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Chondrodysplasia of Texel Sheep

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Abstract

Chondrodysplasia of Texel sheep is a newly described recessively inherited disorder distinct from other chondrodysplasias described in sheep. Phenotypically normal at birth, affected lambs develop microscopic lesions as early as 9 days of age, and usually demonstrate gross deformities and markedly reduced rates of bone growth by 2 to 3 weeks. Individual bone growth rates are most severely affected in the proximal bones of the forelimbs. Chondrodysplastic lambs typically have short stature, angular limb deformities, a barrel-shaped chest and a wide-based stance. Gross lesions include tracheal narrowing and contortion, enlarged costochondral junctions, and erosion of articular cartilage in major limb joints. Microscopic lesions are confined to hyaline cartilage, and are characterised by degeneration of the interterritorial matrix and dense perichondrocytic rings consisting predominantly of type VI collagen. These lesions are identical in appearance to those in achondrogenesis 1b and diastrophic dysplasia, two diseases caused by defects of the diastrophic dysplasia sulphate transporter (DTDST) in human beings.

An investigation to measure the uptake of radiolabelled sulphate by dermal fibroblasts *in vitro* did not provide evidence of a defect in the DTDST in chondrodysplastic Texel sheep. A linkage disequilibrium study of ovine chromosomes 1, 5, 6, 13 and 22 using microsatellite DNA markers was unable to identify evidence of a mutation causing this form of chondrodysplasia. Capillary electrophoresis of unsaturated chondroitin sulphate disaccharides demonstrated a relative reduction in the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate in affected animals of all ages. This biochemical feature enables the potential determination of the phenotype of newborn lambs prior to the emergence of gross or microscopic lesions.

The pathology of the disease, combined with the findings of the genetic, biochemical and *in vitro* studies, suggest that a mutation may be present in the *CHST11* gene. This gene is a good candidate for future studies aimed at discovering the genetic defect in chondrodysplasia of Texel sheep and developing a test to identify heterozygous animals.

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Table of Contents

List of Figures....ix

List of Tables....xi

Glossary....xii

1 Introduction, literature review and study objectives....1

1.1 Introduction....2

1.2 Composition and function of cartilage....3

1.2.1 Extracellular structure....3

1.2.2 Sulphation of matrix proteoglycans....7

1.2.3 Endochondral ossification....8

1.2.4 Structure of articular cartilage....9

1.2.5 Structure of physeal cartilage....10

1.2.6 Chondrodysplasias in human beings....13

1.2.7 Defects in extracellular structural proteins....14

Defects of type II collagen....14
Defects of type IX collagen....16
Defects of type X collagen....17
Defects of type XI collagen....17
Defects of cartilage oligomeric matrix protein (COMP)....17
Defects in other extracellular structural proteins....18

1.2.8 Defects in metabolic pathways....18

Defects of the diastrophic dysplasia sulphate transporter (DTDST)....18
Defects in other metabolic pathways....20

1.2.9 Defects in the folding and degradation of macromolecules....21

1.2.10 Defects in hormones and signal transduction mechanisms....22

Fibroblast growth factor receptor 3 (FGFR3)....22
Defects in the PTH/PTHrP receptor....23
Others....24

1.2.11 Defects in nuclear proteins and transcription factors....24

1.2.12 Defects in RNA and DNA processing and metabolism....24

1.2.13 Defects in cytoskeletal elements....25

1.2.14 Chondrodysplasias of unknown aetiology....25

Chondrodysplasia punctata group....26
Short rib polydactyly syndromes....26
Spondylometaphyseal dysplasias....26

1.3 Chondrodysplasias of animals....27

1.3.1 Animals as models for human disease....27

1.3.2 Sheep.....28
"Spider lamb syndrome".....28
Ancon mutant.....29
Lethal dwarfism.....30
Texel chondrodysplasia.....30
South Down dwarfism.....31
Ectrodactyly.....31
1.3.3 Cattle.....32
Brachycephalic "snorter".....32
Dexter bulldog.....33
Complex vertebral malformation (CVM).....34
Others.....34
1.3.4 Dogs.....35
Alaskan malamute chondrodysplasia.....35
Canine GM-1 gangliosidosis.....35
Canine MPS I.....36
Canine MPS VII.....36
English pointer enchondrodystrophy.....36
Miniature poodle pseudoachondroplasia.....37
Oculoskeletal dysplasias.....37
Norwegian Elkhound chondrodysplasia.....38
Great Pyrenees chondrodysplasia.....39
Irish Setter chondrodysplasia.....39
Multiple epiphyseal dysplasia of beagles.....39
Bull terrier osteochondrodysplasia.....40
Others.....40
1.3.5 Cats.....40
Feline MPS VI.....40
Feline MPS VII.....41
Scottish Fold osteochondrodysplasia.....41
Others.....42
1.3.6 Pigs.....42
Dwarfism.....42
Danish Landrace chondrodysplasia.....42
Hereditary dwarfism.....42
1.3.7 Deer.....43
1.3.8 Goats.....43
1.3.9 Mice.....43
Achondroplasia (cn).....43
Brachymorphic mouse.....44
Cartilage matrix deficient (cmd) mice.....44
Others.....44
1.3.10 Rabbits.....44
1.3.11 Birds.....45
1.3.12 Non-human primates.....45

1.4 Nutritional chondrodysplasia....45
1.4.1 Manganese deficiency....45
1.4.2 Plant toxicity....47
1.5 Conclusion and study objectives....47
2 Pathology of chondrodysplasia in Texel sheep....49
2.1 Introduction....50
2.2 Materials and methods....51
2.2.1 Animals used and samples collected....51
2.2.2 Tissue processing and analysis....52
2.2.3 Immunohistochemistry....53
2.2.4 Electron microscopy....53
2.3 Results....54
2.3.1 Clinical signs....54
2.3.2 Gross pathology....57
2.3.3 Histological findings....60
2.3.4 Histomorphometry....66
2.3.5 Immunohistochemistry....68
2.3.6 Electron microscopy....69
2.4 Discussion....71
2.5 Summary....78
3 Morphometric studies....79
3.1 Introduction....80
3.2 Materials and methods....81
3.2.1 Animals used and measurements taken....81
3.2.2 Analysis....83
3.3 Results....85
3.3.1 Overview....85
3.3.2 Whole body growth....86
3.3.3 Thoracic growth....88
3.3.4 Mandibular growth....89
3.3.5 Forelimb bone growth....91
3.3.6 Hindlimb bone growth....93
3.3.7 Allometry....96
Bone lengths in relation to crown-rump length....96
Relative limb bone lengths....97
3.4 Discussion....100

3.5 Summary....103
4 Sulphate uptake by dermal fibroblasts....105
4.1 Introduction....106
4.2 Materials and methods....107
4.2.1 Cell culture....107
4.2.2 Radiolabelled sulphate uptake....108
4.2.3 Protein determination....108
4.2.4 Analysis....109
4.3 Results....109
4.4 Discussion....110
4.5 Summary....111
5 Genetics of Texel chondrodysplasia....113
5.1 Introduction....114
5.2 Materials and methods....116
5.2.1 Animals used....116
5.2.2 DNA extraction....116
5.2.3 Microsatellite selection....116
5.2.4 PCR protocol....118
5.2.5 Analysis....119
5.3 Results....120
5.3.1 Pedigree information....120
5.3.2 Microsatellite scoring....120
5.3.3 Chi-squared analysis....121
5.3.4 Half-sib regression interval mapping....122
5.4 Discussion....122
5.5 Summary....124
6 Sulphation of chondroitin disaccharides in cartilage....125
6.1 Introduction....126
6.2 Materials and methods....127
6.2.1 Animals used and samples collected....127
6.2.2 Chondroitin disaccharide extraction....128
6.2.3 Capillary electrophoresis....128
6.2.4 Analysis....129
6.3 Results....129
6.4 Discussion....131

6.5 Summary....134

7 General discussion....135

7.1 Introduction....136

7.2 Development of lesions....136

7.3 Possible aetiology....139

7.4 Importance of chondrodysplasia in Texel sheep....143

7.4.1 Relevance to industry....143

7.4.2 Biomedical research....144

7.5 Limitations to this study....145

7.6 Future studies....146

7.7 Conclusion....148

Appendix 1 - Defects in extracellular structural proteins....150

Appendix 2 - Defects in metabolic pathways....153

Appendix 3 - Defects in the folding and degradation of macromolecules....156

Appendix 4 - Defects in hormones and signal transduction mechanisms....158

Appendix 5 - Defects in nuclear proteins and transcription factors....160

Appendix 6 - Defects in RNA and DNA processing and metabolism....161

Appendix 7 - Defects in cytoskeletal elements....162

Appendix 8 - Chondrodysplasias of unknown aetiology in humans....163

Appendix 9 - Chondrodysplasias of production animals....166

Appendix 10 - Chondrodysplasias of companion animals....170

Appendix 11 - Chondrodysplasias of laboratory animals....175

Appendix 12 - Chondrodysplasias of birds....180

Appendix 13 - Microsatellite primer sequences....182

Appendix 14 - Pedigree information....183

Appendix 15 - Microsatellite results....184

References....186

List of Figures

Figure 1.1 Microarrangement of major collagens within hyaline cartilage matrix....5

Figure 1.2 Proteoglycan aggregate structure of hyaline cartilage.............................6

Figure 1.3 Sulphate transport and activation in chondrocytes................................8

Figure 1.4 Structure of articular cartilage showing the arrangement of cells and matrix components of the various zones...10

Figure 1.5 The organisation of physeal cartilage showing the arrangement of cells and matrix components of the various zones...12

Figure 1.6 Diagram outlining some of the pathways involved in the regulation of endochondral ossification...13

Figure 1.7 Sulphation of cartilage proteoglycans in control and chondrodysplastic Texel sheep...31

Figure 2.1 Chondrodysplastic Texel lambs...56

Figure 2.2 Chondrodysplasia in Texel sheep...57

Figure 2.3 Trachea from a chondrodysplastic Texel lamb....................................58

Figure 2.4 Thoracic cavities from 3-4-month-old control and chondrodysplastic lambs...59

Figure 2.5 Enlarged costochondral junctions in chondrodysplasia of Texel sheep 59

Figure 2.6 Joint lesions in chondrodysplasia of Texel sheep................................60

Figure 2.7 Hydronephrosis in chondrodysplasia of Texel sheep............................61

Figure 2.8 High-power view of control and chondrodysplastic hyaline cartilage. .62

Figure 2.9 Articular cartilage from the proximal humerus of a 30-day-old chondrodysplastic lamb...63

Figure 2.10 Costochondral junctions of normal and chondrodysplastic lambs 6-7 weeks of age...64

Figure 2.11 Physes of control and month-old chondrodysplastic lambs................65

Figure 2.12 Sagittal sections of 3rd-4th lumbar vertebral bodies and intervertebral disc from 3-4-month-old control and chondrodysplastic Texel lambs. .66

Figure 2.13 Tracheal cartilage from normal and chondrodysplastic lambs...........67

Figure 2.14 Immunohistochemical staining of types II and VI collagen in articular-epiphyseal cartilage from control and chondrodysplastic Texel sheep. .70

Figure 2.15 Articular cartilage chondrocytes and pericellular matrix from control and chondrodysplastic Texel lambs...71

Figure 2.16 High power views of pericellular and interterritorial matrix of cartilage from control and chondrodysplastic Texel lambs..................................71

Figure 3.1 Serial measurements taken from control and chondrodysplastic Texel lambs...84

Figure 3.2 Crown-rump length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals.......................87

Figure 3.3 Shoulder-rump length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals......................88
Figure 3.4 Thoracic width and rate of increase in thoracic width in control and chondrodysplastic Texel lambs at different age intervals......................89
Figure 3.5 Thoracic depth and rate of increase in thoracic depth in control and chondrodysplastic Texel lambs at different age intervals......................90
Figure 3.6 Mandible length and growth rate of control and chondrodysplastic Texel lambs at different age intervals..91
Figure 3.7 Humeral length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals..92
Figure 3.8 Ulnar length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals...93
Figure 3.9 Metacarpal length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals..93
Figure 3.10 Femoral length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals..95
Figure 3.11 Tibial length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals...95
Figure 3.12 Tarsal-metatarsal length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals......................96
Figure 3.13 Relative bone lengths in the forelimb of chondrodysplastic and control Texel lambs at 21-30 days of age..99
Figure 3.14 Relative bone lengths in the hindlimb of chondrodysplastic and control Texel lambs at 31-40 days of age..99
Figure 3.15 The change in relationship between tibial and tarsal-metatarsal length in chondrodysplastic and control Texel lambs...................................100
Figure 3.16 Comparison of lengths of equivalent bones in fore and hind limbs of chondrodysplastic and control Texel lambs..100
Figure 4.1 Fibroblast sulphate uptake in chondrodysplastic and control Texel sheep...111
Figure 5.1 Amplification scheme for fluorescently labelled universal primers.....120
Figure 5.2 Typical chromatogram showing amplified microsatellite sequence....122
Figure 5.3 Chi-squared test results for differences between control and chondrodysplastic Texel sheep in heterozygosity, allele frequency and genotype of microsatellite markers..123
Figure 6.1 Typical electropherogram of chondroitin disaccharides from ovine articular cartilage...131
Figure 6.2 Ratios of Δdi-mono4S to Δdi-mono6S in chondrodysplastic and control Texel sheep of different ages...132

List of Tables

Table 2.1 Chondrocyte density expressed as cells per 400X field in cartilage of chondrodysplastic and control Texel lambs....................................68

Table 2.2 Cartilage thickness of chondrodysplastic and control Texel lambs........69

Table 3.1 ANOVA models fitted for the comparison of lengths of limb bones between control and chondrodysplastic Texel lambs..............................85

Table 3.2 Influences other than chondrodysplasia on body length........................88

Table 3.3 Influences other than chondrodysplasia on thoracic dimensions...........90

Table 3.4 Influences other than chondrodysplasia on mandibular length..............91

Table 3.5 Influences other than chondrodysplasia on forelimb bone length..........94

Table 3.6 Influences other than chondrodysplasia on hindlimb bone length.........97

Table 3.7 Bone length and body dimensions influenced by sex, dam, rank, birthweight or status when crown-rump length is a covariate...............98

Table 5.1 List of microsatellite markers chosen for amplification, together with nearby genes related to cartilage or skeletal development....................118

Table 5.2 Optimal annealing temperatures for microsatellite primers.................119

Table 5.3 Half-sib regression interval mapping results for microsatellite data from chondrodysplastic and control Texel sheep..123

Table 6.1 Concentrations of chondroitin sulphate disaccharides from chondrodysplastic and control Texel sheep of varying ages.................132

Glossary

AB	-	alcian blue, histological stain
Abluminal	-	pertaining to the outer portion of a tubular structure
Acanthosis nigricans	-	a form of skin hyperpigmentation
Achondroplasia	-	specific term for failure of cartilage growth, also commonly used to refer to a common form of dwarfism in humans
Acromesomelia	-	shortening of the middle and distal parts of the limbs
Alymphatic	-	lacking lymphatic vessels
Anauxetic	-	without growth
Aneural	-	lacking innervation
Anisospondyly	-	different abnormal shapes of vertebral bodies
Ankylosis	-	bony fusion of a joint
Anlage	-	an embryonic precursor to a structure
ANOVA	-	analysis of variance
Appositional growth	-	growth by the addition of external layers (c.f. interstitial growth)
APS	-	adenosine phosphosulphate
Arthropathy	-	joint disease
Articular-epiphyseal complex	-	the epiphyseal cartilage of young animals consisting of both an articular surface and a zone of growth
ATP	-	adenosine triphosphate
Avascular	-	lacking blood vessels
Basophilic	-	a tissue that stains with a basic dye, such as haematoxylin
Blepharophimosis	-	abnormally narrow palpebral fissure (gap between eyelids)
BMP	-	bone morphogenic protein
Bossing	-	swelling
Brachycephaly	-	having a short (broad) head
Brachydactyly	-	short fingers
Brachygnathia	-	shortened mandible or jaw
Camptodactyly	-	flexural deformity of interphalangeal joints
Cancellous bone	-	has a latticed structure (c.f. compact bone)
CATSHL syndrome	-	a syndrome featuring camptodactyly, tall stature, scoliosis and hearing loss
CDMP-1	-	cartilage derived morphogenic protein-1
CE	-	capillary electrophoresis
CHILD syndrome	-	congenital hemidysplasia with ichthyosiform erythroderma and limb defects
CI	-	confidence interval
Chondroblasts	-	immature cartilage cells
Chondrocalcin	-	the C-propeptide of type II collagen

Chondrocytes	-	mature cartilage cells
Chondrodysplasia	-	an abnormality of cartilage growth or development
Chondrogenic tissue	-	contains cells with the potential to differentiate into cartilage-forming cells
Chondron	-	the functional unit of cartilage
CHST3	-	the gene encoding chondroitin 6-sulphotransferase, adding sulphate to the 6-position of chondroitin
CHST11	-	the gene encoding chondroitin 4-sulphotransferase, adding sulphate to the 4-position of chondroitin
Cisternae	-	cavities or reservoirs
COMP	-	cartilage oligomeric matrix protein
Compact bone	-	has a dense, laminar structure (c.f. cancellous bone)
Coxa vara	-	a hip deformity where the angle between the ball and shaft of the femur is reduced
cpm	-	counts per minute
Cyanosis	-	bluish colour of skin, mucous membranes, etc. due to lack of oxygenated haemoglobin in the blood
Dentigerous cysts	-	follicular tooth-based cysts
Diaphysis	-	the main shaft of a long bone
Diarthrodial	-	a free-moving form of joint articulation
Distal	-	(as in part of a limb) far from the body
DJD	-	degenerative joint disease
DMC	-	Dyggve-Melchior-Clausen dysplasia
DMEM	-	Dulbecco's modified Eagle's medium
Dolichocephaly	-	having a long head
DTDST	-	diastrophic dysplasia sulphate transporter
Dysmorphism	-	abnormality of shape
Dysplasia	-	abnormality of growth or development
Dyspnoea	-	breathing difficulty
Ectrodactyly	-	absence of one or more digits
Elastic cartilage	-	has elastic fibres and lamellae within the matrix
Endochondral bones	-	bones that grow or develop within cartilage
Epiphysis	-	the end of a long bone separated from the main part of the bone by the physis
Epitope	-	the part of a molecule to which an antibody may bind
Erythroderma	-	reddening of the skin
Erythrogenesis (erythropoiesis)	-	the formation of red blood cells
Exophthalmus	-	abnormal protrusion of the eyeball
FAM	-	6-carboxy-fluorescine
Fenestrated	-	containing one or more openings
FGF	-	fibroblast growth factor
FGF2	-	fibroblast growth factor-2
FGFR3	-	fibroblast growth factor receptor-3

Fibrillogenesis - formation of fibrils

Fibrocartilage - contains bundles of type I collagen within the matrix

Genu valgum - valgus deformity at the knees, "knock-knees"

Glaucoma - increased intraocular pressure

H&E - haematoxylin & eosin, common histological stain

Haploinsufficiency - occurs when a single functional gene is unable to produce enough product to maintain a wild type (normal) phenotype

Hepato-splenomegaly - enlarged liver and spleen

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

Homeobox genes - highly conserved genes that regulate bodily segmentation during embryonic development

HPLC - high performance liquid chromatography

Hyaline cartilage - has a homogeneous, amorphous matrix

Hydrocephalus - dilatation of the cerebral ventricles

Hypertrophy - in chondrocytes is a stage late in maturation with increased cell size

Hypertelorism - widely-spaced eyes

Hypocellular - having decreased cell density

Hypoplasia - incomplete development of an organ or tissue

Hypotonia - decreased tone of skeletal muscles

IBD - identical by descent

Ichthyosiform - resembling scaly skin

Ihh - Indian hedgehog

Inclusion - abnormal aggregation of substance, e.g. within a cell

Interstitial growth - growth by internal expansion, e.g. division of cells already within the tissue (c.f. appositional growth)

