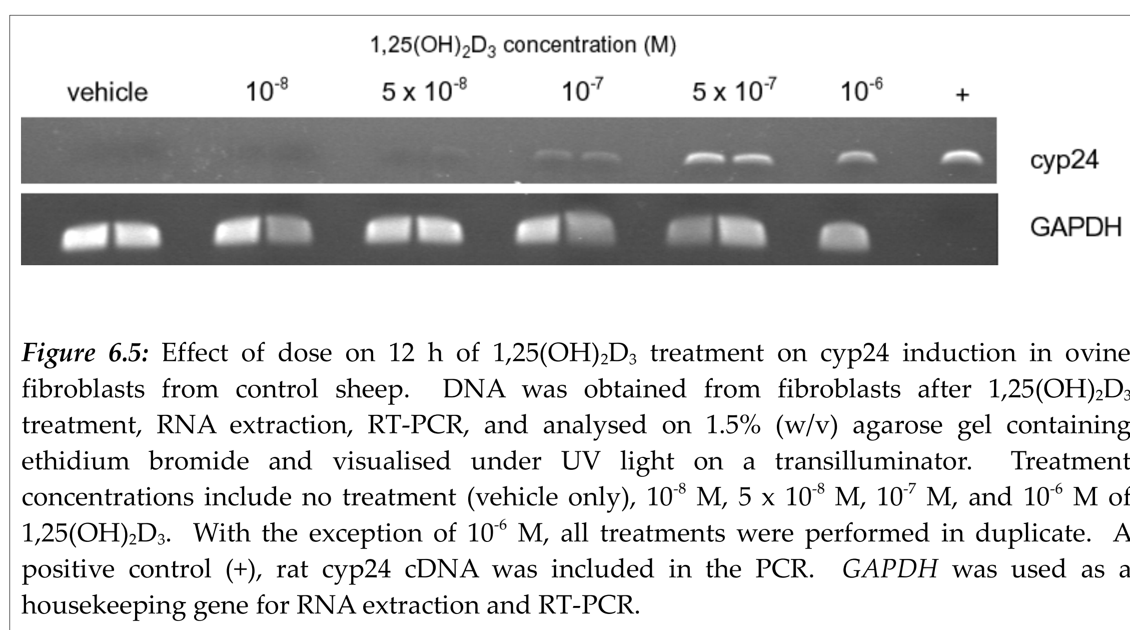


Figure 6.5: Effect of dose on 12 h of $1,25(OH)_2D_3$ treatment on cyp24 induction in ovine fibroblasts from control sheep. DNA was obtained from fibroblasts after $1,25(OH)_2D_3$ treatment, RNA extraction, RT-PCR, and analysed on 1.5% (w/v) agarose gel containing ethidium bromide and visualised under UV light on a transilluminator. Treatment concentrations include no treatment (vehicle only), 10^{-8} M, 5 x 10^{-8} M, 10^{-7} M, and 10^{-6} M of $1,25(OH)_2D_3$. With the exception of 10^{-6} M, all treatments were performed in duplicate. A positive control (+), rat cyp24 cDNA was included in the PCR. *GAPDH* was used as a housekeeping gene for RNA extraction and RT-PCR.

6.3.2.4 EFFECT OF $1,25(OH)_2D_3$ ON CYP24 MRNA LEVELS OF SHEEP WITH INHERITED RICKETS

Fibroblasts from sheep with inherited rickets were treated with 10^{-8}, 5 x 10^{-8}, 10^{-7}, 5 x 10^{-7}, and 10^{-6} M of $1,25(OH)_2D_3$ for 12 h. A response to treatment was seen at all doses of $1,25(OH)_2D_3$ (Figure 6.6). A repeatable finding was the production of CYP24 mRNA in all sheep (although faint in sheep 53), despite the absence of $1,25(OH)_2D_3$ treatment. GAPDH bands were present in all samples.

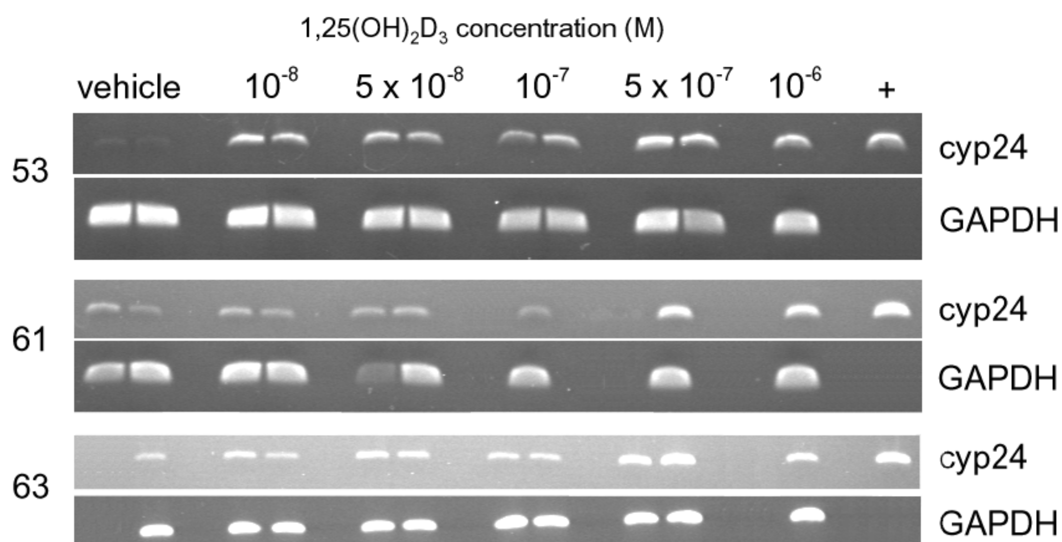


Figure 6.6: Effect of dose on 12 h of $1,25(OH)_2D_3$ treatment on cyp24 induction in fibroblasts from sheep with inherited rickets. DNA was obtained from fibroblasts after $1,25(OH)_2D_3$ treatment, RNA extraction, RT-PCR, and analysed on 1.5% (w/v) agarose gel containing ethidium bromide and visualised under UV light on a transilluminator. Treatment concentrations include no treatment (vehicle only), 10^{-8} M, 5×10^{-8} M, 10^{-7} M, 5×10^{-7} and 10^{-6} M of $1,25(OH)_2D_3$. A positive control (+), rat cyp24 cDNA was included in the PCR. *GAPDH* was used as a housekeeping gene for RNA extraction and RT-PCR.

6.4 DISCUSSION

In this study, a vitamin D-binding analysis was successfully performed and demonstrated that the VDR in fibroblasts from affected sheep had a similar number of binding sites, and affinity for the ligand ($1,25(OH)_2D_3$), as that of control sheep. The production of 24-hydroxylase mRNA was induced in ovine cultured skin fibroblasts using $1,25(OH)_2D_3$ and a dose-response established. The optimal response as measured by CYP24 mRNA production was 12 h and the dose response increased from 5×10^{-8} to 1×10^{-6} M of $1,25(OH)_2D_3$. Sheep with inherited rickets showed induction of CYP24 at a lower concentration than

in control sheep, and 24-hydroxylase mRNA was present in affected sheep even without 1,25(OH)$_2$D$_3$ treatment. In addition, a partial sequence of ovine *25-hydroxyvitamin D$_3$-24-hydroxylase* was identified for the first time.

The partial nucleotide sequence of ovine *24-hydroxylase* showed the greatest similarity with the predicted bovine sequence, as would be expected since both species are ruminants, and the next closest similarity with the pig (a fellow ungulate). The nucleotide sequences of the ox, pig and human show four consistent single base differences from the ovine sequence. For three out of four of these base changes (nucleotide numbers 573, 642, 648) the amino acid sequence is conserved. However, the amino acid sequence shows a consistent change in the sheep from a lysine (K) to an asparagine (N) at position 230 (nucleotide 690) in the cow, pig and human. Lysine is a basic amino acid, while asparagine has a polar carboxamide side chain. Therefore, if this amino acid is in a critical position it may alter the conformation or function of ovine 24-hydroxylase compared to other species. In order to create consensus primers, the region of the ovine *CYP24* gene sequenced was specifically chosen from an area with a high level of identity between the rat *CYP24* gene and the human equivalent. It seems likely therefore, that there would be considerable homology between this partial ovine sequence and the corresponding sequence in other species.

Vitamin D-binding analyses performed on vitamin D receptors from human fibroblasts use a protein concentration of 1 mg/mL and a dose rate of 0.1-1.0 nM [^{3}H]1,25(OH)$_2$D$_3$ (Griffin and Zerwekh, 1983; Malloy *et al*., 1989; Malloy *et al*.,

1990). A ten-fold decrease in protein concentration combined with a ten-fold increase in treatment concentration, as compared with humans, was required in sheep in order to obtain saturation of the VDR with $[^3H]1,25(OH)_2D_3$ (Griffin and Zerwekh, 1983; Malloy *et al.*, 1989; Malloy *et al.*, 1990; Malloy *et al.*, 2004). This is reflected in the 1000-fold higher K_d and B_{max} values obtained in sheep (Malloy *et al.*, 1997; Malloy *et al.*, 2002a). These results correspond with the higher dose of $1,25(OH)_2D_3$ required to observe a response in CYP24 mRNA production in the sheep. In the human, doses as small as 10^{-12} M $1,25(OH)_2D_3$ induce 24-hydroxylase production in fibroblasts, and doses of 10^{-9} and 10^{-8} M show a strong response (Malloy *et al.*, 2002b; Nguyen *et al.*, 2004; Tashiro *et al.*, 2004). The human studies used northern blotting for quantification of CYP24 mRNA production in response to $1,25(OH)_2D_3$. In this ovine study, RT-PCR, a technique that is considered to be more sensitive to small quantities of RNA (Nguyen *et al.*, 2004) was used, but a response to $1,25(OH)_2D_3$ was not observed until $1,25(OH)_2D_3$ concentrations reached 5×10^{-8} M, a higher concentration than that required in other species. Similarly, the incubation times for the vitamin D-binding analysis, and induction of CYP24 experiments were shorter in sheep than in humans. Vitamin D-binding analyses in humans have a $[^3H]1,25(OH)_2D_3$ incubation time of 16-24 h (Hirst *et al.*, 1985; Malloy *et al.*, 1989), whereas in sheep an incubation time of only 12 h was considered optimal. This correlates with the 12 h $1,25(OH)_2D_3$ treatment time for optimal CYP24 induction in the sheep.

The time course of $1,25(OH)_2D_3$ induction in sheep appears to be more compact than in other species. In this study, CYP24 mRNA production only occurred at high doses after 6 and 16 h, but after 12 and 14 h the mRNA production

occurred over a range of lower doses. It appears that a faster and longer response of induction occurs at high doses, such as 10^{-6} M, than at lower doses. In humans, 24-hydroxylase mRNA levels plateau at 12-24 h, with a 20,000-fold induction seen at 12 h (Chen *et al*., 1994; Tashiro *et al*., 2004). As with the sheep, a response in CYP24 mRNA production in humans was seen at 5-6 h (Nguyen *et al*., 2004; Tashiro *et al*., 2004). In porcine renal proximal tubule cells, CYP24 induction has been measured at 2 h after treatment (Zierold *et al*., 2000). The band observed at 6 h was very faint in the sheep in this study; therefore it seems unlikely that measurable induction occurs much earlier.

The absence of a response to low doses of $1,25(OH)_2D_3$ with an extended treatment period of 16 h may indicate that ovine 24-hydroxylase mRNA has a shorter half-life than CYP24 mRNA from human fibroblasts and keratinocytes, which remained constantly elevated until 24 h after treatment (Chen *et al*., 1994; Nguyen *et al*., 2004). In mice injected with $1,25(OH)_2D_3$, CYP24 mRNA levels maintained a steady state for 18-72 h (Akeno *et al*., 1997). Similarly, opossum kidney cells treated with $1,25(OH)_2D_3$ had increased CYP24 promoter activity 24 h after treatment (Armbrecht *et al*., 2003).

Induction of 24-hydroxylase by $1,25(OH)_2D_3$ has also been performed in renal tubular cells of the rat, pig, opossum and mouse (Akeno *et al*., 1997; Armbrecht *et al*., 2003; Furuichi *et al*., 1998; Zierold *et al*., 2000). In AOK-B50 cells (porcine kidney proximal tubule cell line) the pig demonstrates substantial induction of $1,25(OH)_2D_3$ at 10^{-8} M (Zierold *et al*., 2000). In mice injected with $1,25(OH)_2D_3$, CYP24 mRNA was most abundant in the kidney and intestine, with smaller

amounts in the skin (Akeno *et al*., 1997). In sheep injected with 0.4 μg/kg BW $1,25(OH)_2D_3$, there was a 7-fold increase in 24-hydroxylase activity in kidney homogenates above control levels (Engstrom *et al*., 1986). It is possible that $1,25(OH)_2D_3$ treatment of cultured renal cells from sheep may show induction of CYP24 mRNA at a lower dose than is required for ovine fibroblasts.

The difference in the dose of $1,25(OH)_2D_3$ required by ovine fibroblasts to induce CYP24, the shorter time during which CYP24 mRNA levels remain elevated in sheep, and the lower protein concentration and higher $[^3H]1,25(OH)_2D_3$ dose required to obtain saturation of the VDR when compared with humans, may reflect the adaption of sheep to life in an environment where they are exposed to increased ultraviolet radiation. Sheep may have evolved with a requirement for a higher concentration of $1,25(OH)_2D_3$ in order to obtain VDR saturation and activation.