Kyphoscoliosis - abnormal curvature of the spine both dorsoventrally and sagittally

Lacuna - the space surrounding chondrocytes caused by an artefact of fixation

Lamellar bone - mature bone with a lamellar arrangement of collagen fibres

LD - linkage disequilibrium

Lordosis - inward curvature of part of the spine

MATN3 - the gene encoding matrilin-3, a protein involved in the homeostasis of cartilage and bone

Megalocephaly - abnormally enlarged head

Membranous bones - growing or developing as a result of direct differentiation of osteoblasts from mesenchyme without a cartilaginous anlage

Mesenchyme - embryonic cells capable of developing into connective tissues or vasculature

Metachromasia - the staining of a tissue a different colour from that of the dye used

Metaphysis - the junction between the physis and diaphysis, containing abundant trabecular bone and a relatively thin cortex

Microdontia - abnormally small teeth

Micrognathia - abnormally small jaw or mandible

Micromelia - abnormally small limbs

Microphthalmia	-	abnormally small eyes
MPS	-	mucopolysaccharidosis
Mydriasis	-	excessive dilation of the pupil of the eye
Myopathy	-	disease of muscle tissue
Myopia	-	short-sightedness
Myotonia	-	increased muscle irritability or spasming
Odontoid hypoplasia	-	underdevelopment of the odontoid process, leading to cervical spine instability
OMIA	-	online mendelian inheritance in animals
OMIM	-	online mendelian inheritance in man
Organogenesis	-	the formation and development of bodily organs
OSMED	-	oto-spondylometa-epiphyseal dysplasia
Ossification	-	the process of bone formation
Osteoblasts	-	immature bone cells
Osteochondro-dysplasia	-	abnormal growth or development of cartilage and bone
Osteocytes	-	mature bone cells
Osteopenia	-	deficiency of bone tissue
Osteophyte	-	a small abnormal bony growth, especially at joint margins
Osteoprogenitors	-	cell with the potential to differentiate into bone-forming cells
Osteosclerosis	-	abnormal hardening of bone
PAP	-	phosphoadenosine-phosphate
PAPS	-	phosphoadenosine-phosphosulphate
PAPSS	-	phosphoadenosine-phosphosulphate synthase
PAS	-	periodic acid-Schiff, a technique used in histology to identify glycogen
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
Pectus carinatum	-	protrusion of the sternum causing "pigeon-breast"
Pectus excavatum	-	retrusion of the sternum causing a "caved-in" chest
Perichondrium	-	dense connective tissue surrounding non-articular cartilage containing an outer fibrous layer and an inner chondrogenic layer
Periosteum	-	dense connective tissue surrounding bone containing an outer fibrous layer and an inner cambium layer containing osteoprogenitor cells
Peroxins	-	peroxisomal assembly proteins
Physis	-	the cartilaginous growth plate in an immature endochondral bone
Platyspondyly	-	having flattened vertebral bodies
Pleomorphic	-	having multiple forms
Polydactyly	-	the presence of more than five digits on hands or feet
Polymorphic	-	having many forms
Primary spongiosa	-	the initial trabecular network in the metaphysis immediately adjacent to the physis consisting of osteoid overlying calcified cartilage
Proximal	-	(as in part of a limb) close to the body
PTH	-	parathyroid hormone
PTHrP	-	parathyroid hormone-related protein

QTL	-	quantitative trait linkage
Rarefaction	-	thinning, becoming less dense
rER	-	rough endoplasmic reticulum
Retinopathy	-	disease of the retina
Rhizomelia	-	abnormally short proximal limb-bones
RHT	-	ruthenium hexammine trichloride
RMRP	-	RNA component of mitochondrial RNA processing endoribonuclease
ROX	-	6-carboxyl-X-rhodamine
SADDAN	-	severe achondroplasia with developmental delay and acanthosis nigricans
Sclerosis	-	hardening
Scoliosis	-	lateral curvature of the spine
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sedlin	-	endoplasmic reticulum protein with unknown function
SLC26A2	-	the gene encoding the DTDST
SNPs	-	single nucleotide polymorphisms
Spondylolisthesis	-	displacement of vertebrae or the vertebral column in relation to vertebrae below
Secondary spongiosa	-	persisting trabeculae of the primary spongiosa that have been remodelled to become lamellar bone
SHOX	-	short-stature homeobox
SLS	-	spider lamb syndrome
SMC	-	Smith-McCort dysplasia
Splanchnocranium	-	the part of the skull connected with the sense organs
Spondylo-	-	involving the spine
Spongiosa	-	cancellous bone consisting of a mesh of trabeculae
STAT	-	signal transducer and activator of transcription
Stenosis	-	abnormal narrowing of a tubular organ or structure
Synostosis	-	fusion of bone
Talipes equinovarus	-	flexural deformity causing "clubbed foot"
TB	-	toluidine blue, a histological stain
TD	-	thanatophoric dwarfism
TDT	-	transmission disequilibrium testing
TGF-β	-	transforming growth-factor-β
Tris	-	tris-(hydroxymethyl)-aminomethane
Valgus	-	abnormal lateral (outward) curvature of a bone or joint
Varus	-	abnormal medial (inward) curvature of a bone or joint
Woven bone	-	immature bone with a random arrangement of collagen fibres
Δdi-mono4S	-	chondroitin 4-sulphate disaccharide
Δdi-mono6S	-	chondroitin 6-sulphate disaccharide

1 Introduction, literature review and study objectives

1.1 Introduction

Skeletal dysplasias are generalised disorders of bone formation (Mundlos & Olsen, 1997). The large number and variety of genetic and environmental diseases with skeletal manifestations reflects the complexity of skeletal formation, growth and homeostasis (Erlebacher *et al.*, 1995). Skeletal dysplasias can be divided into osteodysplasias, involving abnormal bone deposition or reduced bone mineral density, or chondrodysplasias, which are characterised by disruption of the growth and/or development of cartilage (Mundlos & Olsen, 1997). Because most bones in the body develop by endochondral ossification, chondrodysplasias are often characterised by severe skeletal abnormalities (Rimoin *et al.*, 1976; van der Harten *et al.*, 1988; Ala-Kokko *et al.*, 1990), although other tissues may also be affected (Horton & Hecht, 2002b). These abnormalities frequently result in disproportionate dwarfism, while proportionate forms of dwarfism are more usually caused by hormonal disorders such as growth hormone deficiency (Sande & Bingel, 1983). The estimated prevalence of chondrodysplasias in human beings is 1 in 4000 births, and over 100 different clinical phenotypes have been described (Horton & Hecht, 2002b).

The growth and development of endochondral bones is regulated by numerous systems, the characterisation of which is a rapidly expanding field. To this purpose, valuable information has been gained from the study of naturally occurring chondrodysplasias. Techniques in modern molecular biology have become more refined so that some disorders have been characterised on a biochemical and genetic level, along with gross, radiographic and histological levels (Superti-Furga *et al.*, 2001). Where possible, this becomes the basis of our understanding of the pathogenic mechanisms involved. Unfortunately, there are still many diseases in which the genetic defect, or the way in which a gene mutation results in identifiable lesions, is unknown (Rimoin *et al.*, 1998). In an effort to source enough tissue to thoroughly characterise and investigate these rare disorders, animals models are often used. Most typically, these are small transgenic rodents, which have the advantages of relatively cheap maintenance and a short generation interval. In skeletal disorders, however, larger animals with a more similar mass to human beings have been recognised as better models due to their more similar physiology and the comparable effects of weightbearing on the development of lesions

(Barsoum *et al.*, 2000). This may be more important when investigating theraputic options.

A recently identified recessively inherited chondrodysplasia of Texel sheep in New Zealand shows similarities to a group of disorders in human beings (Thompson *et al.*, 2005). This thesis will present a detailed investigation of pathology, biochemistry and genetics in order to explore whether chondrodysplasia in Texel sheep could be a suitable model for some heritable diseases in human beings, and to assist in the development of a genetic test for carrier animals to benefit breeders of Texel sheep in New Zealand.

This literature review will provide an overview of the organisation and regulation of cartilage development and endochondral ossification, describe the major forms of chondrodysplasia in human beings and animals, and conclude with an outline of the course this study will take.

1.2 Composition and function of cartilage

Cartilage is found in many parts of the body, and is involved in multiple functions, such as providing support and flexibility to the airways, pinna and nose, acting as a low-friction shock absorber for joint articulation and enabling elongation of endochondral bones (Hamilton & Mossman, 1972). Three distinct types of cartilage exist: fibrocartilage (forming joint menisci and fibrous joints), elastic cartilage (found in the epiglottis, pinna and nose) and hyaline cartilage (in diarthrodial joints and physes, or growth plates) (Martin *et al.*, 1998). Hyaline cartilage is of most interest in chondrodysplastic disease because of its importance in skeletal development and articulation.

1.2.1 Extracellular structure

Adult hyaline cartilage is normally aneural, alymphatic and avascular (*c.f.* the vascular canals found in fetal or newborn cartilage), consisting of chondrocytes surrounded by an amorphous matrix of collagen and sulphated proteoglycans (Hamilton & Mossman, 1972; Archer *et al.*, 1996; Lefebvre *et al.*, 1997; Martin *et al.*, 1998). Each chondrocyte is surrounded by a pericellular envelope consisting of a thin layer of proteoglycans continuous with the cell membrane (Eggli *et al.*, 1985; Luyten *et al.*, 1997), and in non-superficial layers is surrounded by a thin fibrous capsule (Poole *et al.*, 1987; Enomoto-Iwamoto *et al.*, 1998). This capsular structure

and its contents are referred to as the chondron, which is considered to be the functional unit of cartilage and protects the cell and its microenvironment during physiological compression (Poole *et al.*, 1987; Poole, 1997a; Naski *et al.*, 1998). Owing to its relatively high concentration of sulphated proteoglycans, matrix adjacent to the chondron stains more densely with cationic dyes such as toluidine blue (TB) or azure, compared with the matrix furthest from cells (Ross *et al.*, 1995). The latter areas are known as the territorial and interterritorial matrices, respectively, and stain less intensely with cationic dyes especially as age increases, due to their reduced proteoglycan content (Oohira & Nogami, 1980). Usually seen in microscopic sections, the space between the chondrocyte and the adjacent territorial matrix is an artifact of fixation, but has been referred to variously as the lacuna, moat, halo, corona, or capsule. Fixation methods that preserve proteoglycans, such as those using ruthenium hexammine trichloride, prevent formation of this artifact (Eggli *et al.*, 1985; Grimsrud *et al.*, 1999).

Ultrastructurally, the chondron is surrounded by a “felt-like” fenestrated capsule of fibres with a polar arrangement in articular and physeal cartilage, such that the lateral parts and pole nearest the articular surface are denser than the pole nearest the diaphysis (Poole *et al.*, 1987; Healy *et al.*, 1999). At the pole nearest the diaphysis, chondrocytes have a primary cilium that interacts with matrix components and is required for normal skeletal patterning and matrix synthesis (McGlashan *et al.*, 2007). In the physeal columnar zone, the territorial matrix adjacent to the chondron has an outer layer containing a sheath of tightly packed, longitudinally arranged fibrils, uniting chondrons within each column (Eggli *et al.*, 1985; St-Jacques *et al.*, 1999). These layers are not distinguishable in transverse septae. The remaining matrix consists of proteoglycans and randomly oriented bundles of collagen fibrils, which are generally thicker in the interterritorial matrix (Noonan *et al.*, 1998; DeLise *et al.*, 2000). Mineralisation, where it occurs, is restricted to interterritorial matrix (Eggli *et al.*, 1985).

Collagen accounts for approximately two thirds of the dry weight of cartilage and is responsible for the material strength of the tissue (Ferguson *et al.*, 2000; Eyre, 2002). The collagen is predominantly type II interacting with types IX and XI (Kühn, 1987; van der Eerden *et al.*, 2000; D'Angelo *et al.*, 2001), although several other collagen types also have vital functions in cartilage (Eyre & Wu, 1987; Olsen, 1996; Grimsrud *et al.*, 2001; Asamura *et al.*, 2005). The microarrangement of the major types of collagen in hyaline cartilage matrix is illustrated in Figure 1.1. Type

II collagen exists in a continuous triple helix, typically of a smaller diameter and more highly glycosylated than type I collagen, and without the tendency to form parallel bundles (Kühn, 1987; Huang *et al.*, 2001). The C-propeptide of type II collagen is known as chondrocalcin (Alini *et al.*, 1992). Type IX collagen is covalently linked to the surface of type II collagen fibrils (Eyre, 2002; Tuan, 2003; Yang *et al.*, 2003) and is involved in the limitation of type II collagen fibril diameter (Eyre & Wu, 1987; Akiyama *et al.*, 2004; Ferguson *et al.*, 2004; Seki & Hata, 2004; Asamura *et al.*, 2005) in a concentration-dependent manner. Type XI collagen is intimately co-polymerised with type II collagen (Eyre, 2002). Type X collagen is synthesised predominantly by hypertrophic chondrocytes at sites of endochondral ossification and is more rapidly degraded by collagenase than type II collagen (Schmid & Linsenmayer, 1987; Kobayashi *et al.*, 2005). In hyaline cartilage, type I collagen is expressed only by the most superficial articular chondrocytes (Sasano *et al.*, 1996; Minina *et al.*, 2002). Type III collagen is a minor but consistent component of hyaline cartilage, linked to type II collagen (Wu *et al.*, 1996; Li *et al.*, 2003). Type VI collagen has a relatively short triple-helical domain, and is virtually ubiquitous in extracellular matrices, including cartilage (Timpl & Engel, 1987). Both type VI and IX collagens are concentrated in the pericellular matrix (Poole *et al.*, 1988b; Poole *et al.*, 1992; Poole *et al.*, 1997b; Hilton *et al.*, 2005b). The functions of collagen types XII and XIV, also found within cartilage, are currently unknown (Eyre, 2002). The *SOX9* gene is involved in the expression of collagen types II, IX, XI and the core proteoglycan, aggrecan (Bi *et al.*, 1999).

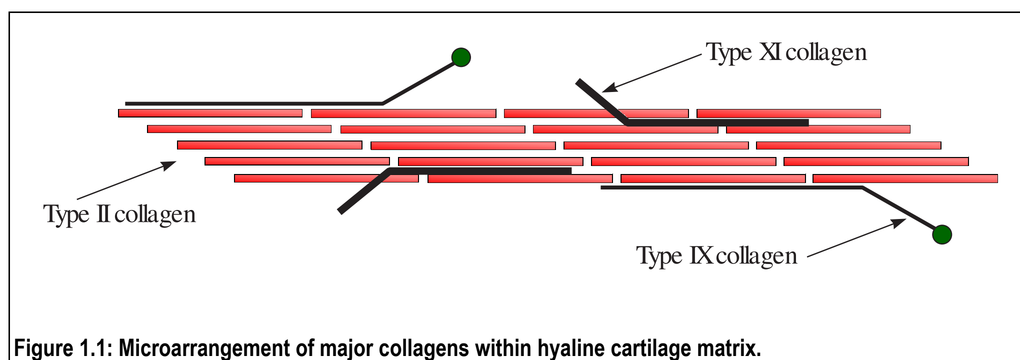


Figure 1.1: Microarrangement of major collagens within hyaline cartilage matrix.

The proteoglycan component of hyaline cartilage matrix comprises sulphated glycosaminoglycans (chondroitin 4-sulphate, chondroitin 6-sulphate, and keratan sulphate) attached in a bottle-brush fashion to a central core protein, aggrecan, which forms large macromolecular aggregates with hyaluronan, assisted by link

protein (Sandy *et al.*, 1997; Martin *et al.*, 1998). This arrangement is shown in Figure 1.2. Hyaluronan is a large linear proteoglycan that may reach a molecular mass of $4x10^6$Da, featuring hydrophilic and hydrophobic faces (Hascall & Laurent, 1997). Link protein is thought to be required for formation of proteoglycan aggregates and organisation of hypertrophic chondrocytes (Watanabe & Yamada, 1999). Decorin, biglycan and fibromodulin are small proteoglycans that influence cell adhesion and fibrillogenesis, appearing to regulate processes involved in connective tissue repair (Sandy *et al.*, 1997). All three small proteoglycans have been located in the articular surface of mature cartilage, but decorin has also been found in the interterritorial matrix and biglycan in the pericellular matrix of both immature and adult cartilage (Archer *et al.*, 1996). Cartilage perlecan is a regulator of endochondral bone growth (SundarRaj *et al.*, 1995; Hassell *et al.*, 2002), and the chondroitin sulphate in perlecan has been shown to bind to collagen fibrils, accelerating fibril formation (Kvist *et al.*, 2006). Heparan sulphate in cartilage is mostly cell-associated, and is involved in ligand-receptor stabilisation in the fibroblast growth factor (FGF) signalling pathway (Tyree *et al.*, 1986).

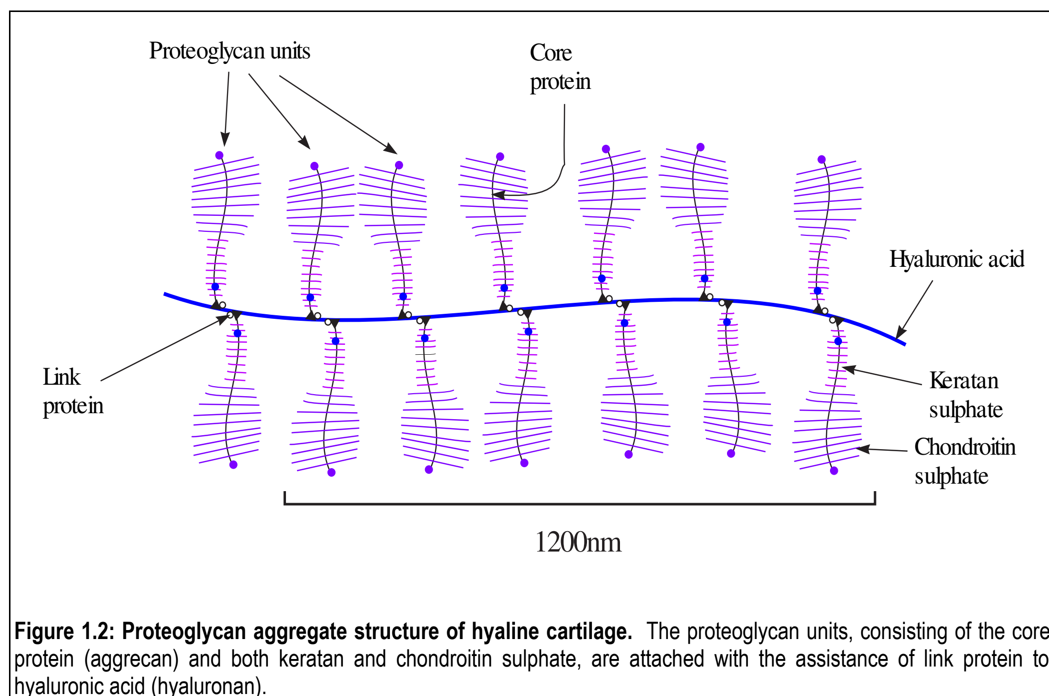


Figure 1.2: Proteoglycan aggregate structure of hyaline cartilage. The proteoglycan units, consisting of the core protein (aggrecan) and both keratan and chondroitin sulphate, are attached with the assistance of link protein to hyaluronic acid (hyaluronan).

The matrilin family consists of 4 proteins that are believed to connect extracellular matrix components such as types II & VI collagen and aggrecan in conjunction with decorin and biglycan to form macromolecular networks (Winterbottom *et al.*, 1992;

Hauser *et al.*, 1996; Wiberg *et al.*, 2003). Matrilin-1 (cartilage matrix protein) and matrilin-3 are plentiful only in cartilage, while matrilin-2 and matrilin-4 can be found in a wider range of tissues (Kleemann-Fischer *et al.*, 2001). Matrilin-3 appears to be required for the appropriate timing of chondrocyte differentiation (van der Weyden *et al.*, 2006). Matrilin-3 is mainly expressed in the proliferative and maturation zones in physeal cartilage (Zhang & Chen, 2000), and can form a heterotetramer with matrilin-1 (Wu & Eyre, 1998).