Elevated serum $1,25(OH)_2D_3$ concentrations in sheep with inherited rickets initially suggested a defect in end-organ responsiveness, likely associated with the VDR. However, the vitamin D-binding analysis showed that receptor function in sheep with inherited rickets was normal. Normal VDR function has been shown in a human patient with clinical HVDRR, but induction of 24-hydroxylase activity by $1,25(OH)_2D_3$ in fibroblasts of the patient was minimal (Hewison *et al*., 1993). In this case, the resistance was due to constitutive overexpression of heterogenous nuclear riboprotein (hnRP) that competed with the VDR-RXR heterodimer for binding to vitamin D-response elements (Chen *et al*., 2006; Chen *et al*., 2003).

The results obtained from the induction of 24-hydroxylase by $1,25(OH)_2D_3$ in fibroblasts from sheep with inherited rickets were unexpected. While CYP24 has high constitutive expression in renal cells of normal animals (Jones, 2007), this does not appear to be the case for other cells in which CYP24 is expressed. *Hyp* mice, a model for X-linked hypophosphataemic rickets of humans, have increased 24-hydroxylase expression and activity in renal cortical cells, leading to increased degradation of $1,25(OH)_2D_3$ (Roy *et al*., 1994; Tenenhouse *et al*., 1988). This increased 24-hydroxylase activity is thought to be specific to the renal 24-hydroxylase (Roy *et al*., 1994). The *ob/ob* leptin deficient mouse model also has increased mRNA expression and activity of renal 24-hydroxylase in response to $1,25(OH)_2D_3$ treatment (Matsunuma *et al*., 2004).

The presence of 24-hydroxylase mRNA in the absence of $1,25(OH)_2D_3$ suggests that 24-hydroxylase may be inappropriately overexpressed in sheep with inherited rickets. One could hypothesise that $1,25(OH)_2D_3$ is broken down by 24-hydroxylase before it has a chance to bind to the VDR and alter expression of vitamin D-responsive genes. A transgenic rat model with constitutive expression of 24-hydroxylase has been developed (Kasuga *et al*., 2002). The transgenic rats had decreased $24,25(OH)_2D_3$ levels and normal $1,25(OH)_2D_3$ levels when compared with wild-type rats (Kasuga *et al*., 2002) and developed glomerulonephritis leading to albuminuria, and loss of vitamin D-binding protein and $25(OH)D_3$ into the urine (Hosogane *et al*., 2003; Kasuga *et al*., 2002). It was thought that the loss of $25(OH)D_3$ resulted in the reduction of plasma $24,25(OH)_2D_3$ (Kasuga *et al*., 2002). The renal protein wasting may therefore

confound the effects of constitutive expression of 24-hydroxylase, suggesting that these transgenic rats may not be a suitable model for 24-hydroxylase constitutive expression.

The breakdown of $1,25(OH)_2D_3$ by an overexpressed 24-hydroxylase would be expected to led to low serum $1,25(OH)_2D_3$ concentrations, in contrast to the elevated $1,25(OH)_2D_3$ concentrations measured in the Corriedale sheep with inherited rickets. However, the immunoextraction technique used by commercial enzyme and radioimmunoassays for the measurement of $1,25(OH)_2D_3$ also recovers other 1α-hydroxylated vitamin D metabolites (Hollis, 1995). The increased serum $1,25(OH)_2D_3$ measured in Corriedale sheep may therefore be an artifact caused by interference from other 1α-hydroxylated metabolites produced by 24-hydroxylase.

Further work is required to examine the mechanism of inherited rickets in Corriedale sheep. The induction of other vitamin D-responsive genes such as osteocalcin, TRPV (transient receptor potential vanilloid) 5 and 6 calcium channels and collagen by $1,25(OH)_2D_3$ should be examined. While 24-hydroxylase mRNA is present in the absence of $1,25(OH)_2D_3$, the enzyme may not be active, and the activity of 24-hydroxylase should therefore be investigated. A simple way to examine this would be to measure 24-hydroxylated vitamin D metabolites in serum. Sequencing of the sheep *24-hydroxylase* gene in affected and control sheep may reveal a mutation leading to the inappropriate overexpression of this gene in sheep with inherited rickets and form the basis for a diagnostic test for heterozygous animals.

6.4.1 SUMMARY

This study has established for the first time a dose-response for induction of 24-hydroxylase in ovine fibroblasts. Although differences exist between normal human and ovine CYP24 induction, both show significant induction of the gene in response to 1,25$(OH)_2D_3$. Therefore the sheep may be a useful large animal model for further investigation of vitamin D metabolism.

The vitamin D-binding analysis showed that the VDR of sheep with inherited rickets and control sheep had similar affinities for 1,25$(OH)_2D_3$. An unexpected finding from this study was the presence of 24-hydroxylase mRNA in the absence of 1,25$(OH)_2D_3$ treatment, suggesting inappropriate overexpression of this enzyme as a possible mechanism for inherited rickets in Corriedale sheep, rather than a defect in end-organ responsiveness

Chapter 7

Treatment Experiment

7.1 Introduction

Corriedale sheep with inherited rickets have normal 25-hydroxyvitamin D_3 ($25(OH)D_3$) and high 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) suggesting a defect in end-organ responsiveness (Thompson *et al.*, 2007). Humans with hereditary vitamin D-resistant rickets (HVDRR) also have a defect in end-organ responsiveness, generally due to mutations in the gene encoding the vitamin D receptor (VDR). In some cases, high oral doses of either $1,25(OH)_2D_3$ (Castells *et al.*, 1986; Kruse and Feldmann, 1995) or vitamin D_2 (Takeda *et al.*, 1989) have resulted in remission of the disease. Alternatively high oral doses of 1α-hydroxyvitamin D (Takeda *et al.*, 1987) may also be used to treat HVDRR. Long term nocturnal intravenous calcium infusion appears to be the most successful treatment for patients resistant to all forms of vitamin D and is used routinely by some clinics if children with HVDRR do not respond to oral calcium and

vitamin D therapy (Hochberg *et al*., 1985; Malloy and Feldman, 1999). Therapy for X-linked hypophosphataemic rickets (XLH) involves phosphate replacement and high doses of vitamin D_3 or $1,25(OH)_2D_3$ (Glorieux, 1999).

As part of an investigation into the pathogenesis of inherited rickets in Corriedale sheep, it was decided to conduct a treatment experiment using high oral doses of vitamin D. Sheep have a vitamin D requirement of 60 IU/kg/day for growth, and the recommended treatment for nutritional rickets is 10-20 times the daily requirement (Booth and McDonald, 1982). Van Saun, 2004, used a dose of 1500-2000 IU/kg (25 times daily requirement) to successfully treat neonatal lambs with rickets (Van Saun, 2004). Vitamin D in high doses can however be toxic. The safe upper dietary level of vitamin D in sheep is estimated to be 25,000 IU/kg if given for less than 60 d and 2,200 IU/kg if given for more than 60 d (Subcommittee on Vitamin Tolerance *et al*., 1987). Weekly dosing of sheep for 4 wk with 40,000 IU/kg of vitamin D intramuscularly caused hypercalcaemia, in addition to cardiovascular and renal calcification (Simesen *et al*., 1978).

The purpose of this trial was to assess the effect on bone mineral content (BMC), bone mineral density (BMD) and serum chemistry parameters of a high oral dose of vitamin D in Corriedale sheep with inherited rickets.

7.2 Materials and Methods

7.2.1 Animals Used and Experiment Design

The subjects were seven, 1-year-old Corriedale sheep (five female and two male) with inherited rickets resulting from the embryo transfer programme discussed in Chapter 2. Four animals were randomly chosen to be in the treatment group, and the remaining three assigned to the placebo group. Vitamin D_3 (500,000 IU/g)[2], was in powdered form and reconstituted in sterile water to give an oral dose of 1,000,000 IU per sheep (20,000-25,000 IU/kg). The animals were dosed weekly for 12 weeks. Sheep in the placebo group were given an oral dose of sterile water. Blood was collected via jugular venipuncture into an evacuated tube with no additive prior to the first treatment, and then weekly. Serum total calcium and phosphate concentrations were measured by a commercial laboratory (New Zealand Veterinary Pathology, Palmerston North) using a Roche Hitachi 911 chemistry analyser (Roche Diagnostics, USA) .

7.2.2 Dual-Energy X-Ray Absorptiometry (DEXA)

DEXA is a non-invasive method for measuring BMD in humans and animals, including rats, dogs, pigs and non-human primates (Mas *et al*., 2007; Mitchell *et al*., 2001; Okudan *et al*., 2006; Wren *et al*., 2005; Zotti *et al*., 2004). DEXA has been useful in humans for monitoring BMD improvement with treatment for

2 Kindly donated by Bruce Vautier from BOMAC Laboratories, Ltd., New Zealand.

osteomalacia and rickets (Bhambri *et al.*, 2006; El-Desouki and Al-Jurayyan, 1997).

DEXA examination was performed prior to the first vitamin D treatment, and one week after the last dose. Whole body scans were performed on a Hologic Discovery A (Hologic Inc., USA) using auto whole body fan beam mode. Animals were premedicated with 0.01 mg/kg atropine (Phoenix Pharmaceuticals, USA). Anaesthesia was induced with 10 mg/kg ketamine (Phoenix Pharmaceuticals, USA) and 0.5 mg/kg diazepam (Parnell Laboratories Pty Ltd, Australia), followed by endotracheal intubation. Top-up doses of ketamine and diazepam were given as required. The animals were placed in ventral recumbency with the fore and hindlimbs outstretched (Figure 7.1). Prior scans with cadaver specimens had determined good repeatability of bone mineral density measurements in this position. In addition to whole body BMC and BMD, the BMC and BMD of lumbar vertebrae 1-5, the right metacarpal bone, right and left tibia were also measured.

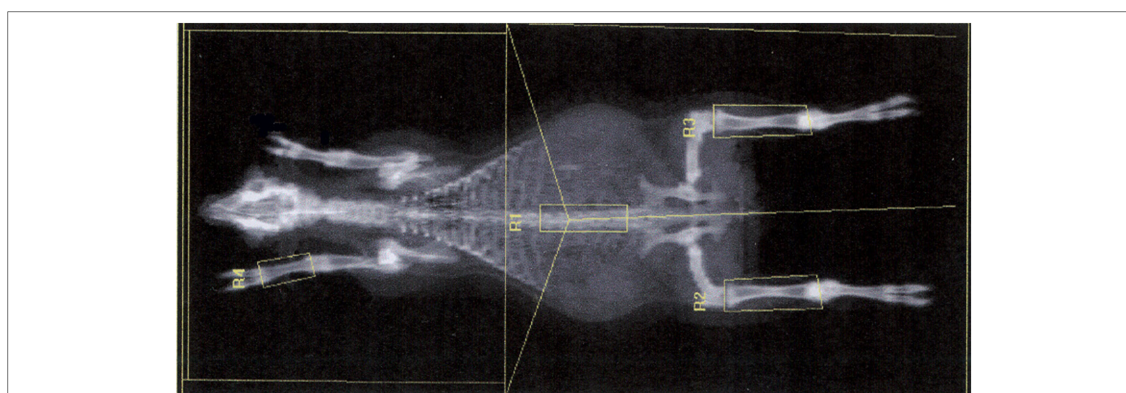


Figure 7.1: Dual energy x-ray absorbtiometry (DEXA) scan showing position of sheep in ventral recumbancy with legs outstretched.

Results were analysed with the Mann-Whitney two-sample test using GraphPad Prism™ 3.0 (GraphPad Software Inc., USA) and Minitab 15 statistical software (Minitab Inc., 2008, USA).

7.3 RESULTS

7.3.1 SERUM CHEMISTRY

No significant differences between treated and non-treated groups in the mean serum phosphate concentrations were detected during the 12 week treatment period (Figure 7.2). Mean serum calcium concentrations at weeks 2 and 8 were significantly greater in treated animals ($P<0.05$), but given the absence of a difference in the other weeks this may have been due to chance (Figure 7.2). The mean serum calcium and phosphate concentrations for the treated and non-treated groups followed similar fluctuations with time (Figure 7.2).

7.3.2 BONE MINERAL CONTENT AND DENSITY

Prior to treatment with vitamin D there was no difference in mean BMD and BMC between the two groups (Table 7.1). The treatment group had a mean increase of 314 g in whole body BMC, which was significantly greater ($P=0.06$) than the increase of 222g in non-treated animals (Table 7.1). Similarly, as a percentage of initial whole body BMC, treated animals had an increase of 36.6% in BMC which was significantly greater than the 24.9% increase in non-treated animals. The gain in whole body BMD was greater in the treated group than in the control group (Table 7.1, $P=0.14$). Treated animals had a 24.9% increase in