1.2.2 Sulphation of matrix proteoglycans

The highly sulphated nature of cartilage matrix creates a strong negative charge density, resulting in the accumulation of positive ions (mainly H+ and Na+) and the retention of water within the matrix. The expansive action of the water acting against type II collagen fibrils results in the useful mechanical characteristics of hyaline cartilage, enabling reversible deformation and resilience to compressive forces (Sandy *et al.*, 1997). When cartilage loses this compressive stiffness, as occurs normally with age, degenerative joint disease ranging from asymptomatic lesions to severe crippling or even fusion of joints may occur (Olsen *et al.*, 2000).

In fibroblasts and chondrocytes, sulphate ions are taken up by an anion exchange mechanism called the diastrophic dysplasia sulphate transporter (DTDST), in which intracellular chloride ions are exchanged for extracellular sulphate ions (Vincourt *et al.*, 2003). Chondrocytes are mostly dependent on extracellular sulphate for proteoglycan sulphation (Ito *et al.*, 1982).

In animals, cytoplasmic sulphate is activated by a single bifunctional enzyme called phosphoadenosine-phosphosulphate synthase (PAPSS) (Superti-Furga, 2001). The first step uses the enzyme's adenosine triphosphate (ATP) sulphurylase activity to synthesise adenosine-phosphosulphate (APS) from sulphate and ATP. The second step in sulphate activation uses APS kinase activity to synthesise phosphoadenosine-phosphosulphate (PAPS) from APS and ATP (Schwartz *et al.*, 1998). PAPS is the sulphate donor for most sulphation reactions in the cytoplasm, and a PAP/PAPS antiporter is required to transport PAPS to the golgi or endoplasmic reticulum prior to the sulphation of secreted or membrane-bound products (Ozeran *et al.*, 1996; Schwartz *et al.*, 1998). This process is summarised in Figure 1.3.

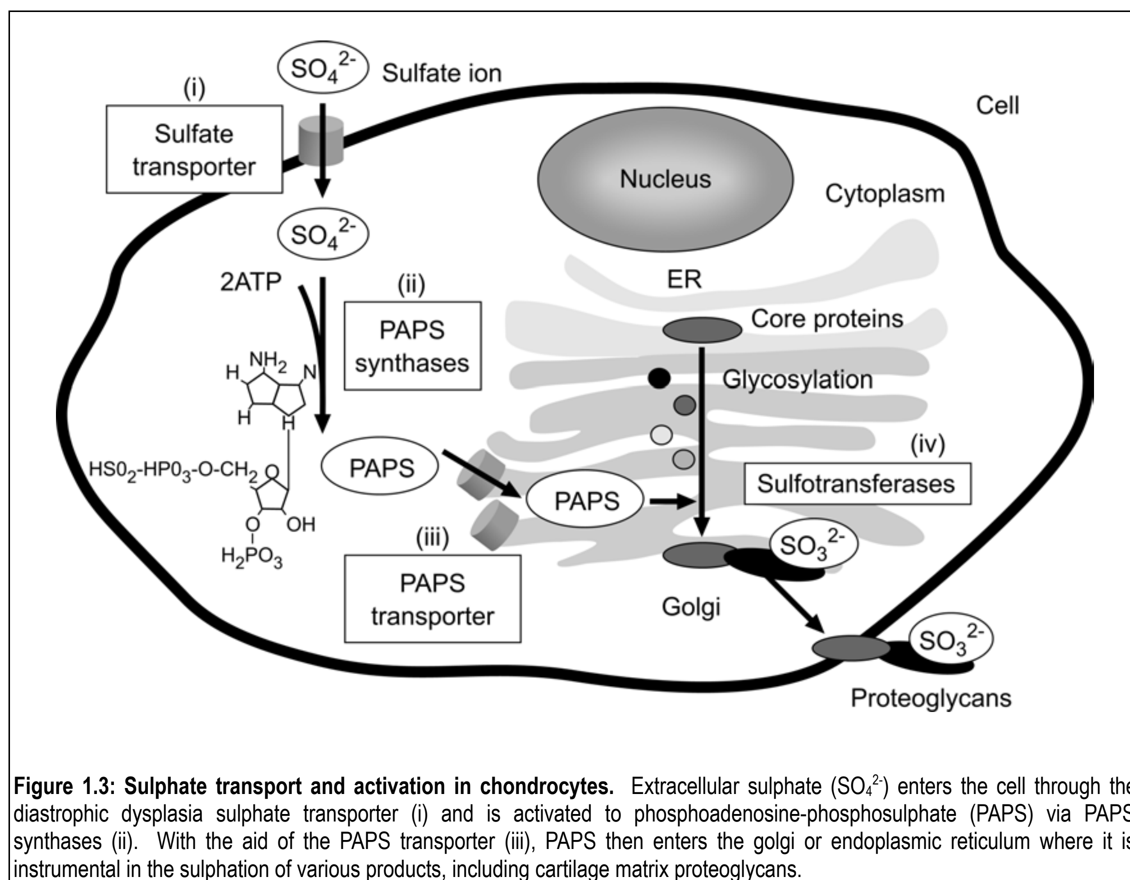


Figure 1.3: Sulphate transport and activation in chondrocytes. Extracellular sulphate (SO_4^{2-}) enters the cell through the diastrophic dysplasia sulphate transporter (i) and is activated to phosphoadenosine-phosphosulphate (PAPS) via PAPS synthases (ii). With the aid of the PAPS transporter (iii), PAPS then enters the golgi or endoplasmic reticulum where it is instrumental in the sulphation of various products, including cartilage matrix proteoglycans.

1.2.3 Endochondral ossification

Abnormalities of hyaline cartilage may affect the formation of endochondral bones including limb bones, ribs, vertebrae and parts of the skull. These bones begin development as crude cartilaginous anlagen in the embryo, as opposed to membranous bones (such as the flat bones of the skull and pelvis) which form by the direct differentiation of osteoblasts from condensations of embryonic mesenchymal cells (Olsen *et al.*, 2000). Membranous bone formation is usually unaffected by abnormalities in cartilage development.

The process of endochondral ossification begins with the condensation of embryonic mesenchymal cells and their differentiation into chondroblasts, which form the cartilaginous anlage (Erlebacher *et al.*, 1995). Pattern formation and organogenesis during embryo development are regulated by homeobox genes, which encode a large family of transcription factors (Gehring *et al.*, 1994).

While the cartilage anlage increases in size due to both interstitial and appositional growth, the diaphyseal chondrocytes differentiate into hypertrophic chondrocytes

with mineralisation of the surrounding matrix, and osteoprogenitor cells from the vascular perichondrium growing over the diaphysis differentiate into osteoblasts that produce a bony collar surrounded by periosteum (Olsen *et al.*, 2000). The perichondrium is continuous with the periosteum, with the exception of the articular surfaces and cartilage-bone interfaces, and consists of an outer fibrous layer and an inner chondrogenic layer. This inner layer blends with the subperichondrial cartilage. A vascular bud from the periosteum penetrates both the bony collar and the mineralising cartilage in the centre of what will become the diaphysis, admitting septoclasts (chondroclasts) and osteoblasts. Septoclasts are cathepsin-B-synthesising mononuclear cells associated with the growing portion of invasive capillaries (Lee *et al.*, 1995). The septoclasts break down the mineralised cartilage matrix, causing lacunae to coalesce into primary areolae, which are rapidly occupied by osteogenic tissue. The remaining spicules of mineralised cartilage become lined by osteoblasts, which deposit osteoid. This is later mineralised to form the primitive cancellous bone of the primary ossification centre, which is then remodelled by the combined action of osteoclasts and osteoblasts (Hamilton & Mossman, 1972). In a similar manner, secondary centres of ossification are established in the epiphyses at the bone ends, leaving growth plates, or physes, which allow continued longitudinal bone growth (Olsen *et al.*, 2000). When bones reach their mature length the physes are no longer required and "close" by ossification. Remodelling of this synostosis eventually leads to continuity between the epiphysis and the metaphysis (Martin *et al.*, 1998).

The following sections describe the architecture of the remaining articular cartilage and the physis prior to ossification.

1.2.4 Structure of articular cartilage

The cellular and fibrous arrangement of articular cartilage is highly organised, as illustrated in Figure 1.4. Directly adjacent to the articular surface lies the acellular lamina splendens, comprised of a superficial afibrillar layer and a deeper fibrillar layer (Kumar *et al.*, 2001). Beneath this lies a tangential zone with collagen fibrils surrounding flattened chondrocytes with their long axes parallel to the articular surface. Deeper to this region is a transitional zone where the chondrocytes are rounded and seemingly randomly organised. Collagen fibrils in this zone are larger and angled obliquely. Below this is a radial zone in which cells are aligned in short, irregular columns with parallel collagen fibres, and a calcified zone rich in

hydoxyapatite (Mankin & Radin, 1997; Martin *et al.*, 1998). The junction between the calcified and non-calcified matrix is visible histologically in sections stained with haematoxylin and eosin (H&E) as an undulating basophilic line, referred to as the "tidemark". This zone of calcified cartilage is firmly anchored to the subchondral bone.

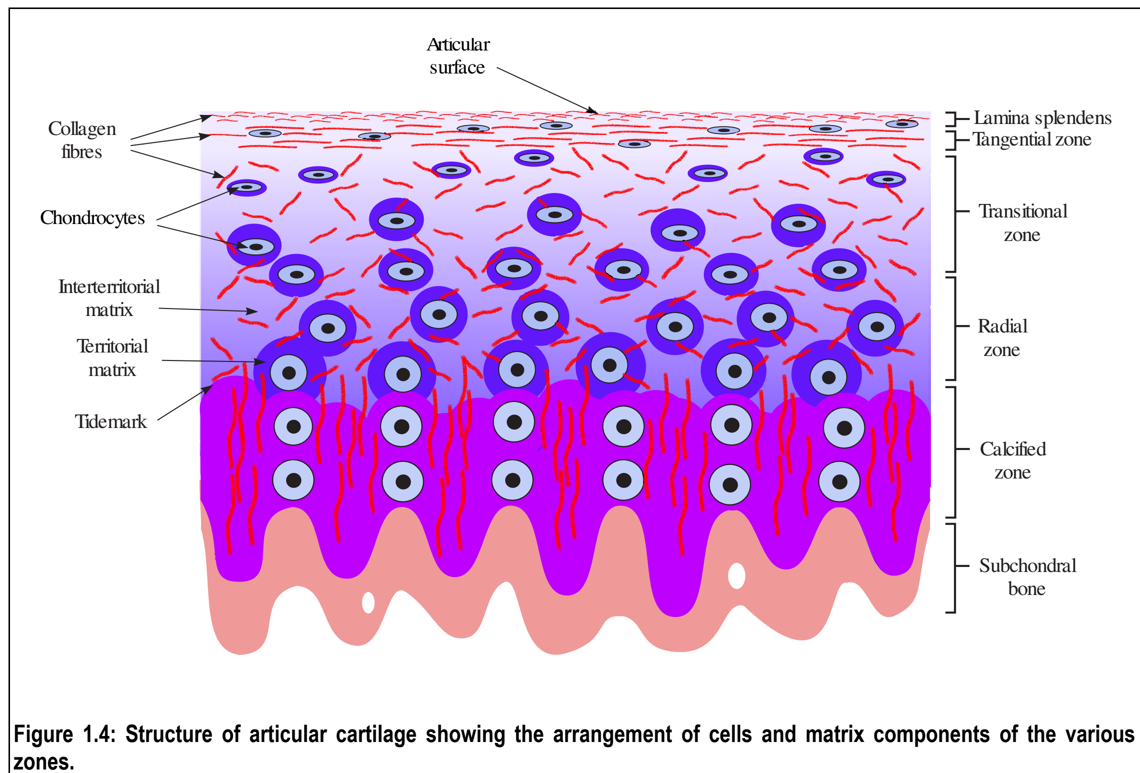


Figure 1.4: Structure of articular cartilage showing the arrangement of cells and matrix components of the various zones.

1.2.5 Structure of physeal cartilage

Physeal cartilage consists of several distinct zones (Ballock & O'Keefe, 2003), as shown in Figure 1.5. Closest to the joint space and anchored to the epiphysis is a resting zone consisting of moderately sized, slowly dividing chondrocytes scattered in a random manner (Martin *et al.*, 1998). In this zone there is a relatively high ratio of extracellular matrix to cell volume (Ballock & O'Keefe, 2003). Adjacent is a proliferative zone, in which chondrocytes divide rapidly to form columns of disk-like cells (Martin *et al.*, 1998). This columnar arrangement is more obvious in the physes of fast growing bones, while slower growing bones tend to have cells arranged in elongated clusters (Morris *et al.*, 2002). Fibroblast growth factor-2

(FGF2) promotes chondrocyte proliferation and blocks further differentiation (Weksler *et al.*, 1999).

Following cell division in the proliferative zone, chondrocytic enlargement begins (Breur *et al.*, 1994), leading to the prehypertrophic (or maturational) stage, during which chondrocytes actively secrete and become separated by matrix. This leads into the hypertrophic zone where the chondrocytes are large and spherical with increased rough endoplasmic reticulum (rER) and Golgi apparatus (Farquharson & Jefferies, 2000). Associated with the hypertrophic phenotype are a range of metabolic alterations, including increased activity of plasma membrane alkaline phosphatase, synthesis of type X collagen, accumulation of glycogen (Martin *et al.*, 1998), down-regulation of type II collagen, secretion of osteonectin and osteopontin, expression of vitamin D receptors (Farquharson & Jefferies, 2000), and secretion of matrix vesicles that act as niduses for mineralisation (Ballock & O'Keefe, 2003). Matrix vesicles are anchored to the collagenous matrix by the collagen binding protein anchorin CII, which also binds collagen types II and X (Kirsch & Pfäffle, 1992). The zone of provisional mineralisation consists of degenerating hypertrophic chondrocytes surrounded by calcified matrix (Ballock & O'Keefe, 2003). The hypertrophic chondrocytes dissolve some of their surrounding matrix and the cartilage septum remaining at the bottom of each chondrocyte column is resorbed by septoclasts (Martin *et al.*, 1998). For any single chondrocyte, this series of developmental events takes place in one site over time, but due to bone growth these temporal events are represented spatially in the physis (Morris *et al.*, 2002).

Capillary loops from the metaphysis enter the cavities left by dead hypertrophic chondrocytes. Most remaining transverse septae of mineralised matrix are actively resorbed by septoclasts (Ballock & O'Keefe, 2003). Osteoblasts deposit a layer of osteoid which is then mineralised to form bone on the remaining trabeculae, creating the primary spongiosa (Martin *et al.*, 1998). As the layer of bone tissue becomes thicker, the trabculae of the primary spongiosa are gradually remodelled and realigned, forming the secondary spongiosa (Ballock & O'Keefe, 2003). Continued cell division at the circumference of the physis, the zone of Ranvier, allows the physis to increase in diameter as the bone grows in length (Martin *et al.*, 1998).

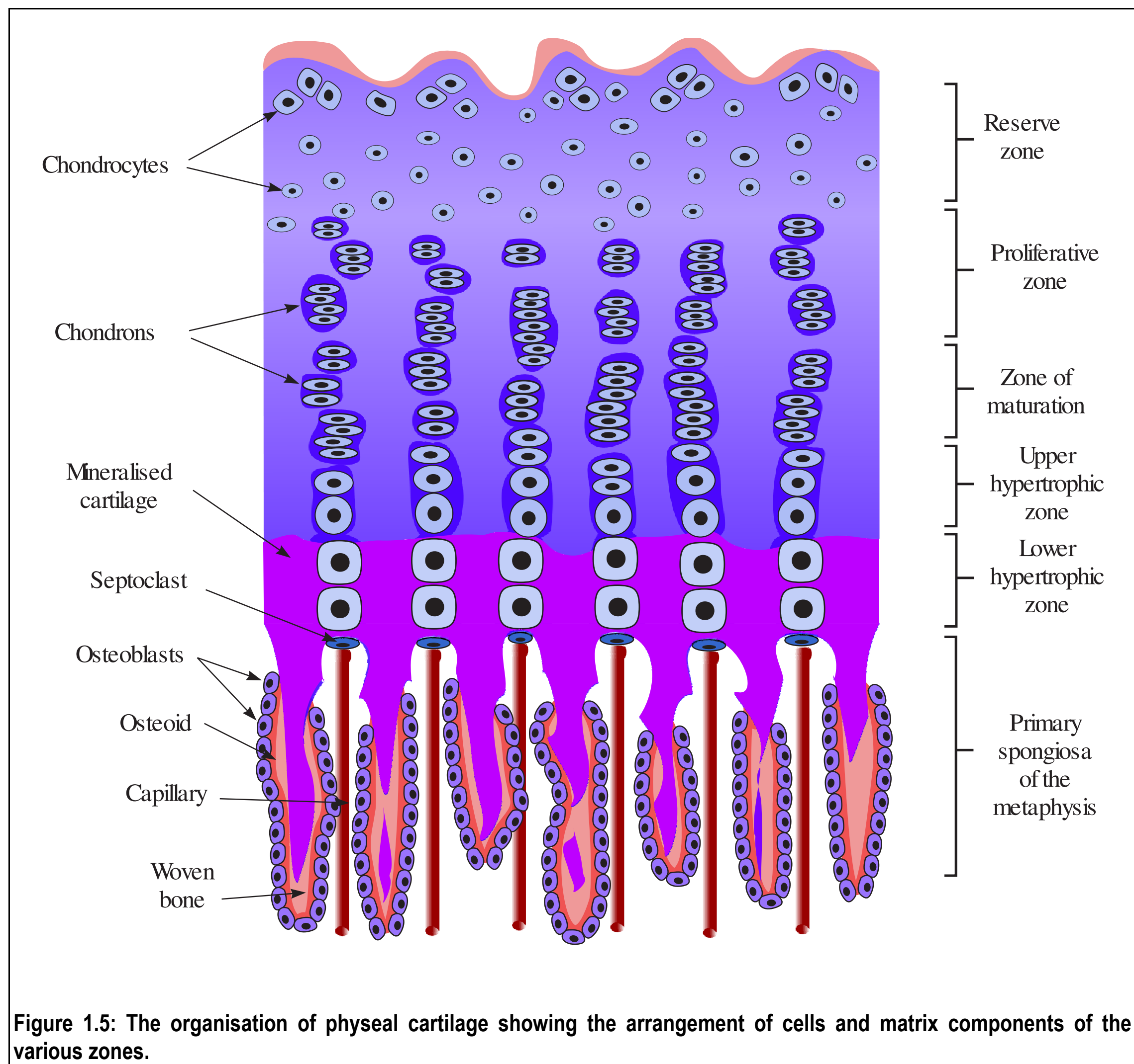


Figure 1.5: The organisation of physeal cartilage showing the arrangement of cells and matrix components of the various zones.

Many local and systemic growth factors are involved in the regulation of endochondral bone growth (Weksler *et al.* 1999), including bone morphogenic proteins (BMPs), FGFs, the Wnt glycoproteins, Indian hedgehog (Ihh)/parathyroid related protein (PTHrP) signalling, cell adhesion proteins and extracellular matrix molecules (Tuan, 2003). The method by which these factors interact to coordinate and regulate endochondral ossification is only partially understood. An outline of some of these pathways is shown in Figure 1.6.

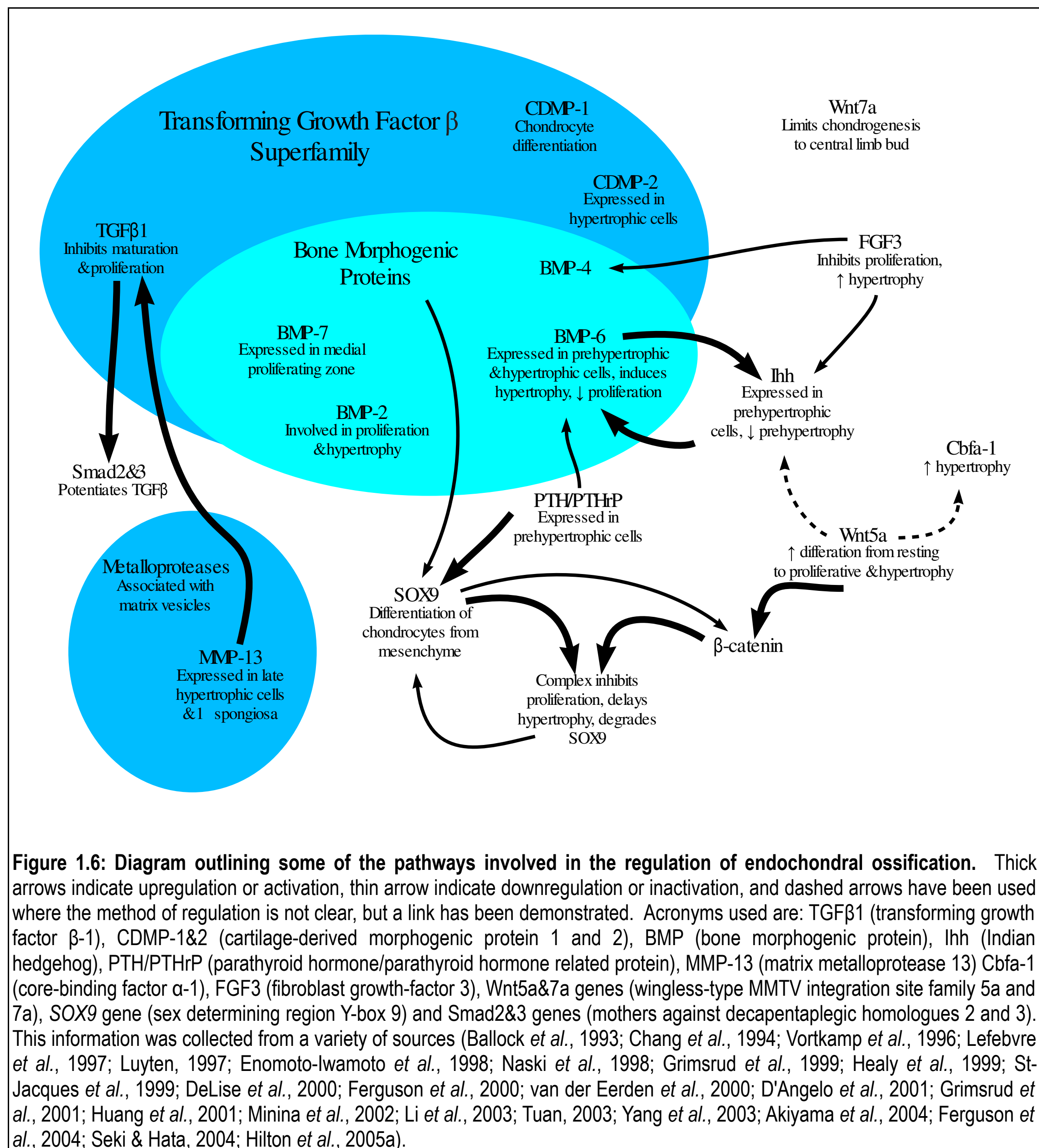


Figure 1.6: Diagram outlining some of the pathways involved in the regulation of endochondral ossification. Thick arrows indicate upregulation or activation, thin arrow indicate downregulation or inactivation, and dashed arrows have been used where the method of regulation is not clear, but a link has been demonstrated. Acronyms used are: TGFβ1 (transforming growth factor β-1), CDMP-1&2 (cartilage-derived morphogenic protein 1 and 2), BMP (bone morphogenic protein), Ihh (Indian hedgehog), PTH/PTHrP (parathyroid hormone/parathyroid hormone related protein), MMP-13 (matrix metalloprotease 13) Cbfa-1 (core-binding factor α-1), FGF3 (fibroblast growth-factor 3), Wnt5a&7a genes (wingless-type MMTV integration site family 5a and 7a), *SOX9* gene (sex determining region Y-box 9) and Smad2&3 genes (mothers against decapentaplegic homologues 2 and 3). This information was collected from a variety of sources (Ballock *et al.*, 1993; Chang *et al.*, 1994; Vortkamp *et al.*, 1996; Lefebvre *et al.*, 1997; Luyten, 1997; Enomoto-Iwamoto *et al.*, 1998; Naski *et al.*, 1998; Grimsrud *et al.*, 1999; Healy *et al.*, 1999; St-Jacques *et al.*, 1999; DeLise *et al.*, 2000; Ferguson *et al.*, 2000; van der Eerden *et al.*, 2000; D'Angelo *et al.*, 2001; Grimsrud *et al.*, 2001; Huang *et al.*, 2001; Minina *et al.*, 2002; Li *et al.*, 2003; Tuan, 2003; Yang *et al.*, 2003; Akiyama *et al.*, 2004; Ferguson *et al.*, 2004; Seki & Hata, 2004; Hilton *et al.*, 2005a).

1.2.6 Chondrodysplasias in human beings

The original classification of skeletal dysplasias in human beings consisted of two groups – short trunked ("Morquio syndrome") or short limbed ("achondroplasia") (Unger, 2002). As knowledge improved, disorders of the skeletal system came to be classified primarily by their clinical and radiographic features. Because some chondrodysplasias caused by mutations in different genes appeared to be the same disease (Superti-Furga *et al.*, 2001), many diseases are now known by more than one name (Hall, 1978; Rimoin *et al.*, 1998). As diagnostic tools have become more advanced, especially with respect to the identification of causative gene mutations,

these older classification systems have been superceded. A recently developed molecular-pathogenic classification system divides skeletal diseases into the following groups: defects in extracellular structural proteins, defects in metabolic pathways (including enzymes, ion channels and transporters), defects in the folding and degradation of macromolecules, defects in hormones and signal transduction mechanisms, defects in nuclear proteins and transcription factors, defects in oncogenes and tumour suppressor genes, and defects in RNA and DNA processing and metabolism (Superti-Furga *et al.*, 2001). Since the publication of this classification system, some chondrodysplasias of previously unknown aetiology have been found to be caused by abnormalities in cytoskeletal elements (Krakow *et al.*, 2004; Beales *et al.*, 2007). Consequently, defects in cytoskeletal elements will be included as a separate group for the purpose of this review. With the exception of defects in oncogenes and tumor suppressor genes, all of these groups contain at least one chondrodysplastic disease.

The following sections of this review focus on chondrodysplasias of human beings and animals. As chondrodysplasias affecting human beings tend to be better described on a molecular-pathogenic level, these diseases will be organised according to the classification system described by Superti-Furga *et al.,* (2001). Chondrodysplasias affecting animals have been organised by species.

1.2.7 Defects in extracellular structural proteins

As discussed above, a variety of extracellular proteins comprise the matrix responsible for the structural integrity of cartilage tissue, and are vital for normal growth and development of all endochondral bones. Defects in any of these components can potentially result in chondrodysplasia.

Defects of type II collagen

Genetic disorders of type II collagen are inherited in an autosomal dominant manner (Hall, 2002), acting through a dominant negative mechanism. Because type II collagen exists as a triple helix composed of three procollagen α chains, all derived from the *COL2A1* gene, the formation of triple helices from a 1:1 mixed population of normal and mutant procollagen chains will result in $^{7}/_{8}$ of collagen molecules containing at least one mutant component (Horton & Hecht, 2002b). Abnormal procollagen may interrupt formation of the triple helix structure (Chan *et al.*, 1995a), leading to reduced stability of the molecule (Bonaventure *et al.*,

1995). This may occur through delayed or atypical folding of the collagen molecule, which can result in excessive post-translational modification, degradation, reduced secretion, and/or accumulation of the product in the rER (Horton & Hecht, 2002a). A reduced number of collagen fibrils would alter the mechanical properties of cartilage, and the interaction of abnormal fibrils with the cell surface may potentially interfere with the diffusion, sequestration and presentation of growth factors to chondrocytes (Horton & Hecht, 2002a).

The genetic disorders of type II collagen in human beings include achondrogenesis type II, Kniest dysplasia, Torrance type lethal platyspondylic skeletal dysplasia (Nishimura *et al.*, 2004; Zankl *et al.*, 2005), spondyloepiphyseal dysplasia congenita (Lee *et al.*, 1989), spondyloepimetaphyseal dysplasia Strudwick type (Tiller *et al.*, 1995), Stickler syndrome type 1 (Williams *et al.*, 1996) and spondyloperipheral dysplasia (Zabel *et al.*, 1996). Achondrogenesis type II and Kniest dysplasia are described below; the remaining defects of type II collagen are listed in Appendix 1. Along with skeletal deformities, type II collagenopathies are often accompanied by ocular disturbances, such as myopia, abnormal vitreous humor and retinal tearing or detachment (Meredith *et al.*, 2007).

Achondrogenesis type II and hypochondrogenesis are caused by mutations in the α1(II) chain of type II procollagen (Bonaventure *et al.*, 1995) and are now considered to be at different ends of a continuous disease spectrum, involving 1 in 40,000-60,000 births (Borochowitz *et al.*, 1986).

Achondrogenesis type II is a lethal chondrodysplasia characterised by short, bowed, long bones with flared and “cupped” metaphyses evident on radiographs, appearing like capless “mushroom stems” (van der Harten *et al.*, 1988). Other radiographic lesions include variable ossification of the spine, axe-head-shaped iliac bones accompanied by unossified ischial and pubic bones, and a hypoplastic, barrel- or bell-shaped thorax (Sillence *et al.*, 1979). Histologically, epiphyseal chondrocytes are densely packed in a reduced extracellular matrix containing numerous cartilage canals with perivascular fibrosis (van der Harten *et al.*, 1988; Gruber *et al.*, 1990). The physis is generally disorganised and hypertrophic chondrocytes extend into the primary and secondary spongiosa, which contain plump and disordered trabeculae. Milder forms have a more organised physeal architecture. Cartilage in affected individuals contains type I collagen rather than type II (Eyre *et al.*, 1986).

Hypochondrogenesis histologically resembles achondrogenesis type II with slightly improved organisation of the hypertrophic zone, but has radiographic features more similar to those of spondylometaphyseal dysplasia (van der Harten *et al.*, 1988). Spondylometaphyseal dysplasia comprises a heterogeneous group of heritable skeletal dysplasias characterised by modifications of the vertebral bodies of the spine and metaphyses of tubular bones (Borochowitz *et al.*, 1988a).

Kniest dysplasia is a relatively uncommon non-lethal disorder characterised by a short trunk and limbs, progressive joint enlargement and a flattened face (Hicks *et al.*, 2001). Cleft palate, tracheal collapse, recurrent otitis media, and severe myopia are variably present (Maumenee & Traboulsi, 1985). Radiographic abnormalities include broad metaphyses, enlarged epiphyses, platyspondyly, progressive kyphoscoliosis and lumbar lordosis (Hicks *et al.*, 2001). Histologically, intracytoplasmic inclusions are present within chondrocytes and there is degeneration of cartilage matrix (Horton & Rimoin, 1979). Abnormally thin, irregular collagen fibrils are visible ultrastructurally, and the epiphyseal extracellular matrix lacks chondrocalcin (Poole *et al.*, 1988a).

Defects of type IX collagen

Multiple epiphyseal dysplasia affects at least 1 in 10,000 people (McKeand *et al.*, 1996) and is caused by mutations in *COL9A1* (Czarny-Ratajczak *et al.*, 2001), *COMP* (Maddox *et al.*, 2000) or *MATN3* (Mäkitie *et al.*, 2004a). This disease has an autosomal dominant mode of inheritance and is characterised by early onset osteoarthritis, a waddling gait, variably short stature, mild distal shortening of the ulnas, and irregular ossification of carpal and tarsal bones (Bönnemann *et al.*, 2000). Stippled calcification of epiphyses is identifiable radiologically at birth, and may either occur in all endochondral bones or be limited to the tarsus (Rasmussen & Reimann, 1973). Joint crepitation, knee joint laxity, scoliosis, subchondral sclerosis, flattened femoral heads, valgus deformity, short femoral necks, flattened and irregular epiphyses of the knees, and bipartate, chondromalacic patellae may also be present (Czarny-Ratajczak *et al.*, 2001).

Intracytoplasmic inclusions are present in chondrocytes, which are surrounded by a slightly fibrillar matrix (Czarny-Ratajczak *et al.*, 2001). Ultrastructurally, the chondrocyte rER is markedly dilated, and contains linear arrays of alternating electron-dense and electron-lucent material. Lesions are more severe in epiphyseal than in physeal cartilage (Czarny-Ratajczak *et al.*, 2001).

Mutations in type IX collagen have also been associated with intervertebral disk disease (Annunen *et al.*, 1999b).

Defects of type X collagen

Schmid metaphyseal chondrodysplasia, the most common type of metaphyseal chondrodysplasia (Lachman *et al.*, 1988), has an autosomal dominant mode of inheritance (Ikegawa *et al.*, 1997). This disease is caused by the synthesis of abnormal procollagen X that cannot be incorporated into mature helical molecules (Chan *et al.*, 1995b), resulting in haploinsufficiency (Chan *et al.*, 1998). The resultant phenotype includes disproportionate dwarfism with bowed legs in early childhood, coxa vara (Bateman *et al.*, 2004), and lumbar lordosis with an anterior pelvic tilt (Chan *et al.*, 1998). Radiology reveals widened, sclerotic and irregular metaphyses (Bateman *et al.*, 2003). Histologically, there is an absence of identifiable hypertrophic chondrocytes and disorganisation of physeal chondrocyte columns (Chan *et al.*, 1995b).

Defects of type XI collagen

Stickler syndrome type II, Marshall syndrome, and both dominant and recessive forms of oto-spondylo-megaepiphyseal dysplasia (OSMED) are caused by mutations in collagen type XI, and feature midface hypoplasia, cleft palate, micrognathia, hearing loss, arthropathy, mild epiphyseal dysplasia, and wide metaphyses (Spranger, 1998). The lesions are evident at birth, but alter with age. See Appendix 1 for further details.

Defects of cartilage oligomeric matrix protein (COMP)

Pseudoachondroplasia and multiple epiphyseal dysplasia are two common chondrodysplasias associated with mutations in *COMP* (Ikegawa *et al.*, 1998). Such mutations in *COMP* can reduce the calcium binding properties of the protein, resulting in an altered conformation (Chen *et al.*, 2000).

Pseudoachondroplasia is an autosomal dominant form of disproportionate dwarfism with joint laxity and moderate brachydactyly, usually appearing at the onset of walking, with a waddling gait and debilitating hip pain in childhood (McKeand *et al.*, 1996; Maddox *et al.*, 2000). A mild form has also been recognised (Manabe *et al.*, 1998). Radiographically, there are small, irregular epiphyses,

delayed ossification, flared metaphyses and anterior beaking of vertebral bodies (Wynne-Davies *et al.*, 1986).

Histologically, prehypertrophic and hypertrophic zones of affected cartilage are disorganised and hypocellular, with many dead cells throughout the growth plate (Hecht *et al.*, 2004). Ultrastructural investigation reveals chondrocytes with dilated rER cisternae containing alternating electron dense and electron lucent granular bands (Pedrini-Mille *et al.*, 1984; Stanescu *et al.*, 1984). Immunoreactivity for COMP and type IX collagen is diminished in the cartilage matrix, but increased within dilated rER cisternae (Hecht *et al.*, 2004).

Defects in other extracellular structural proteins

Mutations in matrilin-3 (*MATN3*) are responsible for some forms of multiple epiphyseal dysplasia (Mäkitie *et al.*, 2004b) and spondyloepimetaphyseal dysplasia (Borochowitz *et al.*, 2004). Silverman-Handmaker dyssegmental dysplasia and Schwartz-jampel syndrome are caused by mutations in the perlecan gene (Nicole *et al.*, 2000; Arikawa-Hirasawa *et al.*, 2001b), which is considered to be involved with the adhesion of chondrocytes to surrounding matrix (SundarRaj *et al.*, 1995). Marfan syndrome is caused by mutations in the gene for fibrillin (Tekin *et al.*, 2007), a component of extracellular microfibrils in many tissues (Sakai *et al.*, 1986). These disorders are listed in Appendix 1.

1.2.8 Defects in metabolic pathways

Defects of the diastrophic dysplasia sulphate transporter (DTDST)

Mutations in the DTDST gene, *SLC26A2*, are responsible for a group of disorders with similar histological features but differing severity, including achondrogenesis 1b (Superti-Furga, 1994), atelosteogenesis type II (Hästbacka *et al.*, 1996b), diastrophic dysplasia (Hästbacka *et al.*, 1994) and recessive multiple epiphyseal dysplasia (Rimoin *et al.*, 1998; Rossi & Superti-Furga, 2001). *SLC26A2* mutations identified in these disorders result in the variable reduction of sulphate uptake by chondrocytes and fibroblasts (Hästbacka *et al.*, 1994; Rossi & Superti-Furga, 2001; Superti-Furga, 2001), causing undersulphation of cartilage extracellular matrix (Superti-Furga *et al.*, 1996a), and abnormal migration of type IX collagen on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Diab *et al.*, 1994). The severity of disease is determined to some degree by the level of

residual transporter function (Karniski, 2001), with the exception of the Finnish diastrophic dysplasia mutation, which occurs in an untranslated exon and results in severely reduced mRNA levels (Hästbacka *et al.*, 1999). Achondrogenesis type 1b and diastrophic dysplasia are described below; atelosteogenesis type II and recessive multiple epiphyseal dysplasia are included in Appendix 2. A transgenic mouse with an *SLC26A2* mutation has been engineered to study this group of disorders (Forlino *et al.*, 2005).

Achondrogenesis type 1b (achondrogenesis Fraccaro type) is a rare autosomal recessive, perinatally lethal chondrodysplasia characterised by short limb bones and a hypoplastic thorax (van der Harten *et al.*, 1988; Superti-Furga, 1994; Hall, 2002). Radiographic investigation of 23 cases by van der Harten *et al* (1988) revealed minimal ossification of vertebral bodies and long bones, accompanied by abundant metaphyseal flaring in the lower limbs. The pelvis was poorly formed, and ribs were shortened with "cupped" metaphyses.

Histological studies exposed loosely arranged epiphyseal chondrocytes surrounded by “collagen rings” and containing cytoplasmic vacuoles (van der Harten *et al.*, 1988; Superti-Furga, 1994). The physes were disorganised with rarefied cartilage matrix containing coarse, “unmasked” collagen fibrils, and hypertrophic chondrocytes extended into plump, disordered, metaphyseal trabeculae.

Originally considered to the be same disease, achondrogenesis type 1b and type 1a can be distinguished histologically. In contrast with achondrogenesis type 1a, the cartilage matrix in type 1b is abnormal, and only in type 1a do the reserve zone chondrocytes contain variably sized periodic acid-Schiff (PAS)-negative intracytoplasmic inclusions that stain green with Gomori’s trichrome procedure (Yang *et al.*, 1976; Superti-Furga, 1996).

Hyaline cartilage in achondrogenesis type 1b was found to be under-sulphated with a reduced sulphate uptake by chondrocytes tested *in vitro*, and little or no residual activity of the DTDST (Superti-Furga *et al.*, 1996a). *SLC26A2* mutations are usually found in both alleles, and generally consist of premature stop codons (Hästbacka *et al.*, 1996b). Further experiments *in vitro* demonstrated that sulphydryl compounds are able to contribute sulphate for proteoglycan sulphation in both achondrogenesis 1b and diastrophic dysplasia chondrocytes, recovering to some degree the level of sulphation lost as a result of the *SLC26A2* mutation (Rossi *et al.*, 2003).