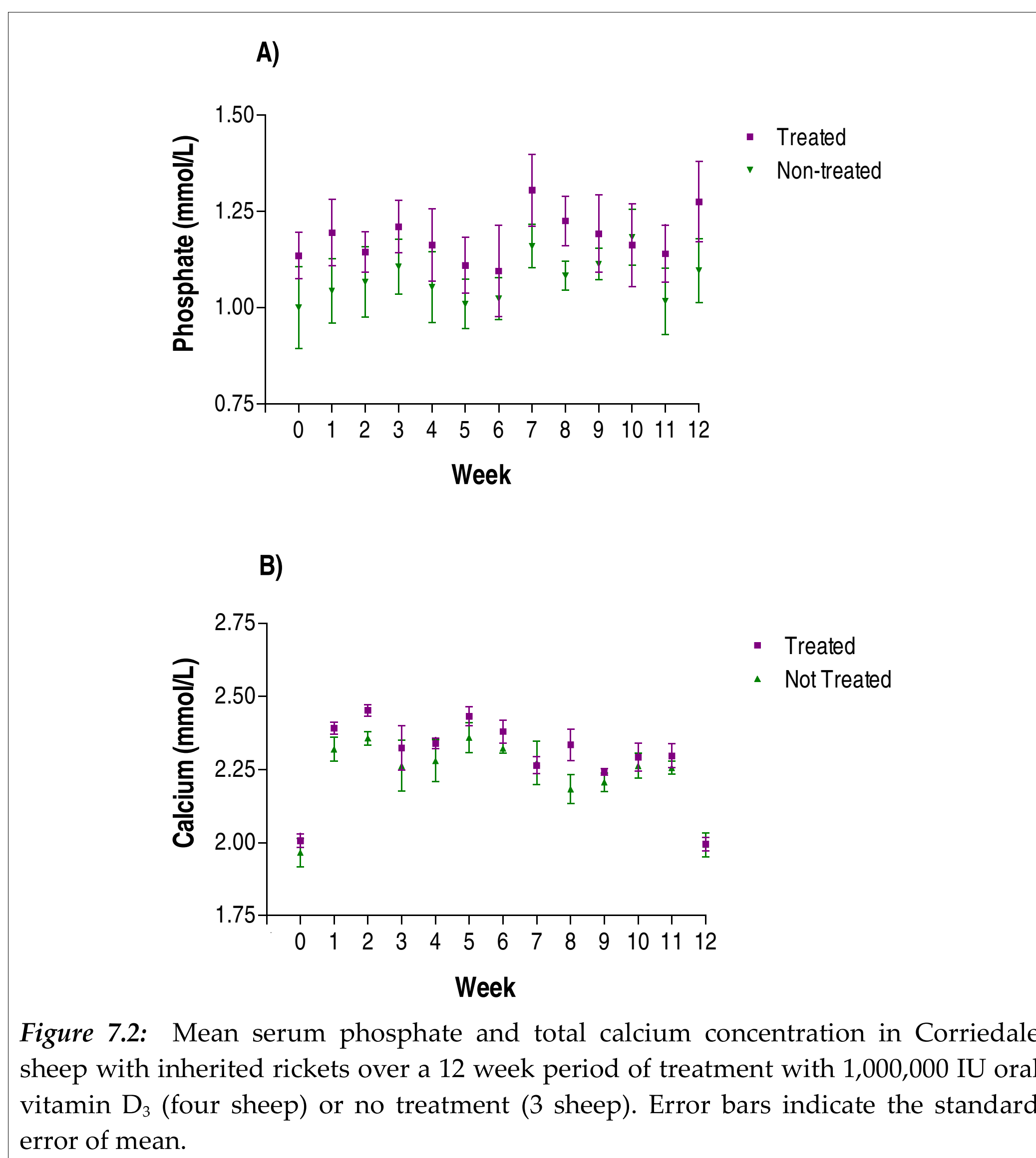


Figure 7.2: Mean serum phosphate and total calcium concentration in Corriedale sheep with inherited rickets over a 12 week period of treatment with 1,000,000 IU oral vitamin D_3 (four sheep) or no treatment (3 sheep). Error bars indicate the standard error of mean.

BMD, compared with the non-treated group which had a 17.3% increase in BMD (P=0.43).

The increases in BMC of lumbar vertebrae 1-5, BMD of lumbar vertebrae 1-5 , and right tibia BMD were significantly greater in treated animals than in controls (Table 7.1, Figure 7.3). The percentage increase in lumbar vertebrae 1-5 BMC and BMD was marked, with treated animals having an increase in BMC

and BMD of 45.8% and 33.3% respectively. This compared with an increase of 13.4% BMC and 19.8% BMD for lumbar vertebrae 1-5 in non-treated animals (Table 7.1). There was no difference between treated and non-treated groups in left tibial and metacarpal BMD and increases in BMD within the two groups were highly variable, particularly for metacarpal BMD (Table 7.1).

Table 7.1: Results of dual energy x-ray absorbtiometry scanning of Corriedale sheep with inherited rickets, either treated (four sheep) or non-treated (three sheep) with 1,000,000 IU oral vitamin D_3 weekly for 12 weeks.

Mean	**Treated ± SEM**	**Non-treated ± SEM**	**P-value**
Whole body initial BMC (g)	903 ± 99	888 ± 15	0.86
Whole body initial BMD (g/cm^2)	0.992 ± 0.075	0.979 ± 0.030	0.60
Whole body BMC gain (g)	314 ± 31	222 ± 19	0.06
% whole body BMC increase	36.6 ± 6.9	24.5 ± 1.8	0.06
Whole body BMD gain (g/cm^2)	0.229 ± 0.036	0.168 ± 0.022	0.14
% whole body BMD increase	24.5 ± 6.2	17.3 ± 2.6	0.43
Spine BMC gain (g)	19.6 ± 4.6	6.1 ± 2.7	0.03
% spine BMC increase	45.8 ± 12.0	13.4 ± 6.8	0.06
Spine BMD gain (g/cm^2)	0.266 ± 0.015	0.163 ± 0.035	0.03
% spine BMD increase	33.3 ± 2.6	19.8 ± 4.5	0.03
Right tibia BMD gain (g/cm^2)	0.237 ± 0.019	0.115 ± 0.033	0.03
% right tibia increase	24.4 ± 3.8	11.8 ± 3.4	0.03
Left tibia BMD gain (g/cm^2)	0.175 ± 0.023	0.189 ± 0.039	0.50
% left tibia BMD increase	17.2 ± 3.0	21.0 ± 4.9	0.86
Metacarpal BMD gain (g/cm^2)	0.123 ± 0.082	0.107 ± 0.052	0.50
% metacarpal BMD increase	23.5 ± 15.0	16.7 ± 7.6	0.50

BMC = bone mineral content; BMD = bone mineral density; Gain = difference between initial BMC or BMD and BMC or BMD after 12 weeks of treatment or no treatment; Spine = lumbar vertebrae 1-5; SEM = standard error of mean. P-value obtained from Mann-Whitney two sample test.

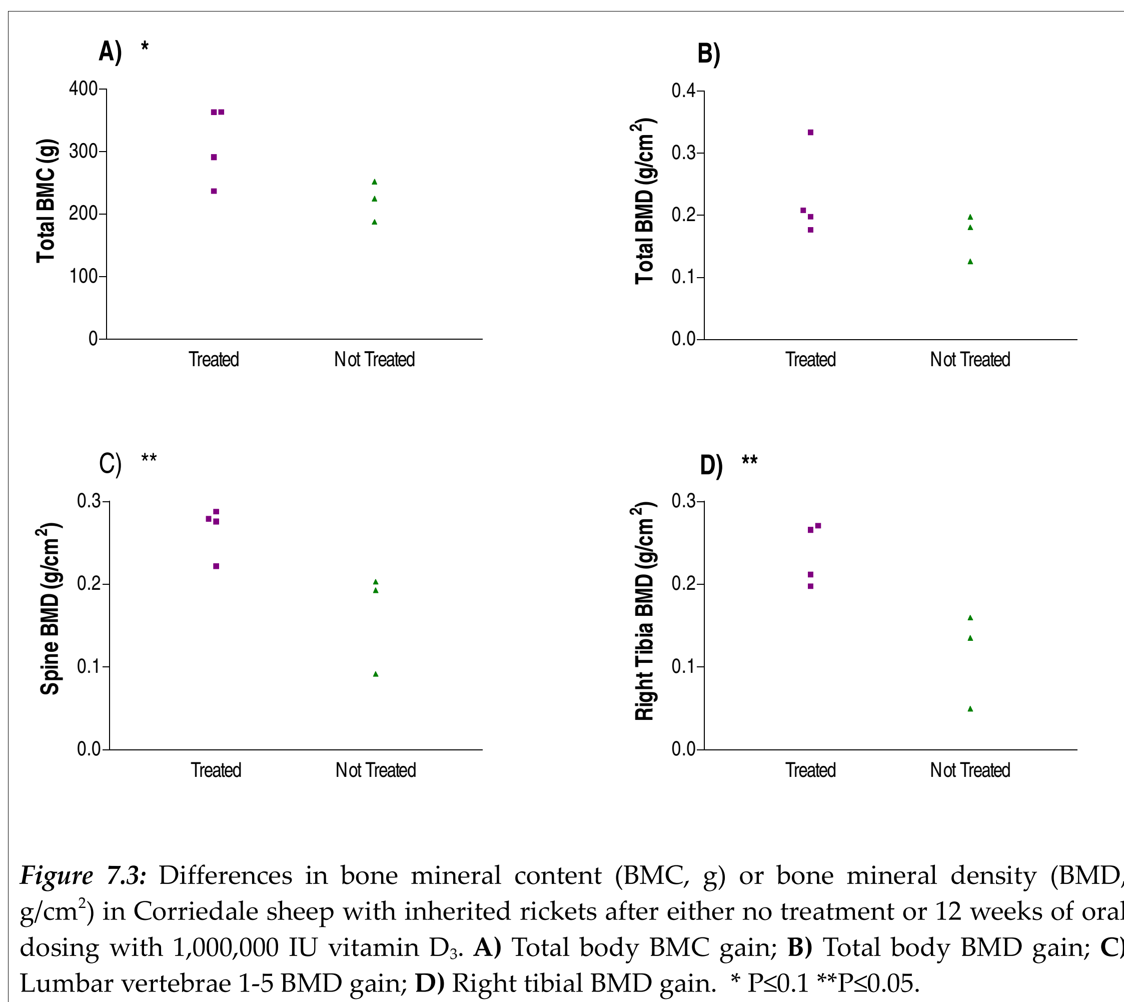


Figure 7.3: Differences in bone mineral content (BMC, g) or bone mineral density (BMD, g/cm^2) in Corriedale sheep with inherited rickets after either no treatment or 12 weeks of oral dosing with 1,000,000 IU vitamin D_3. **A)** Total body BMC gain; **B)** Total body BMD gain; **C)** Lumbar vertebrae 1-5 BMD gain; **D)** Right tibial BMD gain. * P≤0.1 **P≤0.05.

7.4 DISCUSSION

Administration of high doses of vitamin D to Corriedale sheep with inherited rickets had no effect on serum phosphate or total calcium concentrations, but significantly increased BMD and BMC, suggesting that sheep with this form of the disease are capable of responding to vitamin D therapy. In humans with XLH, combined treatment with $1,25(OH)_2D_3$ and phosphate is successful in eliminating lower limb deformities and allowing the affected child to reach normal height, however vitamin D therapy leads to hypercalcaemia, hypercalciuria and nephrocalcinosis (Whyte, 2002). Similarly, humans with

vitamin D-dependent rickets type 1, are also successfully treated with physiologic doses of 1,25$(OH)_2D_3$, and high doses of vitamin D_3 or 25$(OH)D_3$ may also treat the condition (Whyte, 2002). Some patients with HVDRR will respond to very high oral doses of 1,25$(OH)_2D_3$, and older patients may no longer require therapy, or require only low doses later in life (Whyte, 2002).

Initial biochemical testing, as presented in Chapter 5, suggested a defect in end-organ responsiveness as the likely mechanism for inherited rickets of Corriedale sheep. However, results from Chapter 6 showed the VDR was functioning normally, and suggested the 24-hydroxylase enzyme was inappropriately overexpressed. The results from the present study lend further support to a functional VDR. Perhaps the high doses of vitamin D used in this treatment experiment overwhelmed the capacity of the 24-hydroxylase enzyme to break it down, allowing binding of 1,25$(OH)_2D_3$ to the VDR and promoting mineralisation of osteoid, thus increasing BMC and BMD.

The main advantage of DEXA over quantitative computed tomography, another technique used for bone density measurements, is the speed and ease of scanning, as an anaesthetised sheep is particularly prone to regurgitation, aspiration and ruminal distension during prolonged anaesthesia (Taylor, 1991). Another advantage of DEXA is that regions of interest can be followed longitudinally (Pouilles *et al*., 2000), as they were in this study. However, cortical and trabecular regions within a bone cannot be identified by DEXA, and DEXA measurements give no indication of bone strength (Cointry *et al*., 2004; Ferretti *et al*., 2001; Leonard *et al*., 2004).

The results from this study showed high variability between BMDs of the long bones. Other studies have also reported variability, due to the difficulty in scanning the extremities in a repeatable manner in anaesthetised sheep (Turner *et al*., 1995). However, these studies also reported that repeatability and precision of BMC and BMD of the lumbar vertebrae in the dorsoventral position was good (Pouilles *et al*., 2000; Turner *et al*., 1995). In addition, BMC was found to be less reproducible than BMD for the same animal, while both BMC and BMD showed high variability between sheep of different age, weight and breed (Pouilles *et al*., 2000). It is thought that the BMD of lumbar vertebrae 1 to 3 may be more sensitive to hormone status and mechanical load (Turner *et al*., 1995). Given that the Corriedale sheep examined in this study were the same age, breed and similar weights, the BMC and BMD data for lumbar vertebrae 1 to 5 can be relied on, and in spite of the small number of sheep in each group, a significant increase in BMC and BMD was measured following vitamin D treatment.

While DEXA BMD measurements are non-specific for the diagnosis of osteomalacia, studies in humans with nutritional osteomalacia and rickets have shown substantial increases in BMD after treatment with vitamin D (Bhambri *et al*., 2006; El-Desouki and Al-Jurayyan, 1997; Ghose, 2004). One study showed an increase in BMD at the lumbar spine and hip of 50-60% over several months of treatment (Bhambri *et al*., 2006). In that study, there was a 25% increase in lumbar spine BMD after 1.5-3 months, which is similar to the results of a New Zealand case study, where a 24.8% increase in BMD was seen after 2 months of

vitamin D treatment for osteomalacia (Bhambri *et al.*, 2006; Ghose, 2004). These results are greater than the extra 13.5% increase in lumbar spine BMD seen in sheep treated with vitamin D in the present trial. However, the response to vitamin D treatment in inherited forms of rickets cannot necessarily be compared with those of nutritional origin, and data on the response of BMD to vitamin D treatment of patients with hereditary rachitic diseases is not available.