Diastrophic dysplasia is an autosomal recessively inherited disease caused by mutations in *SLC26A2* and is rare, other than its high incidence in the Finnish population (carrier frequency of 1-2%) due to founder effect (Langer, 1965; Hästbacka *et al.*, 1996a; Rimoin *et al.*, 1998; Hästbacka *et al.*, 1999). Clinical features include short limbed dwarfism, swollen ear lobes, severe talipes equinovarus, deformed tarsal and metatarsal bones, and extremely shortened and oval-shaped first metacarpal bones (Langer, 1965). Joint contractures affect the hips, knees and elbows, and are accompanied by metacarpo-phalangeal and interphalangeal joint rigidity, "hitch-hiker's" thumb, and narrowed interpedicular distance of the lower lumbar vertebrae (Shapiro, 1992). Young children affected with diastrophic dysplasia may have double-layered manubrium sterni which fuse to form a normal manubrium in later years (Currarino, 2000). The knees of affected fetuses appear grossly normal initially, but deteriorate with age (Peltonen *et al.*, 1999).

Hyaline cartilage in diastrophic dysplasia contains microscopic foci of matrix degeneration, with prominent fibrillar material traversing cystic areas (Shapiro, 1992), and accumulations of matrix around cells described as "large, multilayered lacunae" (Stanescu *et al.*, 1984). Transmission electron microscopy reveals the fibrillar material to be short, randomly arranged, abnormally wide collagen fibrils with a markedly irregular circumference on cross-section, interspersed with normal collagen fibrils. Many cells have disorganised organelles and cystic regions consistent with premature senescence and degeneration (Stanescu *et al.*, 1984; Shapiro, 1992).

Defects in other metabolic pathways

Loss of chondroitin-6-O-sulphotransferase-1 function results in a severe chondrodysplasia with progressive spinal involvement (Thiele *et al.*, 2004), while mutations in phosphoadenosine-phosphosulphate synthase 2 (*PAPSS2*) are responsible for Pakistani type spondlyoepimetaphyseal dysplasia (Ahmad *et al.*, 1998; ul Haque *et al.*, 1998). Chondroitin biosynthesis is severely impaired in schneckenbecken dysplasia, which is caused by mutations in the nucleotide sugar transporter, *SLC35D1* (Hiraoka *et al.*, 2007).

Inborn errors of cholesterol biosynthesis manifest as X-linked chondrodysplasia punctata types 1 and 2 (Franco *et al.*, 1995; Braverman *et al.*, 1999; Shotelersuk & Tongkobpetch, 2005), and congenital hemidysplasia with ichthyosiform

erythroderma and limb defects (CHILD syndrome) (König *et al.*, 2000). Peroxisomal disorders featuring some degree of chondrodysplasia punctata include rhizomelic chondrodysplasia punctata types 1, 2 and 3 (Braverman *et al.*, 1997; Ofman *et al.*, 1998; de Vet *et al.*, 1999), and Zellweger syndrome (Dodt *et al.*, 1995; Shimozawa *et al.*, 2004). PEX genes encode peroxisomal assembly proteins (peroxins), which import matrix proteins into peroxisomes (Distel *et al.*, 1996). See Appendix 2 for further details of these disorders.

1.2.9 Defects in the folding and degradation of macromolecules

This group of diseases consists of X-linked spondyloepiphyseal dysplasia (SED-XL), caused by mutations in *SEDL*, encoding an endoplasmic reticulum protein of unknown function, Sedlin (Gedeon *et al.*, 1999), abnormalities in the activity of cathepsin-K (Donnarumma *et al.*, 2007), mutations in *MMP13* (Kennedy *et al.*, 2005), and several types of mucopolysaccharidosis (MPS). The individual diseases involved are described in Appendix 3, excluding the types of MPS that do not feature obvious skeletal abnormalities.

The MPS disorders are a group of heritable diseases characterised by the progressive intralysosomal storage of glycosaminoglycans (Leroy, 2002). Several forms exist, each associated with inherited defects in different lysosomal enzymes involved in mucopolysaccharide metabolism (Clarke *et al.*, 1992; Yamagishi *et al.*, 1996; Bunge *et al.*, 1997; Vervoort *et al.*, 1997; Beesley *et al.*, 2001; Litjens & Hopwood, 2001; Leroy, 2002). Proteoglycan synthesis is affected in these diseases, and indigestible substrates accumulate within the cytoplasm of affected cells (Leroy, 2002).

MPS is typically associated with variable dwarfism, together with kyphosis, coarse facial features, corneal opacity (except types IIA, IIB and III), cardiac abnormalities, hepatosplenomegaly and excessive urinary excretion of glycosaminoglycans (Arbisser *et al.*, 1977; Schmidt *et al.*, 1987; Wippermann *et al.*, 1995; Beesley *et al.*, 2001; Leroy, 2002).

Cartilaginous lesions include enlarged chondrocytes containing abundant granular or finely vacuolated cytoplasm. Large areas of loose connective tissue are scattered throughout the reserve zone of physeal cartilage in MPS types I and IV, but the rest of the growth plate remains well organised. MPS type I features wide trabeculae with areas of continuous horizontal connection between longitudinally oriented

spicules. Ultrastructurally, chondrocytes contain numerous large intracytoplasmic vacuoles filled with undegraded glycosaminoglycan (Rimoin *et al.*, 1976).

1.2.10 Defects in hormones and signal transduction mechanisms

While hormones and signal transduction mechanisms are crucial to the control and regulation of endochondral ossification, many pathways are poorly understood. The diseases in this group are listed in Appendix 4.

Fibroblast growth factor receptor 3 (FGFR3)

A family of autosomal dominant chondrodysplasias with variation in severity are associated with mutations in *FGFR3*, which result in ligand-independent activation of the receptor (Rimoin *et al.*, 1998; Vajo *et al.*, 2000; Ballock & O'Keefe, 2003). FGFR3 normally plays an inhibitory role in the lengthening of endochondral bones (McEwen *et al.*, 1999; Aikawa *et al.*, 2001; Rozenblatt-Rosen *et al.*, 2002) (see Figure 1.6). While a partial loss-of-function mutation of *FGFR3* has been recorded to result in camptodactyly, tall stature, scoliosis and hearing loss (CATSHL syndrome) (Toydemir *et al.*, 2006), most *FGFR3* mutations in human beings are characterised by dwarfism.

Achondroplasia is the most common form of dwarfism in humans, affecting 1 in 15,000-40,000 people (Park & Wallerstein, 2003). Although gonadal mosaicism in normal parents with multiple affected children has been identified, more than 90% of cases of achondroplasia are due to sporadic dominant mutations (Mettler & Fraser, 2000). For this reason, nucleotide 1138 of *FGFR3* is currently considered to be the most mutable nucleotide of the human genome (Rousseau *et al.*, 1994; Shiang *et al.*, 1994; Bellus *et al.*, 1995a; Horton, 2006). Early literature on achondroplasia included a variety of different chondrodysplasias, complicating the perceived pathology (Rimoin *et al.*, 1976). Even the name, “achondroplasia” is misleading as it falsely implies a complete failure of cartilage growth. While “achondroplasia” is an inaccurate descriptive term, it has become well established by long usage (Langer *et al.*, 1967).

Achondroplasia is characterised by a reduced rate of endochondral growth (Rimoin *et al.*, 1970), rhizomelia, megalocephaly, frontal bossing with midface hypoplasia, and brachydactyly (Langer *et al.*, 1967). Histologically, growth plates are relatively well organised but cell columns are shortened, even though the number of cells per

column is not decreased (Rimoin *et al.*, 1970). Large collagen fibrils are present in the narrow hypertrophic zone, and the primary spongiosa contains coarse longitudinal septae accompanied by horizontal bridging (Maynard *et al.*, 1981). Homozygosity is usually lethal in this disease due to complications of hydrocephaly and pulmonary insufficiency resulting from thoracic hypoplasia (Pauli *et al.*, 2005).

Hypochondroplasia is a form of short-limbed dwarfism similar to mild achondroplasia, and is rarely diagnosed before 2 years of age (Bellus *et al.*, 1995b; Prinster *et al.*, 1998; Ramaswami *et al.*, 1998; Rimoin *et al.*, 1998; Winterpacht *et al.*, 2000). It features short stature, megalocephaly with mild frontal bossing, micromelia, and lumbar lordosis (Ramaswami *et al.*, 1998).

Thanatophoric dysplasia (TD) is the most common neonatally lethal skeletal dysplasia in humans, affecting 1 in 20,000-50,000 births (Orioli *et al.*, 1986). The *FGFR3* mutations found in TD trigger premature apoptosis through activation of the signal transducer and activator of transcription (STAT) signalling pathway (Tavormina, 1997; Legeai-Mallet *et al.*, 1998; Wilcox *et al.*, 1998). TD is characterised by rhizomelic dwarfism with megalocephaly, platyspondyly, and thoracic hypoplasia. The disorder has been reclassified into type I (most common) and type II based on femoral radiology and the underlying mutation. In TD type I the femurs are curved, while in TD type II the femurs are straight and a clover-leaf skull deformity is often present. Histologically, physeal chondrocyte columns are disorganised, with short, thick primary trabeculae (Delezoide *et al.*, 1997; Wilcox *et al.*, 1998).

Another chondrodysplasia resulting from mutations in *FGFR3* is severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) (Bellus *et al.*, 1999; Tavormina *et al.*, 1999). For more details on these diseases, refer to Appendix 4.

Defects in the PTH/PTHrP receptor

Mutations in *PTHR1*, which encodes the parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP) receptor, are reported in Jansen metaphyseal chondrodysplasia (Schipani *et al.*, 1995; 1997a; 1999; Bastepe *et al.*, 2004) and Blomstrand metaphyseal dysplasia (Karperien *et al.*, 1999).

The mutations involved in Jansen metaphyseal chondrodysplasia result in constitutive activation of the PTH/PTHrP receptor (David, 1998), causing

intracellular accumulation of adenosine 3'5'-monophosphate (Schipani *et al.*, 1997a). Asymptomatic hypercalcaemia and hypophosphataemia also occur (Schipani *et al.*, 1995), despite normal or low levels of PTH and PTHrP (Bastepe *et al.*, 2004).

Studies in transgenic mice with constitutive activation of PTH/PTHrP receptors have revealed a decreased rate of chondrocyte maturation, persistence of hypertrophic chondrocytes, along with delayed vascular invasion and mineralisation of cartilage (Schipani *et al.*, 1997b).

Others

Other defects in signal transduction mechnisms resulting in chondrodysplasia include Grebe/Hunter-Thompson acromesomelic dysplasia and brachydactyly type C, caused by a defect in cartilage-derived morphogenic protein-1 (*CDMP1*) (Stelzer *et al.*, 2003), a member of the TGF-β family that may influence the differentiation of osteo-chondroprogenitor cells to chondrocytes (Luyten, 1997; Tsumaki *et al.*, 1999), and brachydactyly type A1 which is due to a defect in Indian hedgehog signal molecule (Ihh) (Gao *et al.*, 2001).

1.2.11 Defects in nuclear proteins and transcription factors

Mutations in *SOX9* have been identified in campomelic dysplasia (Bi *et al.*, 2001), while short-stature homeobox (*SHOX*) mutations are responsible for Léri-Weill dyschondrosteosis (Belin *et al.*, 1998), and cleidocranial dysplasia is caused by mutations in *RUNX2* (*CBFA1*) (Mundlos, 1999). Ellis-van Creveld chondroectodermal dysplasia is due to mutations in *EVC2* (Galdzicka *et al.*, 2002), which is expressed in physeal prehypertrophic and hypertrophic chondrocytes (Tsuji *et al.*, 2004). These diseases are listed in Appendix 5.

1.2.12 Defects in RNA and DNA processing and metabolism

Defects in this category are listed in Appendix 6. The only forms of chondrodsyplasia included in this category by Superti-Furga *et al.*, (2001) are autosomal recessive diseases caused by defects in the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*). *RMRP* is required for normal assembly of ribosomes, and is involved in cyclin-dependent cell cycle regulation (Thiel *et al.*, 2005). Cartilage-hair hypoplasia is a relatively common disease in Finland, and is characterised by short limbs and hypoplastic hair, along

with abnormal cellular immunity and deficient erythrogenesis (Sulisalo *et al.*, 1993; Harada *et al.*, 2005). Anauxetic dysplasia is a disorder characterised by extremely short stature with small epiphyses and a deficiency of resting and proliferating chondrocytes, accompanied by a reduced hypertrophic zone (Thiel *et al.*, 2005).

While Shwachman-Diamond syndrome, caused by mutations in *SBDS*, is not included in this category in the classification system by Superti-Furga (2001), the disease is believed to involve RNA metabolism (Boocock *et al.*, 2003), so is included here. Shwachman-Diamond syndrome is an autosomal recessive disorder characterised by delayed bone maturation, metaphyseal abnormalities, pancreatic insufficiency and haematologic abnormalities (Dror & Freedman, 2002; Nakashima *et al.*, 2004).

1.2.13 Defects in cytoskeletal elements

Filamins are large proteins that bind actin, stabilising the actin cytoskeleton and linking it to cell membranes (Stossel *et al.*, 2001). Mutations in the gene for filamin-B, *FLNB*, result in the atelosteogenesis-omodysplasia group of disorders, consisting of Atelosteogenesis types I and III, Boomerang dysplasia and Larsen syndrome (Krakow *et al.*, 2004; Bicknell *et al.*, 2005; Bicknell *et al.*, 2007). These diseases range in severity from very mild to lethal, often featuring micromelia, midface hypoplasia, and failure of ossification of some skeletal elements (Sillence *et al.*, 1997; Becker *et al.*, 2000; Hall, 2002). In the more severe diseases, physeal cartilage is hypocellular and contains multinucleate giant cells (Sillence *et al.*, 1982; Sillence *et al.*, 1997). The disorders in this group are listed in Appendix 7.

Asphyxiating thoracic dystrophy 1 (Jeune syndrome) is an autosomal recessive disorder caused by mutations in intraflagellar transport 80, *IFT80* (Beales *et al.*, 2007). Characterised by a narrow thorax, short limbs, polydactyly and pelvic abnormalities, this disease also features renal dystrophy, hepatic fibrosis and pancreatic cysts (Ho *et al.*, 2000).

1.2.14 Chondrodysplasias of unknown aetiology

The underlying defects in many inherited chondrodysplasias have yet to be determined. Such disorders are generally grouped according to their gross, radiological and/or histological features and are briefly described here. Also

included are disorders in which the gene mutation has been identified, but the gene function is not clear. Further details are presented in Appendix 8.

Chondrodysplasia punctata group

This is a group of rare disorders characterised by radiographic stippling of epiphyses and extraepiphyseal cartilage (Rimoin *et al.*, 1976). A lethal form of chondrodysplasia punctata features an abnormal face, rhizomelia, psychomotor and developmental delay, weak respiratory muscles, cervical platyspondyly, and death by 2 years of age (Kumada *et al.*, 2001). Widespread stippled calcifications in areas of endochondral bone formation are visible radiographically.

Short rib polydactyly syndromes

This group of autosomal recessive skeletal dysplasias includes asphyxiating thoracic dystrophy 1, Ellis-van Creveld syndrome (mentioned earlier), and lethal short rib polydactyly syndrome types I to IV (Ho *et al.*, 2000). These disorders typically feature thoracic dystrophy, micromelia, polydactyly, and a variety of non-skeletal abnormalities (Ho *et al.*, 2000; Galdzicka *et al.*, 2002; Hall, 2002; Balci *et al.*, 2003).

Spondylometaphyseal dysplasias

The spondylometaphyseal dysplasias are a collection of distinctly different disorders with similar radiographic findings (Rimoin *et al.*, 1976) and include Sedaghatian type spondylometaphyseal dysplasia, Torrance platyspondylic chondrodysplasia (mentioned previously), achondrogenesis type 1a, and opsismodysplasia. These disorders vary in severity, but are linked by platyspondyly and micromelia (Whitley & Gorlin, 1983; Borochowitz *et al.*, 1988b; Foulds *et al.*, 2003; Neumann *et al.*, 2003;). Dyggve-Melchior-Clausen dysplasia (DMC) and Smith-McCort dysplasia (SMC) are caused by mutations in *DYM*, and differ only by the presence of mental retardation in DMC (Cohn *et al.*, 2003; Paupe *et al.*, 2004; Bayrak *et al.*, 2005).

1.3 Chondrodysplasias of animals

1.3.1 Animals as models for human disease

Chondrodysplasias are a large group of pleomorphic, heterogeneous diseases (Rimoin *et al.*, 1998). Since many forms of chondrodysplasia in human beings are not lethal and are often rare, the accumulation of cases for adequate study is often difficult (Sande & Bingel, 1983; Mundlos, 2001). Animal models of such diseases can be used to provide sufficient material for histological and biochemical investigation, and may be valuable for developing diagnostic assay procedures and testing therapeutic regimens (Olsen *et al.*, 2000). For example, canine MPS type I is considered to be remarkably similar clinically, pathologically and biochemically to the human form of the disease (Shull *et al.*, 1994). Dogs with this form of MPS have been used in enzyme replacement therapy research and in the development of a potential gene therapy using α-L-iduronidase cDNA in order to treat the disease in humans (Shull *et al.*, 1994).

With the advent of genetic manipulation, the creation of specific gene defects is now possible and is most frequently performed in mice due to their small size, relatively easy management, and short generation interval. Despite this, dogs have been considered more appropriate than rodents as models for human disease due to their similar physiology and body size to humans (Barsoum *et al.*, 2000). Large animals also enable long-term studies that would not be possible in laboratory rodents (Casal & Haskins, 2005).

Many strains of transgenic mice have been created for the sole purpose of studying an equivalent human chondrodysplasia, or to increase understanding of the contibution of various genes to chondrogenesis and skeletal development. Discussion in the following section will focus on naturally occurring chondrodypslasias in domestic and laboratory animals. In some situations it is difficult to determine whether a chondrodysplasia has a genetic or environmental aetiology, and nutritional factors such as manganese deficiency or plant toxicity may be involved.

1.3.2 Sheep

"Spider lamb syndrome"

"Spider lamb syndrome" (SLS) occurs mostly in Suffolk and Hampshire sheep but is also reported in the U.S. Southdown, Shropshire and Oxford breeds (Cockett *et al.*, 1999). This disorder is the most common chondrodysplasia of sheep, and is named for the long, spider-like limbs that accompany it. Although the mode of inheritance is generally accepted as autosomal recessive with complete penetrance and variable expression (Oberbauer *et al.*, 1995), a more recent study suggests that skeletal size is increased in mature heterozygous animals (Smith *et al.*, 2006). Selection of large breeding animals may have lead to the high gene frequency for SLS in the United States of America. Suffolk embryos imported from the USA introduced SLS to New Zealand and Australia in 1984 (West *et al.*, 1995). A causal mutation has been identified in the gene encoding FGFR3 (Beever *et al.*, 2006) and a DNA-based test to detect heterozygous sheep is now commercially available (Beever & Cockett, 2001).

In addition to elongated limbs, affected lambs may have kyphoscoliosis, bilateral carpal valgus and skull malformations such as Roman nose, deviated nasal septa, and a reduced dorso-ventral dimension of the calvarium. Cartilage erosions and severe degenerative joint disease also occur, especially in major limb joints (Vanek *et al.*, 1987).

Radiographic abnormalities are evident at birth in affected animals and become more severe with age (Vanek *et al.*, 1989). Typically, there is incomplete development of the proximal ulna with abnormal, multifocal sites of ossification and progressive elbow joint degeneration, along with humeral subluxation due to a shallow glenoid cavity. Other radiographic abnormalities include wedge-shaped vertebral bodies, irregular dorsal spinous processes of cranial thoracic vertebrae, pectus excavatum and cuboidal, misaligned, inconsistently spaced sternebrae with indistinct growth plates (Vanek *et al.*, 1989; West *et al.*, 1995).