Given the increase in whole body and spinal BMC and BMD of treated sheep, it would be expected that the sheep treated with vitamin D would show some increase in their serum calcium and phosphate concentrations, but this was not the case. Vitamin D treatment of nutritional rickets and osteomalacia in humans shows maximum effect in 4 to 10 weeks, and has a prolonged effect of up to 30 weeks after cessation of therapy (Whyte and Thakker, 2005). Successful therapy in humans is indicated by increasing urinary calcium concentration, hypercalciuria generally occurs before hypercalcaemia, and may appear abruptly when the bones cease acting as a sink for calcium (Whyte and Thakker, 2005). In a case series of 26 patients with osteomalacia, a return to normal serum calcium and phosphate concentrations occurred on average in 2.8 months, and was seen prior to a complete return to normal BMD (Bhambri *et al.*, 2006). It may be that in this study on Corriedale sheep, serum calcium and phosphate concentrations did not improve as the skeletal deficit of calcium and phosphate had yet to be completely replenished, and the bones were still actively mineralising.

Further treatment experiments in these sheep could be used to investigate calcium transport in the upper gastrointestinal tract of sheep. Experiments using fistulated sheep have shown that approximately 50% of gastrointestinal calcium absorption in this species occurs in the rumen (Schroder *et al*., 1997). Sheep, unlike goats, do not show vitamin D-dependent calbindin-D_{9k} absorption of calcium across the ruminal wall (Schroder *et al*., 2001), but active calcium transport is thought to occur across the rumen wall by delivery of protons (H^+) from short chain fatty acids and a Ca^{2+}/H^+ exchange mechanism (Schroder *et al*., 1999). Therefore the effects of high doses of oral calcium, not only on bone density but also absorption of calcium across the rumen wall in sheep with inherited rickets could be examined.

7.4.1 SUMMARY

This study has shown that Corriedale sheep with inherited rickets treated with high doses of oral vitamin D show an increase in whole body and lumbar vertebrae bone mineral content and density. These results support the *in vitro* studies (in Chapter 6) that identified a functional vitamin D receptor in affected sheep.

Chapter 8

The Immune System

8.1 Introduction

Two forms of immunity, non-specific or innate immunity and specific or acquired immunity, work together to provide an effective immune system (Snyder, 2007). The innate immune system is the first line of defense, and comprises phagocytic and dendritic cells, plus anatomic (skin, cilia) and physiologic (gastric acid) components (Akira *et al*., 2006; Snyder, 2007). Phagocytic cells like neutrophils, monocytes and tissue macrophages contain membrane receptors known as pattern recognition receptors that recognize pathogen components (Akira *et al*., 2006; Snyder, 2007). Toll-like receptors (TLRs) are pattern recognition receptors, that when bound to microbial components, trigger the activation of signaling cascades leading to activation of genes involved in host defense (Akira *et al*., 2006). Acquired immunity on the

other hand, is the second line of defense and is characterised by antigen specificity and self/non-self recognition (Snyder, 2007).

The acquired immune system can be roughly divided into two parts; the humoral response with B-lymphocytes defending against extracellular pathogens and toxins, and the cell-mediated response with T-lymphocytes defending against intracellular pathogens (Snyder, 2007). T-lymphocytes may be further subdivided into CD4+ helper T-lymphocytes (T_H) and CD8+ cytotoxic T-lymphocytes (T_C) (Snyder, 2007). T_H1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and tissue necrosis factor-β (TNF-β) cytokines and are responsible for classic cell-mediated responses such as delayed-type hypersensitivity and activation of cytotoxic T-lymphocytes (Kindt *et al*., 2007). T_H2 cells produce IL-4, IL-5, IL-6 and IL-10 cytokines and function as a helper for B-lymphocyte activation, which is required for bacteria and parasite immunity (Kindt *et al*., 2007).

The role of vitamin D in the immune system has been the subject of considerable research over the last few decades (DeLuca and Cantorna, 2001). Peripheral lymphocytes, macrophages, promyelocytes and thymic tissue all have been shown to express the vitamin D receptor (VDR) (DeLuca and Cantorna, 2001). Of these, CD8+ T-lymphocytes have the highest concentration of VDR, while CD4+ T-lymphocytes and monocytes/macrophages contain low concentrations (Veldman *et al*., 2000). T-lymphocyte-mediated immune responses are reduced in the absence of 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), but are also diminished when $1,25(OH)_2D_3$ concentrations are

high (DeLuca and Cantorna, 2001). Active vitamin D leads to decreased production of the cytokines IFN-γ and IL-2 (Mahon *et al*., 2003) and up-regulates the production of IL-4 (Cantorna *et al*., 2000a), thus suppressing T_H1 responses while up-regulating T_H2 responses (Adams *et al*., 2007). Consequently, vitamin D has been used as an immunosuppressive treatment for certain experimental autoimmune diseases, including autoimmune encephalomyelitis (a model for multiple sclerosis), rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease (Cantorna *et al*., 1996; Cantorna *et al*., 2000b; DeLuca and Cantorna, 2001). A high calcium intake is required for vitamin D to produce immunosuppression in these disease conditions (Cantorna *et al*., 1999; DeLuca and Cantorna, 2001).

Vitamin D also has a role in the innate immune system. Activation of TLRs on monocytes/macrophages leads to increased expression of the VDR and 1α-hydroxylase enzyme, which in turn induces the production of antimicrobial peptides (Liu *et al*., 2006a). These TLR-stimulated macrophage responses are strongly dependent on serum 25-hydroxyvitamin D concentrations, and are shown to be reduced in serum from vitamin D-deficient individuals (Liu *et al*., 2006a). Activated macrophages and dendritic cells express a 1α-hydroxylase (CYP27B1) activated by IFN-γ and TLR agonists, unlike the renal isoform that is regulated by Ca^{2+} (White, 2008). However, vitamin D appears to inhibit TLR expression and decrease responsiveness to pathogen-associated molecular patterns (Sadeghi *et al*., 2006).

Nutritional rickets in human patients has been associated with an increased incidence of infections, possibly due in part to increased lymphocyte apoptosis (El Hodhod *et al*., 2006) and decreased cell-mediated immunity (Yang *et al*., 1993). CD4+ T-lymphocytes from *VDR* null mice produce more IFN-γ and less IL-2, IL-4, IL-5 than those from wild-type mice (Froicu *et al*., 2003), but when *VDR* null mice were normocalcaemic, no immune function defects were identified (Mathieu *et al*., 2001). It may be that the function of $1,25(OH)_2D_3$ in the immune system is redundant, and the immune dysfunction is the result of low blood calcium, an essential intracellular messenger in the immune system (Mathieu *et al*., 2001)

During this investigation of inherited rickets in Corriedale sheep, affected animals appeared to be more susceptible than normal sheep to diseases caused by ectoparasites and endoparasites, and to dermatophilosis. Although this may reflect a breed difference or non-specific stress-induced immunosuppression associated with developing skeletal lesions, it was decided to conduct a pilot study investigating aspects of immunity in affected sheep.

8.2 MATERIALS AND METHODS

8.2.1 ANIMALS

The subjects of this experiment were two male and six female Corriedale sheep with inherited rickets (aged 1 ½ - 3 ½ years) and eight female age-matched mixed breed controls from the same property. Blood was collected into lithium heparin evacuated tubes by jugular venipuncture and processed within 2 h of

collection. Additional plain evacuated tubes were collected at the same time, centrifuged and the serum stored at -80°C until processed.

A complete blood count was performed at a commercial laboratory (New Zealand Veterinary Pathology Ltd., Palmerston North) using an ADVIA 120 Hematology Analyser (Siemens Healthcare Diagnostic Inc., USA). Serum cortisol concentrations were also measured at a commercial laboratory (New Zealand Veterinary Pathology Ltd.) using a DPC Immulite Analyser (Diamond Diagnostics Inc., USA).

8.2.2 PHAGOCYTOSIS OF FLUORESCENT *ESCHERICHIA COLI* BY NEUTROPHILS

Heparinised whole blood (100 µL) from affected and control sheep was added to fluorescent-activated cell sorting (FACS) tubes (Becton, Dickenson and Co., USA) on ice, followed by 50 µL fluorescein isothiocyanate (FITC) labelled *Escherichia coli* (4 x 10^6 bacteria, non-opsonised K12 strain, Bioparticles, Fluorescein conjugate, Invitrogen Ltd., USA). The following five control tubes were prepared: free non-quenched *E. coli*, free *E. coli* quenched with 50µL trypan blue 0.4% solution in phosphate-buffered saline (PBS), blood only, blood with *E. coli* on ice and free quenched *E. coli* mixed with blood just prior to reading. All tubes except the ice control were incubated in a 37°C water bath for 10 min prior to lysis with FACS lysis buffer (Becton, Dickenson and Company, USA). The tubes were then incubated in the dark for 15 min at room temperature. Subsequently, the tubes were centrifuged at 450 *g* for 10 min at 18°C. The supernatant was discarded and the pellet resuspended in 2 mL of

PBS. The tubes were again centrifuged at 450 *g* for 10 min at 18°C. The supernatant was discarded, and the pellet resuspended in 500 µL of 1% paraformaldehyde in PBS, to which 50 µL of trypan blue was added.

Samples were immediately acquired on a BD FACSCalibur using CellQuest software (Becton, Dickenson and Company, USA). Blood neutrophils were gated on by forward and side scatter characteristics. For each sample, 10,000 gated events were acquired whenever possible. The lower threshold for positive-staining (*E. coli* containing) cells was determined using a negative control consisting of fixed white blood cells mixed with fixed, trypan blue-quenched *E. coli*, to exclude neutrophils with externally bound *E. coli*. Background phagocytosis levels were determined by incubating blood samples with *E. coli* on ice for 10 min rather than at 37°C, and then following the above lysis procedure. Neutrophils with fluorescence levels above the background fluorescence in the latter tube were considered to be positive for phagocytosis.

8.2.3 CELL SURFACE MARKER ASSAY

The mouse anti-ovine CD4:RPE antibody (0.1 mg/ml IgG2a conjugated to R-phycoerythrin, AbD serotec, ALS, Australia) and mouse anti-ovine CD8:RPE antibody (0.1mg/ml IgG2a conjugated to R-phycoerythrin, AbD serotec, ALS, Australia) were titrated in order to determine the optimal concentration of antibody for the experiment. The following concentrations were tested for each antibody: 1 µg/100 µL, 0.5 µg/100 µL, 0.25 µg/100 µL, 0.125 µg/100 µL, 0.0625 µg/100 µL and 0.03125 µg/100 µL. As determined by flow cytometry, the

optimal CD4:RPE and CD8:RPE antibody concentrations were 0.1 μg/100 μL and 0.25 μg/μL respectively.

Six control tubes were prepared for each experiment: blood with control FITC (mouse IgG2a:FITC, BD Pharmingen, USA) and control PE (mouse IgG2a:PE, BD Pharmingen, USA), blood with control FITC and CD4:RPE, blood with control FITC and CD8:RPE, blood with annexin-V:FITC (rh annexin-V conjugated to FITC, Bender Medsystems, ALS, Australia) and control RPE, blood with annexin-V:FITC and CD4:RPE, and blood with annexin-V:FITC and CD8:RPE.

Each CD4-annexin-V assay FACS tube contained 100 μL of blood from either an affected or control sheep, 5 μL annexin-V:FITC, 1 μL CD4:RPE (0.1 μg/100μL), 94 μL of PBS and 100 μL of annexin-binding buffer (10 mM Hepes/NaOH, ph 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Each CD8-annexin-V assay FACS tube contained 100 μL of blood from either an affected or control sheep, 5 μL annexin-V:FITC, 2.5 μL CD8:RPE (0.25 μg/100μL), 92.5 μL of PBS and 100 μL of annexin-binding buffer. The tubes were then placed in a refrigerator, protected from light, for 30 min. Subsequently, 2 mL of FACS lysis buffer was added and the tubes incubated at room temperature, protected from light, for 15 min. PBS (2 mL) was added and the tubes were centrifuged at 450 *g* for 5 min at 4°C. The supernatant was discarded and 2 mL of PBS added to resuspend the pellet. The tubes were centrifuged again, at 450 *g* for 5 min at 4°C. The supernatants were again discarded. The pellet was resuspended in 300 μL of PBS and read immediately on a BD FACSCalibur flow cytometer (Becton, Dickenson and Co.,

USA). Lymphocytes were gated on by forward and side scatter characteristics, and a minimum of 10,000 events acquired per sample. Positive fluorescence staining was identified by setting the lower threshold at the level observed in blood stained with irrelevant isotype-matched control antibodies.

CD4+ and CD8+ counts were derived by determining CD4+ and CD8+ cell frequency in the blood and multiplying by the total lymphocyte count to obtain absolute CD4+ and CD8+ cell numbers per volume of blood.

8.2.3.1 Treatment of Leucocytes with Ethanol

Heparinised whole blood (1 mL) from each control and affected sheep was treated with 100 mM ethanol (Kapasi *et al.*, 2003). The treated blood was incubated overnight at 37°C, 5% CO_2, and then processed as for the cell marker assay.

Heparinised whole blood (1 mL) from a control sheep was treated with 100 mM, 200 mM, 300 mM and 400 mM of ethanol, and a negative control treated with PBS, to examine the effect of increasing ethanol concentration on leucocyte apoptosis. The treated blood was incubated overnight at 37°C, 5% CO_2, and then processed as for the cell marker assay.

8.2.4 Measurement of Serum IgG Concentration

Serum immunoglobulin G (IgG) concentration was measured using a direct enzyme-linked immunosorbent assay (ELISA). Serum was diluted to 1 in

200,000 in PBS and added in triplicate to a 96-well plate. A nine-point standard (ChromPure Ovine IgG whole molecule, Jackson Immunoresearch Laboratories, Inc., USA - 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, 15.6 ng/mL, 7.8 ng/mL, 3.9 ng/mL) diluted in PBS was also added to triplicate wells of the plate. Three wells had PBS only to serve as blanks. The plate was incubated overnight at 4°C then washed three times with PBS/Tween buffer and blocked with 100 μL PBS/3% bovine serum albumin (BSA) for 1 h at room temperature. The plate was again washed three times with PBS/Tween buffer, and incubated for 90 min at room temperature with 50 μL donkey anti-ovine IgG:horseradish peroxidase (AbD Serotec, ALS, Australia) diluted to 50 ng/mL in PBS/1%BSA. It was then washed five times with PBS/Tween buffer and 50 μL of TMB (TetraMethylBenzidine, Zymed, Invitrogen Ltd., USA) was added. The plate was incubated in the dark at room temperature for 20 min. Sulphuric acid (25 μL of 1 M) was added to stop the reaction and the plates were read at 450 nm on an EL_X 808 ultra microplate reader (Bio-tek Instruments Inc., USA).

8.2.5 Lymphocyte Stimulation Assay

8.2.5.1 Preparation of Peripheral Blood Mononuclear Cells

Heparinised whole blood was diluted 5:1 in cold PBS, then layered onto Histopaque®-1077 (Sigma-Aldrich Co., USA) and centrifuged at 1400 *g* for 20 min at room temperature. The buffy coat layer was removed and washed twice (1400 *g* for 10 min) with complete media comprising of RPMI-1640 (Invitrogen Ltd., USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.3 mg/mL glutamine (Penicillin-Streptomycin-Glutamine, Invitrogen Ltd., USA) and 25 μmol 2-

mercaptoethanol (Invitrogen Ltd., USA). The peripheral blood mononuclear cells (PBMC) were resuspended in complete RPMI-1640 media and adjusted to a final concentration of 2 x 10^6 cells/mL.

8.2.5.2 Lymphocyte Proliferation Assay

For the lymphocyte proliferation assay, 50 μL (1 x 10^5 cells) of cell suspension along with 50 μL of mitogen or medium alone was added to 96-well plates. PBMC from each sheep were cultured in triplicate for each mitogen. The mitogens tested included Con A (10 μg/mL, extract from *Concanavalia ensiformis* type IV-S, Sigma-Aldrich Co., USA), LPS (20 μg/mL, lipopolysaccharides from *Escherichia coli* O55:B5, Sigma-Aldrich Co., USA) and parasite antigen (30 μg/mL, CarLA [carbohydrate larval antigen] from *Trichostrongylus colubriformis* (Harrison *et al.*, 2003)). The plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere for 3 d. On the morning of the 3rd d, 10 μL of MTT (5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich Co., USA) was added to each well. The plates were incubated for a further 4 h, and then 100 μL of acid-isopropanol was added, mixed well, and left for a 3-4 min. The plates were then read at 550 nm on an EL_X 808 ultra microplate reader (Bio-tek Instruments Inc., USA). The proliferation index was calculated as the ratio of proliferation of stimulated cells to the basal proliferation of unstimulated cells.

8.2.5.3 Interferon-γ Cytokine Assay

For the cytokine assay, 500 μL (1 x 10^6 cells) of cell suspension and 500 μL of mitogen was added to 24-well plates. The mitogens tested included Con A,

LPS, and parasite antigen. The plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere for 3 d. Interferon-γ was measured from the cell supernatants using Bovigam™ bovine gamma interferon ELISA (CSL Veterinary Ltd., Australia) as per the manufacturer's instructions. The plates were read on a VersaMax microplate reader with Soft-Max Pro version 4 (Molecular Devices Corp., USA) at a wavelength of 450 nm.

8.2.6 Data Analysis

Results were analysed using Student's two sample t-test with Minitab 15 statistical software (Minitab Inc., 2008, USA).

8.3 Results

8.3.1 Complete Blood Count

Sheep with inherited rickets had a signficantly lower total white blood cell count, lymphocyte number, red blood cell number and haematocrit than age-matched controls (Table 8.1). No significant difference was seen in the mean numbers of monocytes, eosinophils, neutrophils and large unstained cells (Table 8.1).

Table 8.1: Complete blood count results for eight control sheep and eight sheep with inherited rickets.

	WBC	Lympho	Neut	Mono	Eos	LUC	RBC	HCT
Affected	5.471	3.365	1.380	0.330	0.185	0.135	9.424	0.2913
Control	8.731	5.344	1.472	0.978	0.720	0.108	12.964	0.3538
P value	0.001	0.018	0.793	0.088	0.059	0.507	<0.001	0.031

WBC = white blood cells (x 10^9 cells/L); lympho = lymphocytes (x 10^9 cells/L); Neut = neutrophils (x 10^9 cells/L); Mono = monocytes (x 10^9 cells/L); Eos = eosinophils (x 10^9 cells/L); LUC = large unstained cells (x 10^9 cells/L); RBC = red blood cells (x 10^{12} cells/L); HCT = haematocrit (L/L).

8.3.2 Serum Cortisol

The mean serum cortisol concentration in affected sheep (65.3 ± 11 nmol/L) was significantly lower than that of control sheep (122.1 ± 18 nmol/L, P=0.023).

8.3.3 Phagocytosis of Fluorescent *E. coli* by Neutrophils

Mean phagocytosis of *E. coli* by neutrophils from sheep with inherited rickets (74.68 ± 1.39%) was significantly greater (P=0.002) than that of control sheep (54.44 ± 4.46%). As illustrated in Figure 8.1, neutrophil phagocytosis of the control animals had a larger range than that of affected animals. Control and affected animals had similar numbers of neutrophils per volume of blood, indicating that the increased bacterial phagocytosis by neutrophils from affected sheep was not the result of an increased number of neutrophils.

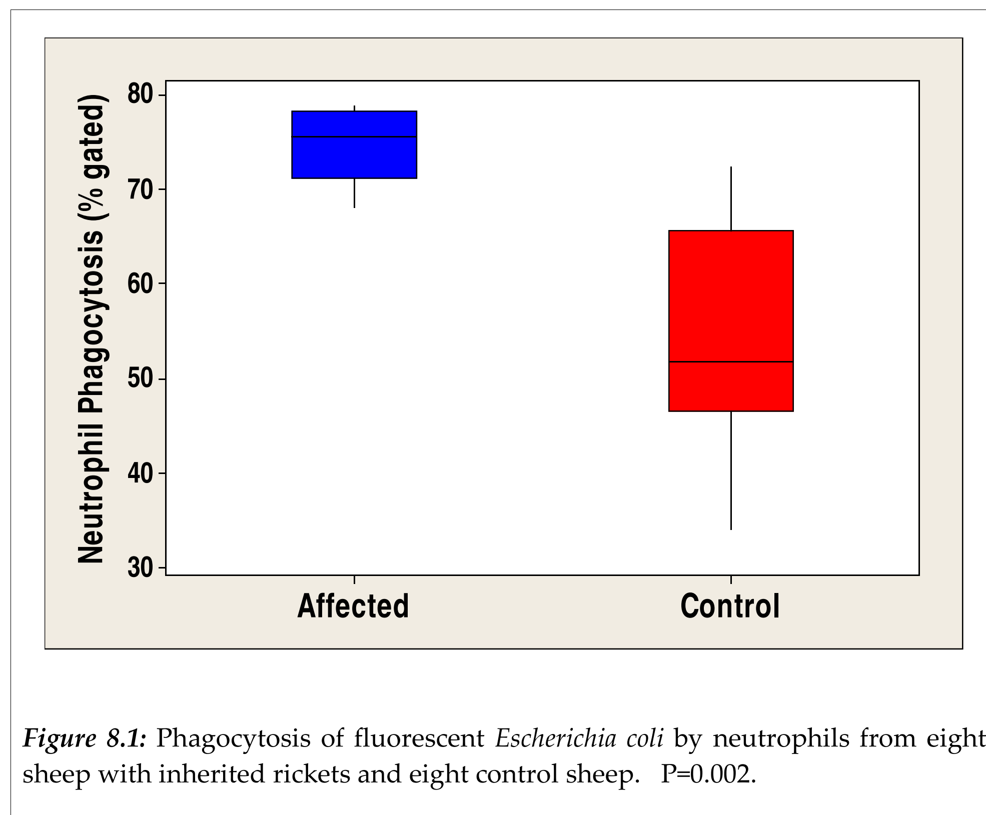


Figure 8.1: Phagocytosis of fluorescent *Escherichia coli* by neutrophils from eight sheep with inherited rickets and eight control sheep. P=0.002.

8.3.4 CELL SURFACE MARKER ASSAY

The mean number of CD4+ and CD8+ lymphocytes was significantly less (P<0.05) in affected animals than in controls, with affected animals having 1.5 and 3.8 times less CD4+ and CD8+ lymphocytes respectively than control animals (Figure 8.2). Of particular note was the difference between affected and control sheep in the ratio of CD4+ to CD8+ lymphocytes (Figure 8.2). Affected animals had 2.5 times more CD4+ than CD8+ lymphocytes, while control animals only had 1.3 times more CD4+ than CD8+ lymphocytes (P=0.001).

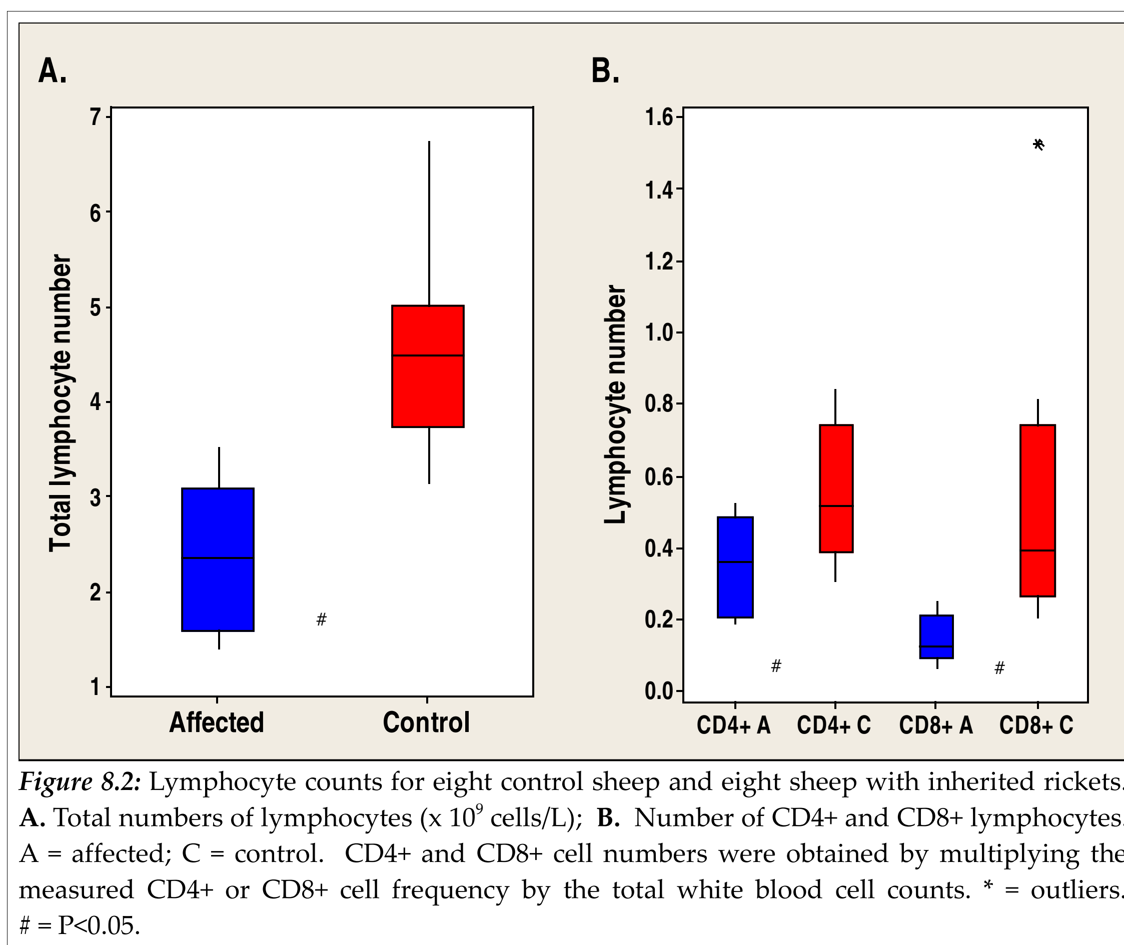


Figure 8.2: Lymphocyte counts for eight control sheep and eight sheep with inherited rickets. **A.** Total numbers of lymphocytes (x 10^9 cells/L); **B.** Number of CD4+ and CD8+ lymphocytes. A = affected; C = control. CD4+ and CD8+ cell numbers were obtained by multiplying the measured CD4+ or CD8+ cell frequency by the total white blood cell counts. * = outliers. # = P<0.05.

The levels of apoptosis were measured in leucocyte subsets. This was identified by the binding of fluorescently labelled annexin-V to phosphatidylserine that had been translocated from the inner to the outer leaflet of the cell membrane during the early apoptotic process (Koopman *et al*., 1994). The number of annexin-positive lymphocytes was small, and did not vary significantly between affected and control animals (Table 8.2). At no point was the number of annexin-positive lymphocytes greater than 0.1%. After overnight treatment with 100 mM ethanol, control animals had higher numbers of annexin-positive lymphocytes, and CD8+ annexin+ lymphocytes than affected animals (P<0.05),

Table 8.2: Results from cell surface marker assay, as measured by flow cytometry, for lymphocytes from eight sheep with inherited rickets and eight control sheep. # = significant at 5% level.

Cell and cell marker	Mean affected (cells/L x 10^9)	Mean control (cells/L x 10^9)	P-value
CD4+ lymphocytes	0.349 ± 0.049	0.547 ± 0.068	0.035 #
CD8+ lymphocytes	0.144 ± 0.023	0.553 ± 0.160	0.036 #
annexin+ lymphocytes	0.0028 ± 0.0007	0.0017 ± 0.0006	0.241
100 mM ethanol CD4+ annexin+ lymphocytes	0.099 ± 0.040	0.142 ± 0.014	0.339
100 mM ethanol CD8+ annexin+ lymphocytes	0.069 ± 0.013	0.136 ± 0.018	0.010 #
100 mM ethanol annexin+ lymphocytes	1.050 ± 0.110	1.567 ± 0.150	0.017 #

although the number of positive cells remained small. No difference between the two groups was seen in the numbers of CD4+ annexin+ lymphocytes.