Articular cartilage of the proximal ulna, humerus and scapula is thinned with local erosions, and cartilaginous tissue is distributed abnormally through the epiphyses and metaphyses of the ulna (particularly the olecranon), humerus, scapula and sternum (Nakano *et al.*, 1994). Histologically, vertebral and long-bone physes

contain thickened and disorganised proliferative and hypertrophic zones (Rook *et al.*, 1988).

Ancon mutant

The ancon (or otter) mutant arose near the end of eighteenth century in Massachusetts, and is considered the earliest recorded mutant among domestic animals (Landauer & Chang, 1949). The strain apparently disappeared in the mid-nineteenth century. The short stature was at the time considered potentially useful for management with a predominance of low stone walls in the area, although there are records of difficulty in driving the sheep to market (Landauer & Chang, 1949).

Limbs were rhizomelic, and photographs of skeletons show varus deformity of the elbow (hence the name ancon *Gr.* elbow) combined with valgus deformity of the carpus (Landauer & Chang, 1949). An autosomal recessive mode of inheritance was considered likely (Landauer, 1950).

In 1919 a similar mutation appeared in a sheep flock in Norway, but although the Massachusetts and Norway ancons appeared superficially similar, they were probably different mutations (Landauer & Chang, 1949).

The deformity in the Norwegian Ancon, described by Chang (1949), was identifiable at birth with femoral bowing, which became more exaggerated with age. The disease featured enlarged long-bone metaphyses, premature closure of the distal tibial and metatarsal physes, and exostosis-like lesions in severe cases. Spines, crests and processes of the bones were more pronounced than normal (especially in males), vertebral fusion was common, and scoliosis and lordosis were also reported. The occipital condyles were sometimes abnormally close together or fused, the diameter of the foramen magnum was often reduced, and extra centres of ossification sometimes occurred at the basi-occipito-sphenoid junction. The severity of lesions was variable between animals and sites within individuals (Chang, 1949).

Histologically, growth plates were usually thin with narrowing of all zones, columnar organization was often irregular, primary trabeculae were relatively few and short, and in cortical bone the Haversian systems were irregular, less compact than normal and often incomplete (Chang, 1949).

A similar form of heritable dwarfism has also been reported in Merino sheep (Shelton, 1968).

Lethal dwarfism

An isolated outbreak of so-called "lethal dwarfism" in mixed breed sheep (Welsh, Radnor and Suffolk cross) has been described (Duffell *et al.,* 1985). Approximately one quarter of the lambs born in the flock during one season were affected. They were born alive and made gasping respiratory movements followed by death within a few minutes. Two experienced rams were used, both siring affected lambs. In some cases only one lamb of twins was affected. A genetic aetiology was not indicated, although an environmental cause could not be found. Congential manganese deficiency or another enviromental factor that was not tested for may have been responsible.

Affected lambs were short and plump with a domed head and short muzzle, short, paddle-like limbs, a narrow thorax with short ribs, and a swollen abdomen. All lambs had cleft palate and scrotal hernia was common. The lungs were hypoplastic and the tracheal diameter was severely reduced. Long bones were short and bowed with large epiphyses and metaphyseal flaring. Primary and secondary ossification centres were reduced in number, and the axial skeleton was short with partial ossification of vertebrae (Duffell *et al.*, 1985).

Histologically there was an absence of physeal chondrocyte columns, most cells were hypertrophic and the intercellular matrix was reduced to narrow strands (Duffell *et al.*, 1985). Vascular channels were broad and tortuous, and articular surfaces were poorly differentiated from perichondral tissues. Tracheal and bronchial cartilage was similarly disorganised, and all affected lambs had varying degrees of thyroid hyperplasia.

Texel chondrodysplasia

An autosomal recessive chondrodysplasia of Texel sheep featuring decreased sulphation of hyaline cartilage has recently been described (Byrne, 2005; Thompson *et al.*, 2005). The relative undersulphation of cartilage proteoglycans is illustrated in Figure 1.7. Affected lambs appeared normal at birth but developed skeletal lesions and often died by 3-4 months of age as a result of tracheal collapse. Gross lesions included short stature, varus deformity of forelimbs, a wide-based

stance with a 'barrel chest', and erosion of articular cartilage. Histologically there were areas of chondrolysis within cartilage matrix, rings of abnormal, basophilic matrix surrounding chondrocytes, and variable disruption of physeal organisation (Thompson *et al.*, 2005). Chondrodysplasia of Texel sheep is the subject of more detailed investigation in this thesis.

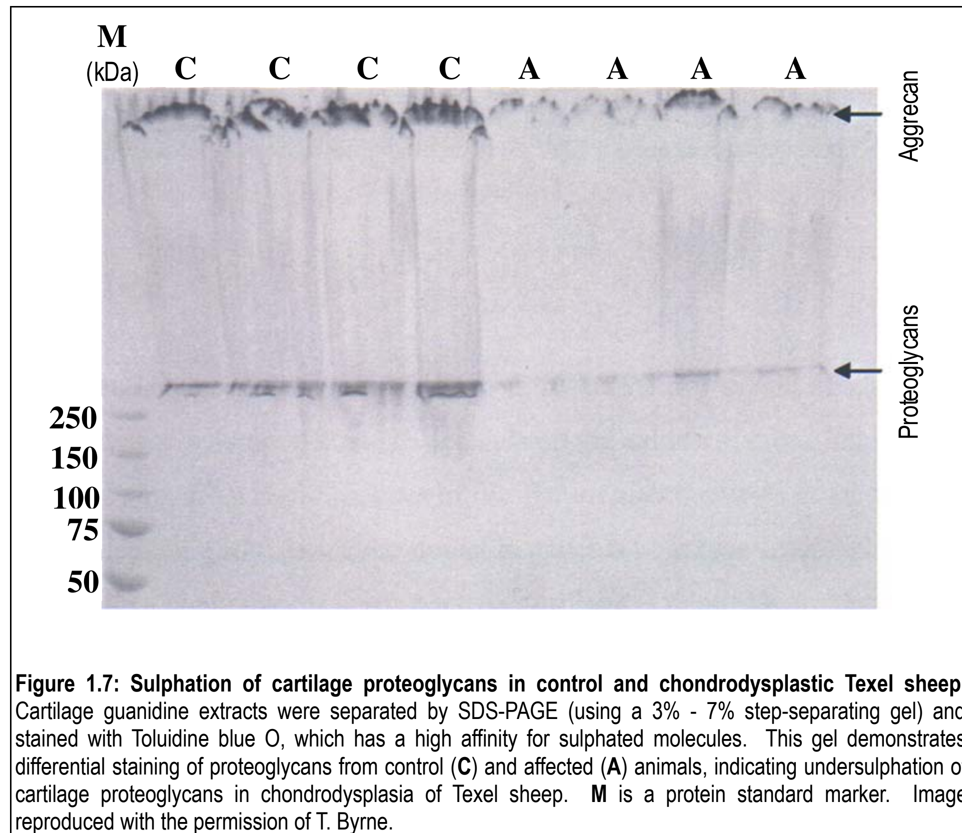


Figure 1.7: Sulphation of cartilage proteoglycans in control and chondrodysplastic Texel sheep. Cartilage guanidine extracts were separated by SDS-PAGE (using a 3% - 7% step-separating gel) and stained with Toluidine blue O, which has a high affinity for sulphated molecules. This gel demonstrates differential staining of proteoglycans from control (**C**) and affected (**A**) animals, indicating undersulphation of cartilage proteoglycans in chondrodysplasia of Texel sheep. **M** is a protein standard marker. Image reproduced with the permission of T. Byrne.

South Down dwarfism

Dwarfism was reported in South Down sheep, causing death in affected lambs between 1 and 2 months of age (Bogart, 1946). The author described grossly shortened limbs, a bulging forehead, a broad appearance through the shoulders, respiratory difficulty due to abnormalities of the palate and throat, and slow growth. Histological examination of thyroids by Bogart (1946) showed that 80% of follicles had no colloid, with connective tissue invading follicle cavities. Lambs improved with thyroxine therapy. While Bogart (1946) suggested a simple recessive mode of inheritance, no evidence was supplied, and the indicated response to therapy makes a genetic aetiology unlikely.

Ectrodactyly

A chondrodysplasia of unknown aetiology and characterised by ectrodactyly has been described in Cheviot sheep (Wray *et al.*, 1971). Clinically, the eyes protruded, the ears and tail were short, forelimbs were shortened and forehooves were replaced by an area of hard skin at the end of the limb. Hindhooves were reduced in size and abnormally shaped, and metapodial bones were slightly distorted at the distal epiphysis. The authors described an abnormal proximal phalanx in the forelimbs and absence of the middle and distal phalanges. The tendons of the superficial and deep digital flexors ended in a mass of fibrous tissue at the distal extremity of each digit in the forelimbs (Wray *et al.*, 1971).

Similar outbreaks occur occasionally on sheep farms in New Zealand, and have been attributed to various non-genetic causes, including nitrate toxicity (Gumbrell, 1990). A case of ectrodactyly and hemimelia in a Najdi sheep has been described (Ramadan, 1993), but no signs of chondrodysplasia were recorded in that occurence.

1.3.3 Cattle

Brachycephalic "snorter"

Brachycephalic "snorter" dwarfism, named for the upper respiratory noises that accompany the disease, was at one time an important autosomal recessive disorder in Hereford cattle (Julian *et al.*, 1957; Jones & Jolly, 1982), but is now uncommon. The disease has also been recorded in the Angus breed (Baker *et al.*, 1951; Bovard & Proide, 1965). Detectable by day 125-130 of gestation, the disease featured disproportionate dwarfism, mandibular prognathia, a characteristically shortened head relative to its width, accompanied by a bulging frontal area due to early closure of the spheno-occipital synchondrosis (in normal cattle full closure is achieved at 24-36 months, while in the dwarf cattle it occurred as early as 5.5 months of age) (Julian *et al.*, 1957). Distal long bones were reduced in length, but with normal diameter, resulting in apparent thickening (Bovard & Hazel, 1963; Jones & Jolly, 1982).

Radiographs demonstrated bony projections into the cranium, especially from the posterior intra-occipital synchondrosis, ventrally flattened lumbar vertebrae with an undulating ventral surface and a concave dip in the dorsal surface, and

shortened lumbar transverse processes with forward hooking at the ends (Jones & Jolly, 1982).

Histologically, chondrocyte columns in the physes were shortened and irregular (Julian *et al.*, 1957). Ultrastructural investigation revealed chondrocytes occasionally containing dilated endoplasmic reticulum enclosing an amorphous, slightly granular material (Jones & Jolly, 1982).

Dolichocephalic dwarfism and Comprest dwarfism have been linked to the brachycephalic snorter and may be part of the same complex (Gregory & Carroll, 1956; Julian *et al.*, 1959). Dolichocephalic dwarfism was characterised by a very long head relative to width, and was recognised in Aberdeen-Angus, Hereford and Shorthorn cattle (Julian *et al.*, 1959).

The Comprest breed was a small-type Hereford. Dwarf calves with or without crooked legs, and occasional severe lethal forms, occurred when breeding Comprest with Comprest, and also Comprest with carriers of non-Comprest dwarfism (Chambers *et al.*, 1954). Some authors suggested that the Comprest dwarf along with the Dolichocephalic dwarf and Brachycephalic Snorter were all forms of the same dwarfism complex (Gregory, 1956; Julian *et al.*, 1959). Stonaker, a stockworker from Colorado Agricultural and Mechanical College, considered the Comprest Hereford to be the result of a gene acting with partial dominance, and that homozygosity resulted in dwarfism (Pahnish *et al.*, 1955).

Dexter bulldog

Chondrodysplasia in Dexter cattle is an example of incomplete dominance, where heterozygous animals develop disproportionate dwarfism while homozygous affected animals are non-viable "bulldog" calves (Usha *et al.*, 1997). The disease is caused by mutations in *ACAN* which codes for the cartilage core protein, aggrecan (Cavanagh *et al.*, 2007). Bulldog calves have become more common due to a recent resurgence of the Dexter breed amongst lifestyle farmers. Affected calves are often aborted prior to 7 months of gestation, and feature a short, scoliotic vertebral column, severe rhizomelia, hydrocephalus with open cranial sutures, a retruded muzzle, cleft palate, protruding tongue, and a large ventral abdominal hernia (Berger & Innes, 1948; Harper *et al.*, 1998).

Histologically, fetal limb bones are composed mainly of highly cellular epiphyseal cartilage with a central segment of diaphyseal bone of subperiosteal origin

consisting of dense cancellous bone and some degree of compact bone (Harper *et al.*, 1998). Limbs lack distinct growth plates, with minimal chondrocyte column formation or maturation, while periosteal bone formation appears normal. The histological features and markedly reduced size of the limb bones are consistent with a failure of endochondral ossification following primary ossification of the diaphysis of the cartilage anlage (Harper *et al.*, 1998). Genetic tests are available for detection of the two major disease-causing mutations (University of Sydney, 2007).

A similar condition to the Dexter bulldog has been reported in Jersey cattle (Mead *et al.*, 1946) and Holstein-Friesians (Bowden, 1970). In the Jersey form of the disease, the homozygotes were phenotypically very similar to the Dexter lethal, and the heterozygotes had reduced body and limb length. In contrast, carriers of the Holstein-friesian form showed no abnormalities, and the disease in this breed can be considered to be autosomal recessive. Holstein-Friesian bulldog calves also differed from Dexter bulldog calves in that gestation was completed to full term (Agerholm *et al.*, 2004b). The authors described a shortened axial skeleton, marked rhizomelia, a short maxilla and bilateral exophthalmus. Radiographic lesions included a ventrally rotated splanchnocranium, small and irregular diaphyses with prominent, rubbery epiphyses causing spinal cord compression in the vertebral column, a stenotic trachea, a narrow thorax containing hypoplastic lungs, and bilateral ventricular hypertrophy (Agerholm *et al.*, 2004b). Histologically, the physes were poorly organised and remnant cartilage cores extended into the metaphyses (Agerholm *et al.*, 2004b).

A similar autosomal recessive chondrodysplasia was reported in British Friesians, in which gestation lasted only 200-300 days (Jones *et al.*, 1978). Affected calves had shortened limbs and muzzle, and had either hydrocephalus and cleft palate or an oedematous body.

Complex vertebral malformation (CVM)

Although not a form of chondrodysplasia, CVM is mentioned here because it is an important lethal disorder in Holstein cattle. This disease has an autosomal recessive mode of inheritance and is caused by a mutation in *SLC35A3*, for which a genetic test is available (Ministeriet for Fã~Devarer, Landbrug og Fiskeri Danmarks Jordbrugsforskning, international patent WO 02/40709 A2, 2003). CVM features multiple hemivertebrae, synostoses and misshapen vertebrae, scoliosis and

arthrogryposis. Cardiac malformations are also associated with CVM, and 75% of affected fetuses are aborted prior to 260 days of gestation (Agerholm *et al.*, 2001; Nagahata *et al.*, 2002; Agerholm *et al.*, 2004a).

Others

Chondrodysplasia of Guernsey Island cattle, the Telemark lethal mutation, American Angus dwarfism, chondrodysplasia of Japanese Brown cattle, the Ayrshire cartilage defect, Stumpy in Shorthorn cattle, and Shorthorn disproportionate dwarfism are described in Appendix 9. A form of proportionate dwarfism with an autosomal dominant mode of inheritance complicated by incomplete penetrance has been identified in commercial Angus herds in Australia (Latter *et al.*, 2006). As this disease is not considered to be a chondrodysplasia, it is not discussed further in this review.

1.3.4 Dogs

Alaskan malamute chondrodysplasia

Alaskan Malamute chondrodysplasia is generally regarded as having an autosomal recessive mode of inheritance (Sande *et al.*, 1982), although the anaemia detectable in both heterozygous and homozygous animals may suggest an incomplete dominant rather than recessive mode of inheritance (Fletch & Pinkerton, 1973; Fletch *et al.*, 1975). While affected pups do not show gross signs of chondrodysplasia until 5-6 weeks of age, radiographic evidence of abnormalities are evident as early as 7-10 days, and include flattening of the distal ulnar metaphysis and delayed ossification of the accessory carpal bone (Sande *et al.*, 1974). In time, the costochondral junctions develop flaring and cupping, and the antebrachium becomes shortened and curved (Sande *et al.*, 1974). Histologically, the physes appear normal at birth (Sande *et al.*, 1982). As the lesions develop, chondrocyte columns in the proliferative zone become irregular and are separated by a broad core of matrix. The primary and secondary spongiosa contain a reduced number of large, irregular spicules, with irregular thickening of physes occasionally associated with trabecular microfractures (Sande *et al.*, 1982). A reduction in the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate in physeal cartilage has been reported (Bingel *et al.*, 1985).

The Alaskan Malamute club of America operates a breeding and certification programme in order to eliminate the disease (The Alaskan Malamute Club of Canada *et al.*, 2003).

Canine GM-1 gangliosidosis

Chondrodysplasia occurs in English Springer Spaniels and Portugese Water Dogs affected by canine GM-1 gangliosidosis, a progressive autosomal recessive disease caused by mutations in *GLB1* (Yamato *et al.*, 2002), resulting in a deficiency in the activity of lysosomal β-galactosidase (Alroy *et al.*, 1992; Müller *et al.*, 1998). Radiographic lesions include irregular intervertebral disc spaces and disturbed vertebral physeal ossification. Affected English Springer Spaniels also show proportionate dwarfism, with frontal bossing and hypertelorism. As the disease progresses, pups develop central nervous signs (Alroy *et al.*, 1992). In Alaskan Huskies, the terminal stages of the disease occur at approximately 7 months of age, and are associated with tonic-clonic convulsions (Müller *et al.*, 2001).

Blood smears from affected dogs contain vacuolated lymphocytes and eosinophils. Histologically, neurons are enlarged, containing pale foamy cytoplasm with inclusions that stain with toluidine blue. Cells from many other tissues, including chondrocytes, show cytoplasmic vacuolation (Alroy *et al.*, 1992).

Canine MPS I

MPS I (α-L-iduronidase deficiency) due to mutations in *IDUA* (Menon *et al.*, 1992) occurs in Plotthounds, and is likely to have an autosomal recessive mode of inheritance (Shull *et al.*, 1982). Affected dogs demonstrate stunted growth, progressive lameness with severe degenerative joint disease in multiple limb joints, and poor vision (Shull *et al.*, 1982). Right ventricular heart enlargement and/or heart valve thickening is a constant finding (Alroy *et al.*, 1992).

Histologically, synovial cells show marked cytoplasmic granulation due to accumulation of acid mucopolysaccharide (Shull *et al.*, 1982), and hepatocytes are swollen with a finely vacuolated, granular, eosinophilic cytoplasm (Shull *et al.*, 1984). Ultrastructural examination reveals dilated vesicles containing a fine granular internal matrix in corneal, dermal and synovial cells. Fibroblast cultures from one affected dog accumulated S-mucopolysaccharide, which returned to normal upon addition of α-L-iduronidase (Shull *et al.*, 1982).

Canine MPS VII

Canine MPS VII is a disorder with an autosomal recessive mode of inheritance, caused by a deficiency of β-glucuronidase due to mutations in *GUSB* (Haskins *et al.*, 1991; Ray *et al.*, 1998; Ray *et al.*, 1999). Affected animals show a reduced growth rate and develop a broad, short face, broad chest and corneal clouding. Cytoplasmic vacuolation occurs in cells of many tissues, including chondrocytes. Radiographs demonstrate severe progressive epiphyseal dysplasia and bilateral hip subluxation or luxation. Cardiac abnormalities, hepatomegaly and a misshapen, narrow trachea with overlapping rings are additional features, and there is excessive urinary excretion of mucopolysaccharides (chondroitin 4- and 6-sulphate and dermatan sulphate) (Haskins *et al.*, 1991).