Overnight treatment of lymphocytes from control and affected sheep with 100 mM ethanol had less effect on annexin staining than higher doses of ethanol. Blood from a control sheep was treated with increasing ethanol concentrations and, as the dose of ethanol increased, so did the numbers of all annexin-positive cells. In particular, there was a large increase in the number of annexin-positive cells as the ethanol dose increased from 200 mM to 300 mM (Figure 8.3). At the 400 mM ethanol dose, a large number of the cells were already dead. Monocytes appeared to be particularly sensitive to the effects of ethanol, at 200 mM ethanol 3.4% monocytes were annexin-postive, while at 300 and 400 mM ethanol, monocytes were non-existent.

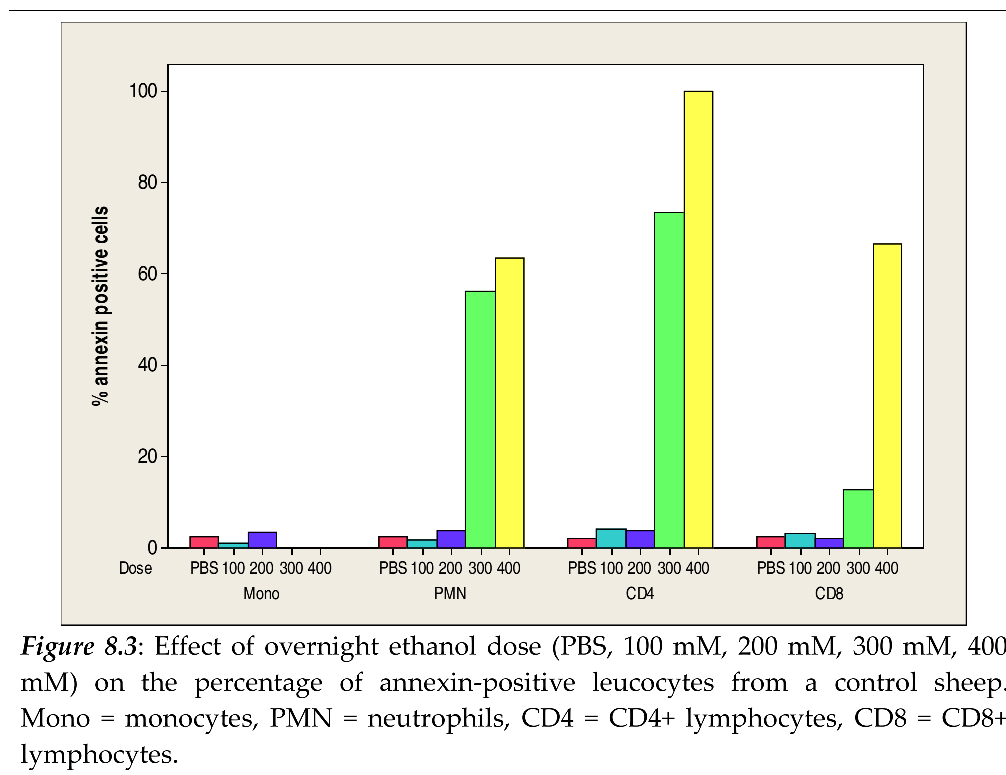


Figure 8.3: Effect of overnight ethanol dose (PBS, 100 mM, 200 mM, 300 mM, 400 mM) on the percentage of annexin-positive leucocytes from a control sheep. Mono = monocytes, PMN = neutrophils, CD4 = CD4+ lymphocytes, CD8 = CD8+ lymphocytes.

8.3.5 Serum IgG Concentration

The mean serum IgG concentration in affected sheep (24.0 ± 3.4 mg/mL) was greater than in control sheep (17.9 ± 2.3 mg/mL), but the difference was not considered significant (P=0.163).

8.3.6 Lymphocyte Stimulation Assays

8.3.6.1 Lymphocyte Proliferation Assay

Basal proliferation rates as measured by MTT were significantly less (P=0.033) in affected sheep (0.2733 ± 0.019) than in control sheep (0.3574 ± 0.028). Sheep with inherited rickets had a higher mean lymphocyte proliferation index (PI) in response to ConA (2.515 ± 0.22) and LPS (1.507 ± 0.13), than control sheep

(ConA 2.031 ± 0.18, LPS 1.277 ± 0.078). The mean lymphocyte PI in response to ConA (P=0.056) and LPS (P=0.082) was significantly different at the 1% level, however as shown in Figure 8.4 there is substantial overlap in PI between the two groups. Parasite antigen in the form of CarLA from *Trichostrongylus colubriformis*, a small intestinal parasite of sheep, was used to examine the hypothesis that Corriedale sheep with inherited rickets had increased susceptibility to gastrointestinal parasites. The mean lymphocyte PI in response to parasite antigen showed no significant difference (P=0.711) between affected and control sheep.

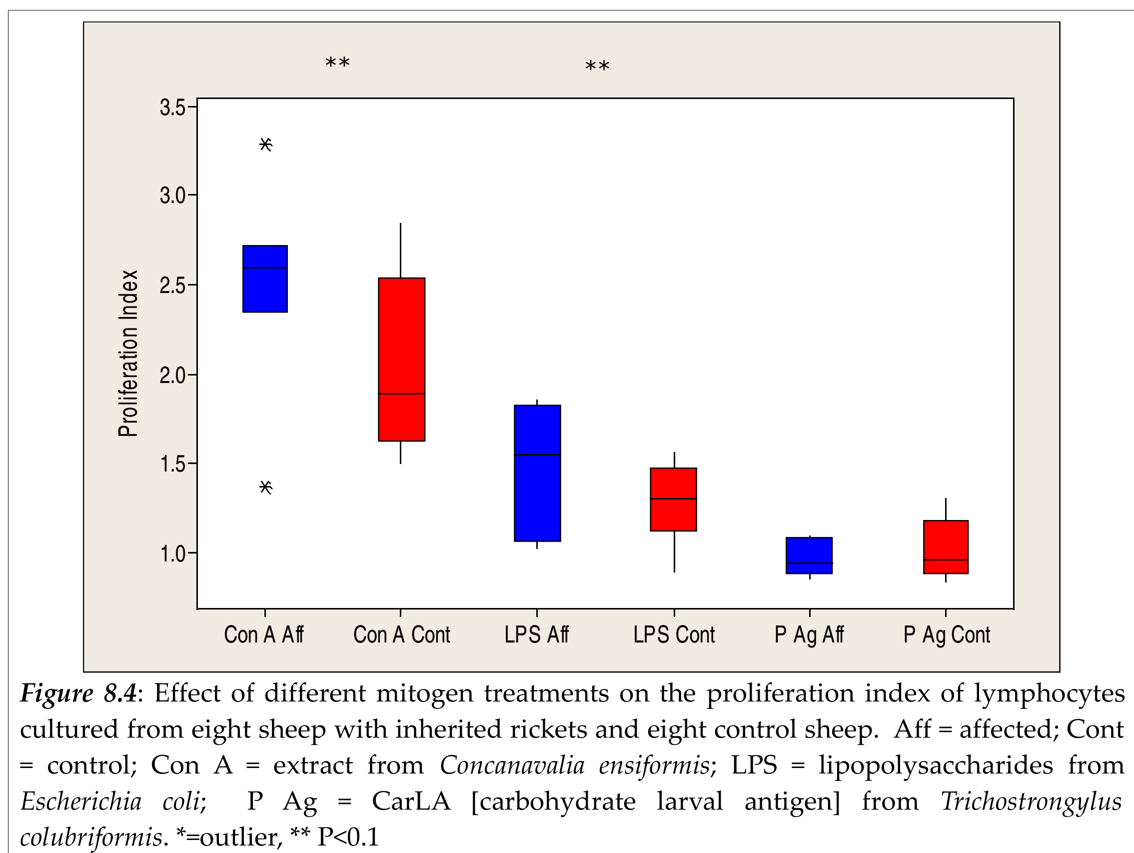


Figure 8.4: Effect of different mitogen treatments on the proliferation index of lymphocytes cultured from eight sheep with inherited rickets and eight control sheep. Aff = affected; Cont = control; Con A = extract from *Concanavalia ensiformis*; LPS = lipopolysaccharides from *Escherichia coli*; P Ag = CarLA [carbohydrate larval antigen] from *Trichostrongylus colubriformis*. *=outlier, ** P<0.1

8.3.6.2 INTERFERON-γ CYTOKINE ASSAY

IFN-γ production by lymphocytes stimulated with the mitogens ConA, LPS and parasite antigen was significantly greater ($P<0.05$) than the background level present in media. No difference was seen between affected and control sheep in the mean OD_{450} when stimulated by ConA and LPS (Table 8.3). However, the mean OD_{450} for IFN-γ production by lymphocytes stimulated with parasite antigen was significantly less in affected sheep than in control sheep ($P=0.060$, Table 8.3).

Of the 1 x 10^6 cells added to each well for the interferon-γ cytokine assay, based on complete blood count and flow cytometry results, in affected animals 9.5% were CD4+, 3.9% CD8+, 77.7% CD4-CD8- and 8.9% were monocytes. In comparison, control animals had 8.65% CD4+, 8.75% CD8+, 67.13% CD4-CD8- and 15.47% were monocytes.

Table 8.3: Effect of mitogen on interferon-γ production by lymphocytes from eight sheep with inherited rickets and eight control sheep, as measured by commercial ELISA (Bovigam™).

Mitogen	**Disease status**	**OD 450 nm**	**P value**	**OD mitogen / OD media**	**P value**
Media only	Affected	0.0515 ± 0.003	0.391		
	Control	0.0546 ± 0.003			
Con A	Affected	0.640 ± 0.15	0.724	12.41 ± 2.9	0.364
	Control	0.774 ± 0.34		15.20 ± 7.1	
LPS	Affected	0.0658 ± 0.007	0.958	1.28 ± 0.1	0.335
	Control	0.0662 ± 0.004		1.22 ± 0.1	
Parasite Ag	Affected	0.236 ± 0.06	0.060**	4.86 ± 1.4	0.097**
	Control	0.458 ± 0.12		8.33 ± 2.1	

Con A = extract from *Concanavalia ensiformis* type IV-S; LPS = Lipopolysaccharides from *Escherichia coli*. Parasite Ag = CarLA [carbohydrate larval antigen] from *Trichostrongylus colubriformis*. ** significant at 1% level.

8.4 DISCUSSION

In this pilot study, Corriedale sheep with inherited rickets had lymphopenia, a decrease in both CD4+ and CD8+ T-lymphocytes in circulation and an increase in the CD4+:CD8+ lymphocyte ratio. The reason for this combination of changes is not clear. Lymphopenia may be stress-induced, and may therefore be expected in association with chronic disease (Dhabhar *et al*., 1995; Jain, 1993). In Merino sheep, acute stress has been shown to increase the CD4+:CD8+ ratio (Degabriele and Fell, 2001), while chronic stress and depression in humans leads to a decrease in CD4+ lymphocytes (Scanlan *et al*., 1998). However, the lower serum cortisol concentrations in affected sheep suggests that stress is not a likely explanation for the alterations in their lymphocyte populations.

In this study, no difference was seen in the serum IgG concentration between sheep affected with inherited rickets and control sheep, suggesting that in this case B-lymphocyte function was not affected by the defect in vitamin D metabolism. ConA stimulates proliferation of T-lymphocytes, while LPS stimulates proliferation of B-lymphocytes (Boilard and Surette, 2001; Venkataraman *et al*., 1999). Little difference between sheep with inherited rickets and control sheep was seen in lymphocyte proliferation in response to either of these mitogens. Interferon-γ (T_H1) production in response to these mitogens was also similar in affected and control sheep. This suggests that direct T-lymphocyte and B-lymphocyte activation in Corriedale sheep with inherited rickets is functioning normally.

Children with vitamin D-deficient rickets have a significant decrease in their circulating T-lymphocytes and an increase in circulating B-lymphocytes (Yener *et al*., 1995). This may account for the increased susceptibility to infection, particularly respiratory infections, seen in children with rickets (Muhe *et al*., 1997; Najada *et al*., 2004). A mouse model with targeted ablation of *25-hydroxyvitamin D 1α-hydroxylase* and undetectable serum 1,25$(OH)_2D_3$, showed decreased CD4+ and CD8+ lymphocytes compared to wild-type mice (Panda *et al*., 2001). This suggests that the lymphopenia and reduced numbers of CD4+ and CD8+ lymphocytes in sheep with inherited rickets could be the result of a defect in vitamin D metabolism.

Unlike *1α-hydroxylase* null mice and sheep with inherited rickets, *VDR* null mice and humans with hereditary vitamin D-resistant rickets (HVDRR) show no changes in total white cell and lymphocyte counts (O'Kelly *et al*., 2002). Given that 1,25$(OH)_2D_3$ inhibits T_H1 cytokines IFN-γ and IL-12, it is conceivable that the opposite would occur in association with vitamin D-deficiency or malfunctioning VDR. Splenocyte cultures from *VDR* null mice had decreased IFN-γ production (T_H1) and increased IL-4 (T_H2) production, suggesting the possibility that the VDR may be important in T_H1 cell development (O'Kelly *et al*., 2002). In vitamin D-deficient mice, cell-mediated immunity was also suppressed (Yang *et al*., 1993).