English pointer enchondrodystrophy

Last reported in 1984, an enchondrodystrophy with an autosomal recessive mode of inheritance in the English Pointer breed was identified in England and Australia (Whitbread *et al.*, 1983; Lavelle, 1984). Signs of deformity were apparent prior to weaning, and progressed to include flaring of long bone metaphyses and valgus deformity of the distal forelimbs. Marked degenerative changes occurred in many joints, and the thorax appeared dorso-ventrally flattened. In some severe cases kyphosis developed (Whitbread *et al.*, 1983). Histology of the physes demonstrated thickened hypertrophic zones in some bones and a limited, disorganised proliferative zone in others. The transitional zone of articular cartilage underwent degenerative changes, beginning as decreased staining intensity and progressing to the formation of cystic spaces (Whitbread *et al.*, 1983).

Miniature poodle pseudoachondroplasia

Pseudoachondroplastic dysplasia with an autosomal mode of inheritance was identified in Miniature Poodles, associated with variable undersulphation of cartilage matrix proteoglycans (Riser *et al.*, 1980; Bingel *et al.*, 1986). Gross lesions appeared at 10-21 days of age, including brachygnathia superior, thickened tracheal rings, a dorso-ventrally flattened thorax, mild kyphosis and enlarged costochondral junctions (Gardner, 1959; Amlöff, 1961; Riser *et al.*, 1980). Radiographic lesions consisted of enlarged, stippled epiphyses with malformation of both the long bones and vertebral column accompanied by flared metaphyses of long bones. Histologically there was delayed ossification of long bone epiphyses (Riser *et al.*,

1980; Bingel *et al.*, 1986), and most chondrocytes were surrounded by "haloes" of dense matrix. The proliferative zone was narrow and cells in the zone of maturation were arranged in irregular clusters. The primary spongiosa contained sparse, short, thick, poorly calcified trabeculae, often with a central core of hypertrophic chondrocytes (Amlöff, 1961).

Oculoskeletal dysplasias

Oculoskeletal dysplasias with an autosomal recessive mode of inheritance have been described in the Scottish Deerhound (Breur *et al.*, 1989), Labrador Retriever (Carrig *et al.*, 1977; Carrig *et al.*, 1988) and Samoyed (Meyers *et al.*, 1983) breeds of dog. Crossbreeding trials indicated that the Samoyed and Labrador oculoskeletal dysplasias were non-allelic (Du *et al.*, 2000). Interestingly, the ocular lesions in Labrador oculoskeletal dypslasia had an incompletely dominant mode of inheritance. While this may have been the result of two genes that are linked, a single gene with differing action on ocular and skeletal systems was been considered more likely (Carrig *et al.*, 1977). The canine oculoskeletal dysplasias can typically be characterised by progressive dwarfism with bowed limbs and abnormal joint development, in conjunction with ocular defects including mydriasis, lens opacification, areas of pigment swirling and opacification involving the central areas of the cornea, cataracts, hyaloid vascular system remnants, retinal detachment, and cystic degeneration of retinas with hypertrophied pigment epithelial cells (Carrig *et al.*, 1977; Meyers *et al.*, 1983; Breur *et al.*, 1989).

Scottish Deerhounds affected by oculoskeletal dysplasia had distinctive chondrocytes containing periodic acid-Schiff (PAS)-positive, diastase-resistant intracytoplasmic inclusion bodies (Breur *et al.*, 1989). Ultrastructurally, the dilated chondrocytic rER enclosed alternating electron dense and electron lucent lamellae (Breur *et al.*, 1992).

The form of oculoskeletal dysplasia found in Samoyed dogs was accompanied by increased urinary levels of chondroitin sulphate, presumably due to altered cartilage metabolism (Meyers *et al.*, 1983). A family of Samoyeds with haematologic abnormalities as well as ocular and skeletal defects was also described (Aroch *et al.*, 1996).

Norwegian Elkhound chondrodysplasia

A chondrodysplasia characterised by wide metaphyses has been described in Norwegian Elkhounds by Bingel & Sande, (1982). By 3 weeks of age, a band of increased bone density at the metaphyses in the region of the primary spongiosa was evident, but resolved over time. Other radiographic lesions included delayed ossification of carpal bones, cranial bowing of the radius and ulna, flared costochondral junctions, and short vertebral bodies with pleated ventral borders.

Histologically, the distal ulnar physes were thin with an abnormally narrow proliferative zone and variable column formation. Some chondrocytes contained large alcian blue (AB)/PAS-positive inclusions which ultrastructrually consisted of finely granular material and were bounded by a smooth discontinuous membrane. The trabeculae of the primary and secondary spongiosa were coarse, short and disorganised with horizontal bridging (Bingel & Sande, 1982).

Great Pyrenees chondrodysplasia

Great Pyrenees pups with an autosomal recessive form of chondrodysplasia showed gross signs of dwarfism by 10 days of age (Bingel & Sande, 1994). The limbs, trunk and muzzle were abnormally short, some pups were deaf, and all had increased urinary excretion of chondroitin sulphate and triglycerides. In time, radiography showed poorly ossified vertebral bodies with indistinct physes, generally delayed epiphyseal development, thickened long bone metaphyses, and shortened ribs with enlarged costochondral junctions (Bingel & Sande, 1994). Angular limb deformities developed, along with short, broad and excessively curved pelvic ilia.

Histologically, physeal chondrocyte column formation was disordered, and trabeculae of the primary and secondary spongiosa were thickened and irregular with transverse bridging (Bingel & Sande, 1994).

Irish Setter chondrodysplasia

An autosomal recessive chondrodysplasia reported in Irish Setters did not become grossly or histologically apparent until several weeks of age, at which point affected animals developed reduced body and limb length, bowing of the radius and ulna, and carpal valgus (Hanssen *et al.*, 1998).

Histologically, both endochondral and periosteal ossification were well organised, with slight exaggeration of periosteal growth. There was mild variation in height of

chondrocyte columns, and in some puppies the distal radial epiphyses were widened. The trabeculae of the spongiosa were unevenly distributed and thicker than normal (Hanssen *et al.*, 1998).

Multiple epiphyseal dysplasia of beagles

A chondrodysplasia of Beagles in which abnormal movement of the hind limbs with a swaying gait were noted at birth was described by Rasmussen (1971). Radiographically, epiphyses of long bones and vertebrae displayed stippling, formation of additional ossification centres and frayed outlines. These lesions appeared similar to those of multiple epiphyseal dysplasia in children, and resolved by 4-6 months of age (Rasmussen & Reimann, 1973). Only slight lameness remained at maturity, and the adult dogs had radiographic lesions of shallow epiphyses and broad metaphyses, as well as deep erosions in the articular cartilage of the femoral head (Rasmussen, 1971). The tracheal lumen was narrowed in affected dogs due to thickening of the cartilage rings and their overlapping at the dorsal aspect of the trachea (Mango *et al.*, 2004).

Histological lesions of articular-epiphyseal cartilage included acellular areas and abnormal foci of dense calcium deposition in the matrix. Chondrocytes adjacent to abnormal foci were disorganised, but growth plates were unaffected (Rasmussen & Reimann, 1973).

Bull terrier osteochondrodysplasia

Chondrodysplasia has been reported in 4 of 9 Bull Terrier littermates, featuring an abnormal hind limb gait with varus deformity at 3-4 months of age (Watson *et al.*, 1991). Acetabulae were shallow and the femoral heads flattened. Histologically, the femoral necks contained large areas of non-ossified cartilage with areas of fibrillar matrix. Non-ossified foci were also present in the distal ulnar, humeral, and proximal radial metaphyses. The authors suggested that the lesions may be due to failure of chondrocyte maturation leading to impaired vascular invasion, which is a prerequisite for remodelling.

Others

Isolated cases of chondrodysplasia have also been reported in several other breeds of dog, and are presented in Appendix 10. These include Cocker Spaniel

chondrodystrophy, German Shepherd chondrodysplasia, Scottish Terrier achondroplasia and Akita achondrogenesis.

1.3.5 Cats

Feline MPS VI

Chondrodysplasia is described in Siamese cats with MPS VI, which is caused by a recessively inherited deficiency of aryl-sulfatase B (Haskins *et al.*, 1980a) and can be considered an animal model for Maroteaux-Lamy Syndrome in humans (Jezyk *et al.*, 1977). Clinically, at 8 weeks of age affected cats have a small head with a broad, short maxilla, odontoid hypoplasia, small ears, cloudy corneas and pectus excavatum. Skeletal lesions, including vertebral fusion or subluxation, and degenerative joint disease progress in severity with age (Haskins *et al.*, 1980a; Haskins *et al.*, 1981).

Histologically, there is evidence of retarded endochondral ossification with focal necrosis and physeal dysplasia (Orgad *et al.*, 1989). Articular cartilage is hypercellular and supported by a sclerotic subchondral bony plate containing islands of cartilage. Chondrocytes, and cells in many other tissues, are packed with membrane-bound inclusions (Haskins *et al.*, 1980a). Coarse metachromatic granules are present in peripheral blood neutrophils (Haskins *et al.*, 1981), and excessive glycosaminoglycans, mostly dermatan sulphate, are excreted in the urine (Jezyk *et al.*, 1977; Haskins *et al.*, 1980a).

Feline MPS VII

Feline MPS VII is caused by β-glucuronidase deficiency (Fyfe *et al.*, 1999). Lesions are apparent by 2 months of age, and by 6 months of age there is retarded growth, corneal clouding, delayed dental eruption, abdominal enlargement and a shortened maxilla. Radiographic abnormalities include fused cervical vertebrae, a flattened thorax, pectus excavatum, short thoracic and lumbar vertebrae, and bilateral coxofemoral subluxation. Metachromatic granules are present in peripheral blood neutrophils, and there is excessive urinary excretion of chondroitin sulphate (Fyfe *et al.*, 1999).

Scottish Fold osteochondrodysplasia

Scottish Fold cats are known for the typical ear-fold, believed by some to be a result of a cartilage abnormality resulting in inability of the pinna to remain pricked (Malik *et al.*, 1999). The causative "folded-ear" gene acts in a dominant manner. Osteochondrodysplasia in the Scottish Fold breed is characterised by lameness, shortened and misshapen distal limbs, and short, broad caudal vertebrae. Intertarsal and tarsometatarsal joint spaces progressively diminish and periarticular osteophytes form, eventually leading to ankylosis (Chang *et al.*, 2007; Malik *et al.*, 1999). Skeletal lesions were first thought to occur only in cats homozygous for the folded-ear gene (Malik *et al.*, 1999), but later studies indicated milder skeletal lesions may also occur in heterozygous animals (Hubler *et al.*, 2004).

Histologically, articular cartilage is irregularly thickened with necrotic foci, flaking and fibrillation, accompanied by chronic synovitis. Persistent islands of cartilage are evident in epiphyseal bone (Malik *et al.*, 1999).

Others

The Munchkin breed and rare forms of chondrodysplasia in cats such as metaphyseal chondrodysplasia, chondrodysplasia with Pelger-Hüet anomaly and feline congential hypothyroidism are listed in Appendix 10.

1.3.6 Pigs

Dwarfism

A naturally-occurring mutation in type X collagen has been identified in domestic pigs by Nielsen (2000), representing an animals model for Schmid metaphyseal chondrodysplasia in humans. The disease in pigs is characterised by an autosomal dominant mode of inheritance, shortened long-bone with abnormal metaphyses containing tongues of physeal cartilage. Histologically, physes contain a disorganised hypertrophic zone and stain poorly with H&E or TB.

Danish Landrace chondrodysplasia

An autosomal recessive form of chondrodysplasia described in Danish Landrace pigs was characterised by shortening of the limbs, especially the forelimbs, with an apparently normal axial skeleton and skull (Jensen *et al.*, 1984). The joints had

increased mobility and became degenerate over time. The hair coat in affected animals was abnormally long.

Radiographically, long bones were abnormally short and thick, with wide metaphyses and mushroom-shaped epiphyses. Histologically, physeal lesions included reduction of the proliferative zone and an irregular hypertrophic zone (Jensen *et al.*, 1984).

Hereditary dwarfism

Spontaneously occurring hereditary dwarfism has been reported in Large White X Landrace pigs, featuring shortened limbs, muzzle, ribs and spinal column (Mores *et al.*, 1989). Histologically, physes were enlarged with foci of degenerate matrix at the junction of the physis and metaphysis, and metaphyseal trabeculae were irregular.

1.3.7 Deer

Four cases of chondrodystrophy were reported in an inbred herd of fallow deer (Baker *et al.*, 1979). Most bones, but particularly those of the limbs, were reduced in length and there was varus deformity of the forelimbs. Phalanges were less severely affected than the other limb bones. Epiphyses were distorted, and in some areas physeal closure was premature. The sternebrae were enlarged and misshapen. A cleft palate, domed skull and large fontanelles were also noted. Histologically, both the proliferative and hypertrophic zones of the physes were reduced (Baker *et al.*, 1979).

1.3.8 Goats

A form of dwarfism resembling "bulldog" calves was described in an inbred herd of goats (Kaikini & Malkhede, 1969). The disorder featured an overly prominent cranial vault, short, wide facial bones, brachygnathia superior, protruding tongue, short neck, markedly shortened limbs and a ventral hernia.

1.3.9 Mice

Some chondrodysplasias in mice are caused by naturally occurring mutations while many others are the result of intentional manipulations for the purpose of research. Appendix 11 includes a table of chondrodysplasias in transgenic mice. Only naturally occurring chondrodysplasias will be presented here.

Achondroplasia (*cn*)

Achondroplastic mice can be distinguished from normal mice at birth (Bonucci *et al.*, 1976). The authors described grossly shortened long bones and small caudal vertebrae. Sannasgala & Johnson, (1990) identified delayed formation of secondary centres of ossification and thin physes containing abnormally small chondrocytes. Histologically, the interterritorial matrix stained less intensely than normal with either AB or colloidal iron, suggesting a reduced proteoglycan content (Bonucci *et al.*, 1976). Primary spongiosa was scant, with short, thick longitudinal septae (Sannasgala & Johnson, 1990).

The skeletal changes seen in homozygous affected (cn/cn) mice are very similar to those seen in human achondroplasia and the mouse has been considered to be a model of the human disease (Bonucci *et al.*, 1976). The causative mutation for achondroplasia in mice, however is in *NPR2*, the atrial natriuretic peptide receptor-B (Tsuji & Kunieda, 2005), rather than in *FGFR3*.

Brachymorphic mouse

Brachymorphism in mice is an autosomal recessive disease associated with mutations in *PAPSS2* resulting in decreased PAPS synthase activity (Pennypacker *et al.*, 1981; Kurima *et al.*, 1998). Developing during the first 4 weeks of life, gross lesions included shortened long bones and tail, and a domed skull (Orkin *et al.*, 1977; Kurima *et al.*, 1998). Histologically, hypertrophic zones were narrowed and the physes became less organised with time. This disorder is considered to be homologous to Pakistani spondyloepimetaphyseal dysplasia in humans (ul Haque *et al.*, 1998).

Cartilage matrix deficient (cmd) mice

Cartilage matrix deficiency in mice is a lethal autosomal recessive disorder characterised by short, wide, endochondral bones, a short head, thoracic hypoplasia, thickened tracheal rings, and subcutaneous oedema (Bell *et al.*, 1986). Near complete deletion of the aggrecan gene has been identified (Krueger *et al.*, 1999). Kobayakawa *et al* (1985) described epiphyseal cartilage with reduced extracellular matrix and poor physeal organisation, occasionally containing degenerate foci. A deficiency of acid mucopolysaccharides in the cartilage extracellular matrix has been recognised (Bell *et al.*, 1986). Ultrastructurally, abnormally dense networks of thickened, mostly parallel, collagen fibrils were

closely associated with chondrocytes, and the number of matrix vesicles was reduced (Kobayakawa *et al.*, 1985).

Others

Chondrodysplasia "cho", murine brachypodism "bp", Chubby "cby", dispropoprtionate micromelia "Dmm", cartilage anomaly "can", dwarfism, and MPS VII are listed in Appendix 11.

1.3.10 Rabbits

Few chondrodysplasias are described in rabbits. These include chondrodysplasia in the presence of Pelger-Hüet anomaly, where the skeletal lesions have a recessive mode of inheritance while the leukocytic changes demonstrate an incomplete dominant mode of inheritance (Nachtsheim, 1950). Autosomal recessive "da" achondroplasia has been identified in the New Zealand White rabbit (Crary & Sawin, 1952), "ac" chondrodysplasia is an incompletely dominant disorder (Crary *et al.*, 1958) where mitochondria lack one phosphorylation site (Mackler *et al.*, 1972), and "cd" chondrodystrophy is a lethal autosomal recessive disorder that appears similar to metatropic dwarfism in human beings (Fox & Crary, 1975). These diseases are listed in more detail in Appendix 11.

1.3.11 Birds

Several forms of chondrodysplasia have been described in domestic birds. These are summarised in Appendix 12.

1.3.12 Non-human primates

A case of lethal short-limbed dwarfism resembling thanatophoric dwarfism in humans was described in a rhesus monkey (*Macaca mulatta*) (Zeman & Baskin, 1986). Grossly, all limbs were markedly rhizomelic and bones were curved with flared and irregular metaphyses. The head was relatively large, the face was flat with slight protusion of the mandible and the thorax was narrow due to short, flared ribs. A normal female reproductive tract was identified along with vestigial structures of undifferentiated tissue grossly resembling testicles in the inguinal area. Endochondral ossification was markedly disorganised, with no chondrocyte columns in physes and failure of orderly vascular penetration. The authors

described a poorly organised primary spongiosa with thick, irregular and often horizontal trabeculae (Zeman & Baskin, 1986).

1.4 Nutritional chondrodysplasia

1.4.1 Manganese deficiency

Manganese (Mn) is crucial for normal cartilage formation due to its function in two enzyme systems required for chondroitin sulphate synthesis: polymerase enzyme which links UDP-N-acetyl-galactosamine and UDP-glucuronic acid to form the chondroitin polysaccharide, and galactosyltransferase which incorporates galactose from UDP-galactose to the galactose-galactose-xylose trisaccharide linking the polysaccharide and the associated protein (Leach *et al.*, 1969; Tsopanakis & Herries, 1976). Because of this, Mn deficiency causes reversible reduction of the incorporation of sulphate into cartilage and impaired glycosaminoglycan metabolism (Leach & Muenster, 1962; Bolze *et al.*, 1985).

Skeletal abnormalities secondary to Mn deficiency have been reported in several species (Neher *et al.*, 1956; Tsai & Everson, 1967; Leach, 1968; Valero *et al.*, 1990) and typically occur in the offspring of deficient dams (Caskey *et al.*, 1944; Frost *et al.*, 1959; Valero *et al.*, 1990), but can also occur in young growing animals fed a deficient diet (Leach, 1968).

Mn deficiency in adult cattle results in reproductive disorders manifesting as increased returns to service (Rojas *et al.*, 1965). Calves born to deficient dams, however, show generalised chondrodysplasia characterised by short limbs with enlarged joints, enlarged costochondral junctions and excessive cartilage in long bone epiphyses (Valero *et al.*, 1990). Tracheal rings are thickened, and affected calves are prone to tracheal collapse. The animals recover slowly after birth, although in severe cases the legs remain bent and short for several months. Histologically, the costochondral junctions are irregular, with poorly organised proliferating chondrocytes and an absence of hypertrophic chondrocytes (Valero *et al.*, 1990). In many areas the matrix contains numerous fine basophilic fibrils and large areas of rarefaction. "Collagen rings" surrounding chondrocytes appear similar to those seen in achondrogenesis 1b and Texel chondrodysplasia. Many small PAS-positive droplets are present in the matrix close to the physes, consisting of interwoven fibres with a stellate appearance ultrastructurally. Similar changes are seen in articular cartilage of the long bones (Valero *et al.*, 1990), and cartilage

from affected calves has a low mucopolysaccharide content (Dyer & Rojas, 1965). Repeated outbreaks of chondrodysplasia in calves born following periods of drought in Australia are likely to be due to Mn deficiency (McLaren, *et al.*, 2007).