The highest concentrations of VDR are found in CD8+ lymphocytes, with CD4+ lymphocytes and macrophages containing much lower concentrations (Veldman *et al*., 2000). Treatment of T-lymphocytes with 1,25$(OH)_2D_3$ increases

the concentration of VDR in these cells by more than two-fold (Veldman *et al*., 2000). This may indicate that a long-term defect in vitamin D metabolism has a greater effect on CD8+ than on CD4+ lymphocytes, as was seen in this study in Corriedale sheep with inherited rickets.

A study in children with infantile rickets suggested that immune cell dysfunction that occurs in rickets was due in part to increased apoptosis of lymphocytes (El Hodhod *et al*., 2006). However, apoptosis of lymphocytes from both control and affected sheep in the present study was negligible, and is unlikely to have contributed to the lymphopenia.

An interesting result, unrelated to vitamin D, was the effect of ethanol on lymphocyte apoptosis in sheep. Human alcoholics are reported to have lymphopenia, and ethanol has been shown to induce human T-lymphocyte apoptosis (Kapasi *et al*., 2003). Blood from sheep with inherited rickets was treated with ethanol in order to determine if lymphocytes from these animals were more susceptible to apoptosis. Although control animals had a slightly greater number of annexin-positive lymphocytes, the differences were small. However, a clear dose response in apoptosis of white blood cells, irrespective of disease status, was seen as ethanol concentration increased. Monocytes in particular appeared highly susceptible to the effects of ethanol, as no cells survived a dose of 300 mM or higher. At an ethanol dose of 400 mM virtually all CD4+ lymphocytes were annexin-positive and more than 60% of neutrophils and CD8+ lymphocytes were also annexin-positive. A substantial increase in the number of annexin-positive cells occurred between the 200 mM and 300

mM ethanol dose, presumably as the dose of ethanol reached a toxic level. Such an effect of ethanol on white cells has not been previously reported in sheep.

Given anecdotal observations that sheep with inherited rickets were more susceptible to both ectoparasites and endoparasites, the lymphocyte response of affected sheep to parasite antigen (CarLA from *Trichostrongylus colubriformis*) was of particular interest. Immunity to parasites is a T_H2 lymphocyte response, characterised by generation of IL-3, IL-4, IL-5 cytokines, eosinophilia, mastocytosis and IgE production (Riffkin *et al*., 1996). A T_H1 response with IFN-γ production and CD8+ lymphocytes appears to retard the immunity acquired to *Trichostrongylus colubriformis* (McClure *et al*., 1995). Similarly, in a study using monoclonal antibodies to CD4+ and CD8+ lymphocytes, depletion of CD8+ lymphocytes had little effect on immunity to *Haemonchus contortus*, while depletion of CD4+ lymphocytes inhibited the development of immunity (Gill *et al*., 1993). No work appears to have been done on the effect of vitamin D deficiency on susceptibility to parasites in humans.

Lymphocyte proliferation in response to parasite antigen was similar in affected and control sheep, however IFN-γ production was lower in affected animals. The number of CD4+ lymphocytes added to the assay for affected and control sheep was similar, raising two possibilities for the decreased IFN-γ production by lymphocytes from sheep with inherited rickets. Firstly, the lower number of monocytes to act as antigen-presenting cells may have led to decreased IFN-γ production. Secondly, the CD4+ lymphocytes in the affected sheep may have a skewed T_H1:T_H2 ratio compared to normal sheep. Affected and control sheep

responded similarly to the direct T-lymphocyte activator ConA and neither responded to LPS, the direct B-lymphocyte activator. This suggests that the decreased IFN-γ production in response to parasite antigen by T-lymphocytes from affected sheep may be due to deficient or defective monocytes and antigen cell presentation. These are preliminary results and further work would be required in order to determine if these sheep are more susceptible to parasites.

Additional work is needed to fully evaluated the immune system in sheep with inherited rickets. In particular, measurement of IL-4 cytokine production (T_H2) would be of interest since up-regulation of IL-4 by 1,25(OH)$_2$D$_3$ is thought to be a key mechanism in the effect of 1,25(OH)$_2$D$_3$ on immunity (Cantorna *et al.*, 2000a). Measurement of IL-4 may be possible in the future with the recent development of an assay for ovine IL-4 (Hope *et al.*, 2005). Additional studies should include investigation of the effect of 1,25(OH)$_2$D$_3$ on lymphocytes, macrophage function and innate immunity in sheep with inherited rickets.

8.4.1 SUMMARY

In this pilot study investigating possible effects on immunity in sheep with an inherited form of rickets, lymphopenia and depletion of both CD4+ and CD8+ lymphocytes were detected. In addition, affected sheep had decreased IFN-γ production by lymphocytes stimulated with parasite antigen. Lymphocyte proliferation, neutrophil phagocytosis, IgG production were similar in affected and control sheep. The defect in vitamin D metabolism present in Corriedale sheep with inherited rickets makes them a potentially useful model for investigating the effect of vitamin D deficiency on the immune system.

CHAPTER 9

GENERAL DISCUSSION

9.1 INTRODUCTION

The discovery of this inherited form of rickets in Corriedale sheep in New Zealand has presented an opportunity to develop a model for the treatment of similar diseases in human patients, and to investigate aspects of vitamin D metabolism. The primary aims of the present study were to characterise the genetic and phenotypic features of the disease, and to determine the disease mechanism, thus providing a basis for future studies using this ovine model and for developing a diagnostic test for heterozygous animals.

9.2 INHERITANCE

The embryo transfer programme using Corriedale ewes and rams with rickets, and the out-cross-back-cross breeding trial using a heterozygous ram reported

in Chapter 2, indicated that this skeletal disease of Corriedale sheep had simple autosomal recessive inheritance. This is consistent with many of the human hereditary rachitic diseases, including vitamin D-dependent rickets type I (VDDR), hereditary vitamin D-resistant rickets (HVDRR), autosomal recessive hypophosphataemic rickets and hereditary hypophosphataemic rickets with hypercalciuria, although hypophosphataemic rachitic diseases in human patients may also show either X-linked or autosomal dominant inheritance (Feng *et al*., 2006; Whyte, 2002).

The farm of origin had obtained rams from the same breeder for 12 years and results of DNA analysis indicated that four out of ten of the rams used in the 2004 breeding season had sired lambs with the disease (Thompson *et al*., 2007). This shows that the frequency of the defective gene was relatively high, not only on the farm of origin, but also the stud farm from which the farmer had obtained the rams. The Corriedale breed has a relatively small gene pool in New Zealand, and there is the potential for this genetic defect to be widespread amongst the breed in this country. The defect may also have been exported to other countries with the transfer of Corriedale genetics.

The loss of affected lambs in the neonatal period during the present study was contrary to field observations, and suggests that the prevalence of the disease on the original property, and within the Corriedale breed, has been underestimated. Neonatal losses average 15-20% (Bruere and West, 1993) on commercial sheep farms and are seldom investigated in detail for cause of death. Identifying the genetic mutation and developing a test for heterozygous

animals with the mutation would be of significant benefit to the industry. Therapy is not an option under normal pastoral conditions.

9.3 Features of the Disease

The clinical signs, imaging, macroscopic and microscopic lesions were all consistent with rickets. Reduced growth rate, angular limb deformities, enlarged physes and costochondral junctions, persistence of hypertrophic chondrocytes in the metaphyses, microfractures, and osteoid seams are all classic changes of this metabolic bone disease. In addition, secondary hyperparathyroidism as a result of hypocalcaemia led to inappropriate and excessive osteoclastic resorption.

Inherited rickets in Corriedale sheep does not appear to be analogous to any of the hereditary rachitic entities of humans. Humans with hereditary rickets regardless of the form, without treatment, develop angular limb deformities (Whyte, 2002) similar to those seen in sheep with inherited rickets. Humans with HVDRR often have alopecia (Malloy *et al*., 1999) but this is not a feature of the disease in Corriedale sheep. The radiographic changes in sheep with inherited rickets are less severe than those of humans with VDDR I and HVDRR, but similar to those of XLH (Pitt, 1995; Shore and Poznanski, 1999). Another similarity between the disease in Corriedale sheep and XLH is the development of enthesophytes with age (Reid *et al*., 1989). On the other hand, sheep with inherited rickets do not have the renal phosphate wasting, which is a feature of hypophosphataemic forms of rickets (Whyte, 2002). The serum chemistry changes in affected Corriedale sheep most closely resemble those of

HVDRR, where normal 25-hydroxyvitamin D_3 (25(OH)D_3) and elevated 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$$D_3$) is reported (Malloy and Feldman, 1999). The increase in serum 1,25(OH)$_2$$D_3$ concentration in Corriedale sheep with inherited rickets however, is only two-fold, in comparison with the 10-20 fold increase in 1,25(OH)$_2$$D_3$ present in humans with HVDRR.

Humans with hereditary rickets are normal at birth and develop the disease within the first few months of life (Whyte, 2002). Vitamin D-deficient rats, *1α-hydroxylase* null pigs and mice, and *vitamin D receptor* (*VDR*) null mice have normal skeletal mineral content at birth and up to 2 weeks after birth (Kovacs, 2008). After this time, animals develop hypocalcaemia, hypophosphataemia and, by weaning, clinical signs of rickets (Kovacs, 2008). In spite of initial reports from the farm of origin that affected Corriedale lambs appeared normal at birth, the development of pathological fractures during parturition and healed infractions in the bones of an affected fetus, indicated that the disease developed *in utero*. This is supported by lower serum phosphate and calcium concentrations at birth in affected lambs compared with control lambs. It is thought that if calcium and phosphate concentrations are within normal limits, then the 1,25(OH)$_2$$D_3$-VDR complex is not required for bone mineralisation (Panda *et al*., 2004). This is supported by the resolution of clinical signs with intravenous calcium infusions in humans with HVDRR, thereby bypassing vitamin D-regulated intestinal calcium absorption (Hochberg *et al*., 1992). The recipients used in the embryo transfer programme were unrelated clinically normal ewes with adequate calcium, phosphate and vitamin D concentrations. If passive diffusion across the placenta was the primary method of calcium supply for the fetus, as reported in rats (Brommage and DeLuca, 1984; Lester,

1986), then affected fetuses should have been born normal. This was not the case in affected Corriedale sheep, indicating that vitamin D is required for calcium transport across the ovine placenta.

Diagnosis of this inherited form of rickets in live lambs was relatively straightforward. Affected lambs were considerably less active than controls, some suffered rib fractures during birth and all had persistent hypophosphataemia. Radiography and peripheral quantitative computed tomography (pQCT) were also useful for diagnosing inherited rickets in the live animal. The radiographic presence of thick cortices, coarse, blurred, metaphyseal trabeculae and enlarged physes would be diagnostic for this form of inherited rickets in sheep (Chapter 3). Bone mineral density (BMD) of the tibia as measured by pQCT, may also be a useful tool. After 3 months of age, a BMD of less than 1000 mg/cm^3, and increased cortical area, would be suggestive of inherited rickets.

This was the first study reporting pQCT changes in animals with rickets. One of the outstanding macroscopic and radiographic features of inherited rickets in Corriedale sheep was thickening of cortical bone. CT scans suggestesd that these thickened cortices were composed of poorly mineralised bone. While the total bone mineral content of cortices in affected sheep was greater than in controls, the BMD was significantly less, no doubt as a result of the significantly greater cortical area. Macroscopically, many fine red streaks were present in the thickened cortices, and microscopically these were found to be Haversian systems and secondary osteons filled with poorly mineralised osteoid. Whether

this cortical thickening, which is also seen in human patients with XLH (Harrison *et al*., 1976), is a result of decreased osteoclastic resorption of poorly mineralised bone, increased strain on poorly mineralised bone, or a combination of the two is unknown. Excessive osteoclastic resorption was a prominent microscopic feature, suggesting decreased osteoclastic resorption is not the major reason for increased cortical thickness in Corriedale sheep with inherited rickets. Such changes would not be expected in animals with nutritional rickets, where a deficiency of either vitamin D or phosphorus would most likely be present for a much shorter period and the effect on bone formed during cortical remodeling therefore considerably less.

From six months of age, collapse of subchondral bone and subsequent flattening and irregularity of the articular cartilage surface was a feature of the disease. This lesion is not reported in humans with any form of rickets, but is reported in other quadrapeds with vitamin D or phosphorus deficiency rickets (Craig and Davies, 1943; Thompson and Robinson, 1989). This may result from the different centre of gravity present in quadrapeds compared to bipeds (Badoux, 1975). The consistent presence of this lesion in the humeral head is most likely due to the combination of poorly mineralised subchondral bone as a result of the rickets, and the fact that nearly 60% of the body weight of the sheep is carried by the forelimbs (Kim and Breur, 2008). The large range of motion of the shoulder joint (Dyce *et al*., 1996) may also contribute to the development and severity of this lesion.

An outstanding microscopic feature, confirmed by the Tripp & MacKay method (Tripp and MacKay, 1972) and Goldner's trichrome method, was the presence of wide unmineralised osteoid seams lining trabeculae and filling Haversian systems. In a study on humans with XLH, osteoid surface ranged from 32 - 98%, and osteoid thickness from 6.5 - 26.7 μm. These are comparable with the histomorphometric results obtained from Corriedale sheep with inherited rickets with an osteoid surface percentage of 42 - 76% and osteoid seam thickness of 27.8 - 35.8 μm.

In addition to the classically described effects of vitamin D on calcium and phosphate homeostasis, research over the last 20-30 years has identified vitamin D receptors (VDR) in multiple cells and organs (Lips, 2006), including the immune system, where the VDR has been shown to be present in lymphocytes, monocytes/macrophages, promyelocytes and thymus tissue (DeLuca and Cantorna, 2001). An observation that Corriedale sheep with inherited rickets appeared to be more susceptible to parasites led to a pilot study designed to assess aspects of their immune system. Affected sheep were severely lymphopenic, with decreases in both CD4+ and CD8+ lymphocytes. Of particular interest was the decrease in interferon-γ production by lymphocytes from affected sheep stimulated with parasite antigen (CarLA from *Trichostrongylus colubriformis* (Harrison *et al*., 2003)). Although the number of sheep included in this investigation was small, the results suggest the possibility of a defect in immunity, perhaps in antigen presentation by monocytes. Further investigation of the immune system in sheep with inherited rickets would be warranted.

9.4 Possible Disease Mechanism

The presence of normal serum 25(OH)D_3 and elevated serum 1,25$(OH)_2D_3$ concentrations (Chapter 5) in Corriedale sheep with inherited rickets, suggested that a defect in end-organ responsiveness to 1,25$(OH)_2D_3$ was responsible for the disease, as seen in human patients with HVDRR (Malloy *et al*., 1999). As such, a series of experiments was designed in order to test the hypothesis that the skeletal disease in Corriedale sheep was due to a vitamin D receptor (VDR) defect (Chapter 6). A vitamin D-binding analysis performed on fibroblasts from affected sheep showed that $[^3H]$1,25$(OH)_2D_3$ binding to the VDR was similar to that of control sheep. Also, sheep treated with high doses of vitamin D_3 (Chapter 7) showed a greater improvement in bone mineral content and density than untreated sheep, as measured by dual-energy x-ray absorptiometry. These results showed that the VDR was functional in Corriedale sheep with inherited rickets.

An unexpected result was obtained from *in vitro* studies assessing the induction of 24-hydroxylase mRNA by 1,25$(OH)_2D_3$. Fibroblasts from Corriedale sheep with inherited rickets, produced 24-hydroxylase mRNA at lower doses of 1,25$(OH)_2D_3$ than fibroblasts from control sheep, and even produced it in the absence of 1,25$(OH)_2D_3$. These results suggested inappropriate overexpression of the 24-hydroxylase enzyme as a likely mechanism. Overexpression of the 24-hydroxylase enzyme, a multicatalytic enzyme responsible for breakdown of 25(OH)D_3 and 1,25$(OH)_2D_3$ (Prosser and Jones, 2004), would however be expected to result in low serum 1,25$(OH)_2D_3$ concentrations, in contrast to the results presented in Chapter 5.

The immunoextraction technique used in commercial enzyme- and radio-immunoassays may also recover other 1α-hydroxylated metabolites, such as 1,25$(OH)_2D_3$-26,23-lactone, 1,24,25$(OH)_3D$, 1,25,26$(OH)_3D$ and calcitroic acid (Hollis, 1995). All are known metabolites resulting from the breakdown of 1,25$(OH)_2D_3$ by 24-hydroxylase (Miyamoto *et al*., 1997b; Prosser and Jones, 2004). Cell lines that overexpress 24-hydroxylase produce a wide range of intermediates, from 1α-24,25$(OH)_3D$ to calcitroic acid following exposure to 1,25$(OH)_2D_3$ in concentrations from 100 nM to 10 μM (Masuda *et al*., 2004). If 24-hydroxylase is inappropriately overexpressed in Corriedale sheep with inherited rickets, then metabolites of 24-hydroxylase could also be elevated and the increased serum 1,25$(OH)_2D_3$ concentration measured by the commercial laboratory could have been an artifact.

Hydroxylation of 1,25$(OH)_2D_3$ by 24-hydroxylase may occur at the either the C-24 or C-23 site, depending on species (Sakaki *et al*., 2005). In humans, hydroxylation of 1,25$(OH)_2D_3$ by 24-hydroxylase may occur at both the C-23 and C-24 sites, in guinea pigs C-23 hydroxylation predominates, while in rats C-24 hydroxylation is most common (Engstrom *et al*., 1986; Pedersen *et al*., 1988; Prosser *et al*., 2007). The C-24 hydroxylation pathway appears to be the predominant 1,25$(OH)_2D_3$ breakdown pathway in sheep (Engstrom *et al*., 1986). The substitution of a glycine for alanine at the 326 position of human 24-hydroxylase, allows extra space for the side chain of 1,25$(OH)_2D_3$ to move deeper into the pocket and allow 23- rather than 24-hydroxylation (Prosser *et al*., 2007). Also, alternative splicing of 24-hydroxylase has been shown to alter

the activity of the enzyme (Ren *et al*., 2005). The potential exists therefore for a mutation in *24-hydroxylase* DNA to lead to a functional enzyme.

A transgenic rat model with constitutive expression of 24-hydroxylase has been developed, but the development of glomerulonephritis, albuminuria and loss of vitamin D-binding protein through the kidney, make this an inappropriate model (Hosogane *et al*., 2003; Kasuga *et al*., 2002). Naturally occurring mutations leading to consititutive expression of 24-hydroxylase have not been reported. Inherited rickets in Corriedale sheep may therefore be the first example of a metabolic bone disease caused by a mutation in *24-hydroxylase*.

Although mutations in human *24-hydroxylase* leading to metabolic bone disease have not been identified thus far, Corriedale sheep with inherited rickets have considerable potential as a model for metabolic bone disease of humans. No reliable rat or mouse model of either 24-hydroxylase constitutive expression or overexpression exists. The sheep, because of its size and ease of management, is regarded as an excellent model for skeletal diseases in humans (Turner, 2002). Bone mineral composition is similar between humans and sheep (Ravaglioli *et al*., 1996). Furthermore, the sheep is a widely investigated model for osteoporosis and is often used to evaulate the effects of therapeutic drugs on osteoporotic bone (Martini *et al*., 2001). Regulation of gastrointestinal calcium absorption is the main difference between human and ovine calcium metabolism, however if the tested drug is not administered orally, then comparisons between humans and sheep can be considered valid (Martini *et al*., 2001).

In addition to metabolising $1,25(OH)_2D_3$, 24-hydroxylase may also metabolise vitamin D analogs, the metabolites of which may have biological activity in their own right (Hamamoto *et al*., 2006). This sheep model may therefore be useful for investigating not only the biological activity of vitamin D analog metabolites, but also drugs with the potential to inhibit 24-hydroxylase and prolong $1,25(OH)_2D_3$ action (Hamamoto *et al*., 2006; Prosser *et al*., 2007). It may also be useful for investigating further aspects of vitamin D metabolism, particularly with regard to the function of $1,25(OH)_2D_3$ breakdown metabolites, the effects of which are controversial (Boyan *et al*., 2003; Kasuga *et al*., 2002). For example, it is thought that 24R,25-dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) decreases chondrocyte proliferation, and stimulates alkaline phosphatase and proteoglycan sulphation in the growth plate via non-VDR pathways (Schwartz *et al*., 2000). Fracture healing in chicks, and healing of rickets in rats have also been shown to be enhanced by $24,25(OH)_2D_3$ (Atkins *et al*., 1992; Kato *et al*., 1998). Additionally, $24,25(OH)_2D_3$ has been reported to increase bone volume and decrease bone turnover (Nakamura *et al*., 1992a; Nakamura *et al*., 1992b). Further insight into these proposed mechanisms may be possible through studies using a model characterised by overexpression of 24-hydroxylase.

9.5 LIMITATIONS TO THE STUDY

The main limitation of the study was the number of affected sheep available for the morphometric analyses of Chapter 2, the disease mechanism experiments of Chapter 6, the treatment experiment of Chapter 7 and the immune system experiments of Chapter 8. Budgeting constraints limited the number of animals

that could be included in the $1,25(OH)_2D_3$ induction of 24-hydroxylase experiment and the vitamin D-binding analysis, and the high cost of $[^3H]1,25(OH)_2D_3$ meant that duplication of the experiment was not possible.

Due to the low lambing percentage in the second year of the breeding trial (Chapter 2) the morphometric data (Chapter 3) were derived from a small number of animals, therefore caution is required in drawing conclusions due to the low statistical power of the study.

At the time of the vitamin D treatment experiment (Chapter 7), only seven affected animals (four in the treated group, three in the non-treated group) were available. Despite this, a significantly greater increase in lumbar vertebrae 1-5 bone mineral content and bone mineral density was present in treated sheep when compared with non-treated sheep. No differences were present in serum calcium and phosphate concentrations, perhaps due to the under-mineralised bone acting as a sink for calcium and phosphate, but due to the small sample size firm conclusions about the effect of vitamin D treatment on serum calcium and phosphate concentrations of Corriedale sheep with inherited rickets could not be made.

Undemineralised bone sections stained with Goldner's trichrome or Von Kossa methods would have been preferable for measurements of osteoid seam thickness reported in Chapter 4, but the cost per section did not allow this. Instead, the Tripp and MacKay method was used and validated by comparison

with selected undemineralised sections stained with Goldner's trichrome and von Kossa's methods.

The serum chemistry study reported in Chapter 5 would have benefited from additional vitamin D assays. Serum was collected from lambs born to the embryo transfer trial, back-cross breeding trial, and the sheep in the treatment trial for this purpose. However, because of the uncertainty of results obtained from commercial radioimmunoassays, it was decided to use the gold standard technique of liquid chromatography-mass spectrometry (LC-MS) for the vitamin D estimation (Lensmeyer *et al.*, 2006; Vogeser *et al.*, 2004). This technique for measurement of serum $25(OH)D_3$ and $1,25(OH)_2D_3$ is not available in New Zealand and samples are being retained until arrangements can be made to have them tested at a specialist laboratory in the United States of America.

9.6 FUTURE STUDIES

The series of studies reported in this thesis should be considered a first step towards characterising this new disease of Corriedale sheep and establishing it as a potential model for investigating vitamin D metabolism. The main priority for the future is to determine the disease mechanism. Results reported in Chapter 6, suggested inappropriate overexpression of 24-hydroxylase as the likely mechanism of the disease. In order to determine that 24-hydroxylase was the only vitamin D-responsive hormone that was inappropriately overexpressed, *in vivo* induction of osteocalcin mRNA by $1,25(OH)_2D_3$ could be examined. If osteocalcin mRNA production in control and affected sheep was

similar, then this would provide further evidence that the defect involves the 24-hydroxylase enzyme. If the expression of osteocalcin mRNA was greater in affected sheep, in a manner similar to 24-hydroxylase mRNA then the defect likely lies elsewhere.

While it appears that 24-hydroxylase mRNA is inappropriately overexpressed, it is unknown whether this translates to a functional enzyme. Therefore, the activity of 24-hydroxylase in control and affected sheep should be investigated. Also, 24-hydroxylated metabolites in the serum should be determined, and if elevated, would suggest that the enzyme is functional. Measurement of 24-hydroxylated metabolites is challenging, therefore reliable measurement of $1,25(OH)_2D_3$ by LC-MS would be necessary, and if normal or decreased may suggest interference of other 1α-hydroxylated metabolites with the measurements reported in Chapter 5.

In addition to induction of osteocalcin mRNA by $1,25(OH)_2D_3$, ovine *24-hydroxylase* could be sequenced. The sequence of control and affected Corriedale sheep could be compared in order to find a possible mutation in the gene. Should a mutation in the *24-hydroxylase* gene be the cause of inherited rickets in Corriedale sheep, then a test for carriers of the gene could be developed and be offered to breeders as part of a control programme. Other agents however (for example, iRNA, co-regulators, transactivators) may interfere with processes between DNA transcription and translation to a functional protein, therefore determining the disease mechanism may be more complicated than finding a mutation in the DNA sequence.

Another fascinating research topic in these sheep would be to investigate vitamin D and calcium transport across the placenta. As discussed in Section 9.3, dystocic lambs born during the embryo transfer trial (Chapter 2) suffered rib fractures, and a fetus had many healed infraction lines in the metaphyses of long bones. This provided convincing evidence that lesions developed *in utero*. If adequate calcium was being transported across the placenta from the normocalcaemic recipient mother, then bone fragility should not have been apparent in the fetus and neonatal lambs. Little research has been performed examining vitamin D and placental calcium transport in sheep, and Corriedale sheep with inherited rickets may be a useful model for elucidating calcium transport mechanisms of the ovine placenta.

Results from pQCT scanning of tibia from Corriedale sheep with inherited rickets showed that the stress-strain index of affected sheep was similar to that of controls. This result was counterintuitive, given that microfractures in the metaphysis were a common finding microscopically. Biomechanical testing of the bones using such methods as the three-point bending test, compression testing, loading tests and torsion testing (Augat *et al*., 1998; Lind *et al*., 2001), may aid in determining the reliability of stress-strain index as a measure of bone strength in these sheep, and indicate whether other forces, in addition to bending, are contributing to loading of the bone and new bone production.

Finally, as part of an investigation into bone strength, imaging of bone collagen would be useful. Collagen quantity, quality and orientation are important

components of the material properties of bone (Ferretti *et al*., 2001) and soluable collagen has been shown to be increased in rachitic bone from chicks (Paterson and Fourman, 1968). In addition, the collagen α1 gene contains a vitamin D-response element (Lichtler *et al*., 1989). Electron microscopy and atomic force microscopy could both be useful for imaging the structure and orientation of collagen in bones from Corriedale sheep with inherited rickets. The amount of collagen tissue, degree of crosslinkage, hydroxyproline to proline ratio (information on ratio of collagenous to non-collagenous proteins) and amount of denatured collagen (damaged, cleaved and non functional) could be determined experimentally in order to assess the quality of the collagen in bones from Corriedale sheep with inherited rickets.

9.7 SUMMARY

Inherited rickets of Corriedale sheep is a newly discovered form of hereditary rachitic disease. Phenotypically, the disease does not appear to be identical to any of the inherited rachitic diseases of humans. The putative cause is inappropriate overexpression of the 24-hydroxylase enzyme responsible for metabolising $1,25(OH)_2D_3$. Corriedale sheep with inherited rickets have considerable potential for biomedical research, including investigating various aspects of vitamin D metabolism and testing new vitamin D treatments. The genetic defect is likely to be widespread in the New Zealand Corriedale sheep population and could be contributing to economic loss for the sheep industry. The development of a test for heterozygous animals would therefore be of benefit in controlling this disease and remains a priority for future research.

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