An increased susceptibility to convulsions was identified in Mn-deficient rats (Hurley *et al.*, 1963), along with ataxia resulting from abnormal development of the middle ear (Hurley *et al.*, 1960). Mn deficiency has also been associated with ataxia in chickens (Caskey *et al.*, 1944). Although Mn deficiency has not been assessed in the acorn calf abnormality or bovine congenital joint laxity and dwarfism, the history of poor diet and the type of lesions involved are suggestive of this nutritional aetiology (Barry & Murphy, 1964; Ribble & Janzen, 1987).

1.4.2 Plant toxicity

Exposure to certain plant toxins, especially during fetal development, can result in generalised skeletal defects. These can be due to direct action of a toxin or secondary to metabolic disturbance, and plant toxicity is usually a differential diagnosis in outbreaks of skeletal disease in grazing animals. Skeletal deformities resembling crooked calf disease, including arthrogryposis, scliosis, torticollis and cleft palate, can be seen in offspring from dams fed *Conium maculatum* (poison-hemlock), *Nicotiana glauca* (tree tobacco) or *Lupinus formosus* (lunara lupine) during gestation (Panter *et al.*, 1988b; Panter *et al.*, 1990). While primary myopathy is not considered to be responsible for the skeletal lesions (Abbot *et al.*, 1986), altered maternal uterine contraction may be involved in their development. Congenital conium toxicity in lambs results in carpal flexural deformities, angular limb deformities and kinked tails, all resolving by 8 weeks of age (Panter *et al.*, 1988a). Osteolathyrism is a non-congenital skeletal disorder caused by ingestion of *Lathyrus odoratus* (sweet pea) (Bélanger *et al.*, 1959). The resulting interference with lysyl oxidase activity increases connective tissue fragility, leading to skeletal effects that include joint deformities and spontaneous fractures.

Some plants may interfere with mineral absorption either directly or indirectly by concentrating other interacting minerals. For example, if high levels of calcium or phosphorus were present in forage, Mn absorption may be hindered, especially in ruminants (Spears, 2003). Various components of plants may directly interfere with mineral absorption, for example, some varieties of fava bean are able to inhibit the intestinal absorption of Mn and zinc (Rubio *et al.*, 1992), both of which are

minerals required for normal cartilage development and endochondral ossification (Leach *et al.*, 1969; Ortega *et al.*, 2004). Alternatively, mycotoxins produced by some fungi on decomposing plant matter may interfere with growth or development of many tissues (Wangikar *et al.*, 2005).

1.5 Conclusion and study objectives

The roles of matrix components and the pathways involved in the regulation of cartilage growth and endochondral ossification are complex and incompletely understood. Much progress has been made through the study of disorders which accent the effects of an abnormality in a single component of cartilage or its regulation. In order to learn most efficiently from a naturally occurring chondrodysplasia, its character needs to be thoroughly described microscopically, biochemically, and, ideally, genetically. More tools, especially with respect to molecular and genetic studies, are available than ever before and are proving increasingly valuable in improving our understanding of cartilage development and chondrodysplastic disease.

The chondrodysplasia reported recently in Texel sheep has presented an opportunity to study a new hereditary cartilage disorder in sheep, an animal with great potential as a model of skeletal disease in human beings. The preliminary studies already performed on chondrodysplastic Texel sheep have indicated strong similarities to the chondrodysplasias in human beings that involve reduced sulphate uptake. The objectives of this study are:

i) To describe in detail the gross, histological, histochemical and ultrastructural lesions of chondrodysplasia in Texel sheep in order to support the classification of this disorder as a new heritable disease of sheep and to assist in determining the likely mechanism.

ii) To measure the pattern of skeletal growth in chondrodysplastic lambs in order to assess the phenotypic effect of this genetic defect on different skeletal components, and to test the hypothesis that bone lengths and growth rates in chondrodysplastic lambs are less than in controls.

iii) To quantify the transport of sulphate into fibroblasts in order to test the hypothesis that undersulphation of hyaline cartilage in chondrodysplasia of Texel sheep is due to reduced cellular uptake of sulphate.

iv) To use a collection of microsatellite markers on samples from animals of known phenotype in order to test for linkage disequilibrium, which may be able to localise the causative mutation in chondrodysplasia of Texel sheep to an area within a chromosome. This will be the first step in the development of a genetic test capable of identifying carrier animals.

v) To quantify the chondroitin 4-sulphate and chondroitin 6-sulphate dissacharide content in chondrodysplastic and normal lambs of differing ages to (a) compare the disaccharide ratios with those found in disorders of sulphate transport in human beings, to (b) investigate the change in proteoglycan sulphation with age in affected and control animals, and to (c) test the hypothesis that a difference in chondroitin sulphate disaccharides is detectable in affected newborn lambs prior to the appearance of gross or microscopic lesions.

2 Pathology of chondrodysplasia in Texel sheep

2.1 Introduction

Chondrodysplasias are defined as abnormalities in the growth and/or development of cartilage. The aetiology may be genetic or environmental, and disproportionate dwarfism is a common manifestation due to the requirement for normal cartilage in the development of bones by endochondral ossification (Rimoin *et al.*, 1976; van der Harten *et al.*, 1988; Ala-Kokko *et al.*, 1990). Several forms of chondrodysplasia have been described in sheep, most notably "spider lamb syndrome" (SLS) in the Suffolk and Hampshire breeds, and the historical Ancon mutant (Landauer & Chang, 1949; Oberbauer *et al.*, 1995). Others include Cheviot achondroplasia (Wray *et al.*, 1971), Southdown dwarfism (Bogart, 1946), lethal dwarfism (a sporadic outbreak in a mixed breed flock) (Duffell *et al.*, 1985), and the recently identified chondrodysplasia in Texels (Thompson *et al.*, 2005).

Chondrodysplasia in Texel sheep was first recorded in a commercial sheep breeding and lamb fattening property in Southland, New Zealand. Preliminary investigation of affected lambs revealed what appeared to be an autosomal recessive form of chondrodysplasia pathologically distinct from other chondrodysplasias described in sheep (Byrne, 2005; Thompson *et al.*, 2005). Biochemical and histological features bore a strong resemblance to a group of chondrodysplasias caused by mutations in the gene for the diastrophic dysplasia sulphate transporter (DTDST), *SLC26A2*, in human beings. This group includes achondrogenesis type 1b, atelosteogenesis type II, diastrophic dysplasia and recessive multiple epiphyseal dysplasia (Hästbacka *et al.*, 1994; Superti-Furga, 1994; Hästbacka *et al.*, 1996b; Rimoin *et al.*, 1998; Rossi & Superti-Furga, 2001). These disorders are characterised by disproportionate dwarfism, deficient uptake of sulphate by chondrocytes and fibroblasts *in vitro*, and decreased sulphation of cartilage extracellular matrix proteoglycans (Hästbacka *et al.*, 1994; Rossi & Superti-Furga, 2001; Superti-Furga, 2001).

The objective of this investigation was to conduct a detailed morphological study of chondrodysplasia in Texel sheep. This information will support the classification of Texel chondrodysplasia as a new disease, distinct from other ovine chondrodysplasias, and provide an indication of the suitability of chondrodysplasia of Texel sheep as a model for similar disorders in human beings.

2.2 Materials and methods

2.2.1 Animals used and samples collected

The Southland property on which chondrodysplastic Texel sheep were first recognised was stocked with approximately 1100 mixed-breed ewes, including Texel, Perendale and White-faced Marsh breeds grazing improved pasture. Terminal sires included Perendale, Polled Dorset, Texel/Romney, Texel/Suffolk and Texel/Perendale breeds. Animals purchased from the Southland farm to provide the basis for a study at Massey University, Palmerston North, New Zealand, were 6 affected rams, 5 affected ewes, 17 putative carrier ewes, 8 rising two-tooth ewes and 11 lambs. All animals were either phenotypically normal or mildly affected with the disease and considered suitable for transport and future breeding. A research flock of sheep carrying the gene for Texel chondrodysplasia was established by out-crossing 3 affected rams to 221 unrelated phenotypically normal ewes, then back-crossing 123 F1 hogget daughters to the same 3 rams. Of the 83 F2 lambs produced, 46 survived to an age where the phenotype could be confidently determined. Approximately 50% of these lambs were homozygous affected, and 50% were phenotypically normal heterozygous carriers of the gene (Byrne, 2005). Another 22 ewes which either had chondrodysplasia or had previously produced chondrodysplastic offspring were present in the flock. These ewes were excluded from the original breeding trial but provided another 26 lambs for study. Lambs with no gross or microscopic lesions after 14 days of age were treated as controls.

Dystocia, attributed to the production of many heavy lambs combined with hogget mating, accounted for 26 lamb deaths. Another 16 deaths occurred due to poor mothering/exposure or bacterial infection. Severity of chondrodysplasia or other disorder (such as umbilical infection) required the euthanasia of 14 lambs during the first few weeks of life, and 13 more were euthanased at approximately 5 months of age. Other phenotypically normal animals were sold to slaughter at 5 months.

Post mortem examinations included a detailed examination of the skeletal system, focussing on the major limb joints and the thorax. Radiographs were taken from 16 animals, ranging in age from 22 days to older than 5 years. The views taken were cranio-caudal and lateral of the limbs, lateral of the spine, and dorso-ventral and lateral of the skull.

Samples collected routinely for histology included humerus, femur, lumbar vertebrae 3 and 4, costochondral junctions 6 and 7, trachea (a 2-3cm section approximately 5cm from the larynx), larynx, heart, lung, liver, and kidney. Bones from young lambs were fixed entire; those from older animals were sectioned longitudinally prior to fixation.

2.2.2 Tissue processing and analysis

All tissues for histology were fixed in 10% neutral buffered formalin for at least 48 hours, after which bones were cut into 2mm thick slabs using a low-speed band-saw. Bone slabs were decalcified in deltaCAL (Delta Products Groups, Aurora, IL) overnight, followed by rinsing in tap water. All tissues were embedded in paraffin wax using standard techniques, prior to sectioning at 4µm thickness. Sections from all samples were stained with haematoxylin and eosin (H&E), with an additional section from cartilaginous tissues stained with toluidine blue (TB). All tissues were processed in batches containing samples from both affected and control animals.

Chondrocyte density was investigated in the articular-epiphyseal complex, the transitional zone of the articular cartilage, and the central area of the tracheal cartilage in animals aged 14 days and over, by which time their disease status was clear. Sections of proximal humerus and trachea stained with H&E were examined at 400X magnification using a grid. For each of the 3 tissue areas, cells within the grid were counted at 3 random locations. If a point of observation fell in an area of severe chondrolysis or contained a blood vessel, another was selected.

Cartilage thickness was measured using Olympus AnalySIS software. Measurements were taken from the central articular and physeal cartilages of the proximal humerus. The thickness of tracheal cartilage was assessed by measuring the distance between abluminal and luminal perichondrium at the ventral-most point of a sectioned tracheal ring taken approximately 5cm from the larynx. Each measurement was performed in triplicate.

Cell density and cartilage thickness results were divided into the age groups of 2 weeks to 2 months, and 3 months to 5 months in order to account for the expected changes associated with age. These groups also reflected the age pattern of lambs available for study due to required euthanasia and farm management practices. The Student's t-test was used to assess the significance of differences in cell density

and cartilage thickness between groups, using Prism 3.0 (GraphPad Software Inc., 1999).

2.2.3 Immunohistochemistry

Immunohistochemical staining for type II and type VI collagen was carried out by Prof. C. Anthony Poole (Section of Orthopaedic Surgery, Department of Medical and Surgical Sciences, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand). Standard paraffin-embedded sections of articular and physeal cartilage, articular-epiphyseal complex and trachea were taken from a phenotypically normal and a chondrodysplastic lamb at 47 and 50 days of age, respectively. Previous histological investigation revealed that although the severity of lesions in chondrodysplasia of Texel sheep was variable, the nature of the lesions was not. Because of this, and constraints on resources, it was considered that a single representative chondrodysplastic lamb with a similarly-aged control would be acceptable for an immunohistochemical investigation.

2.2.4 Electron microscopy

Tissues for electron microscopy were collected from 16 lambs immediately post-euthanasia, and consisted of tracheal ring, approximately 5cm from the larynx, and articular cartilage from the proximal humerus. Samples were prepared using both the standard Karnovsky's method and a modified technique using ruthenium hexammine trichloride (RHT). The use of RHT in the fixation steps reduces fixation artifact in cartilage by precipitating proteoglycans, causing the cell membrane to remain attached to the pericellular matrix (Eggli *et al.*, 1985; Hunziker *et al.*, 1992). In this method, the samples were transported in phosphate-buffered saline, diced to $1mm^3$, and fixed for 2.5h using 0.7% RHT and 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). Samples were then rinsed in 0.1M cacodylate buffer (pH 7.4), soaked in the same buffer, and further fixed for 2.5h using 0.7% RHT and 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4), followed by another rinsing and soaking step with 0.1M cacodylate buffer (pH 7.4). The specimens were dehydrated through an acetone series then infiltrated in a 1:1 mixture of Spurr's resin and dry acetone overnight. This was replaced by pure resin the following morning and left to infiltrate for at least 4h. Specimens were moulded into pure resin and cured at 60°C for at least 48h. Sections 70nm in thickness were cut, placed on copper grids and stained with uranyl acetate and lead citrate.

2.3 Results

2.3.1 Clinical signs

Affected lambs were phenotypically normal at birth, but began to lag behind their peers in size by 2-3 weeks of age (occasionally as early as 1 week). This was particularly noticeable when only one lamb in a set of twins was affected. The chondrodysplastic lambs typically developed a stocky appearance with shortened neck and limbs, bilateral varus deformity of forelimbs centred on the carpus, flexion of the carpal joints, and a wide-based stance (Figures 2.1 and 2.2). The apparent stockiness was due to both the combination of reduced length of long bones (see Chapter 3) with relatively normal soft tissue growth, and the dorsoventrally flattened thorax creating a "barrel-like" appearance. The pectoral girdle was typically lower than the pelvic girdle in the standing animal, particularly in lambs with severe forelimb varus deformities and carpal flexion. Elbow and carpal joints were enlarged with palpable metaphyseal flaring of the distal radius. Hooves were often deformed, becoming rotated medially so that many animals walked on the abaxial surface of the lateral digit. This was considered to be secondary to the varus limb deformity and abnormal positioning. Lamb with severe varus and foot deformities also had marked hyperextension of the metacarpophalangeal joints (Figure 2.2B).

Chondrodysplastic lambs typically had a "stiff-legged" gait with a side-to-side rocking motion about their long axis. In severe cases, lambs were often reluctant to rise from recumbency. Following exercise, such lambs showed occasional episodes of collapse accompanied by marked dyspnoea and cyanosis. Sudden death occurred sporadically in severe cases. Affected lambs were sometimes observed walking slowly backwards, although no other clinical findings suggested a neurological disturbance.

Occasionally lambs could not be classified as chondrodysplastic until they were greater than 1 month of age. These tended to be relatively slow-growing lambs, possibly as a result of an underlying visceral infection or, in some cases, competition from siblings for milk. Some of these lambs did not develop the characteristic chondrodysplastic appearance until approximately 4 months of age, and tended to survive much longer than the more severely affected lambs. This was a similar pattern of development to that of the dwarf rams used as sires in the back-cross breeding trial (Thompson *et al.*, 2005).

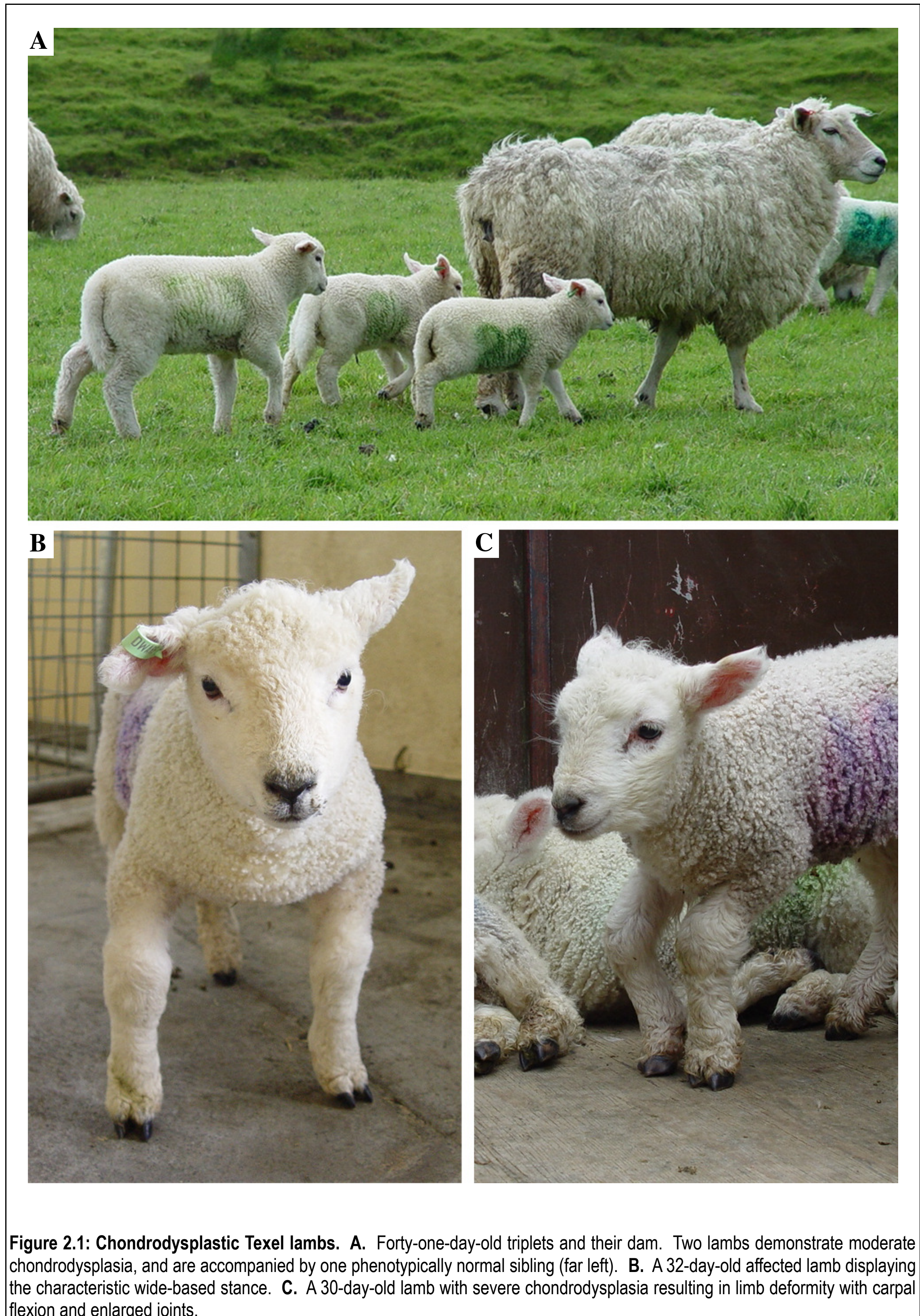


Figure 2.1: Chondrodysplastic Texel lambs. A. Forty-one-day-old triplets and their dam. Two lambs demonstrate moderate chondrodysplasia, and are accompanied by one phenotypically normal sibling (far left). **B.** A 32-day-old affected lamb displaying the characteristic wide-based stance. **C.** A 30-day-old lamb with severe chondrodysplasia resulting in limb deformity with carpal flexion and enlarged joints.

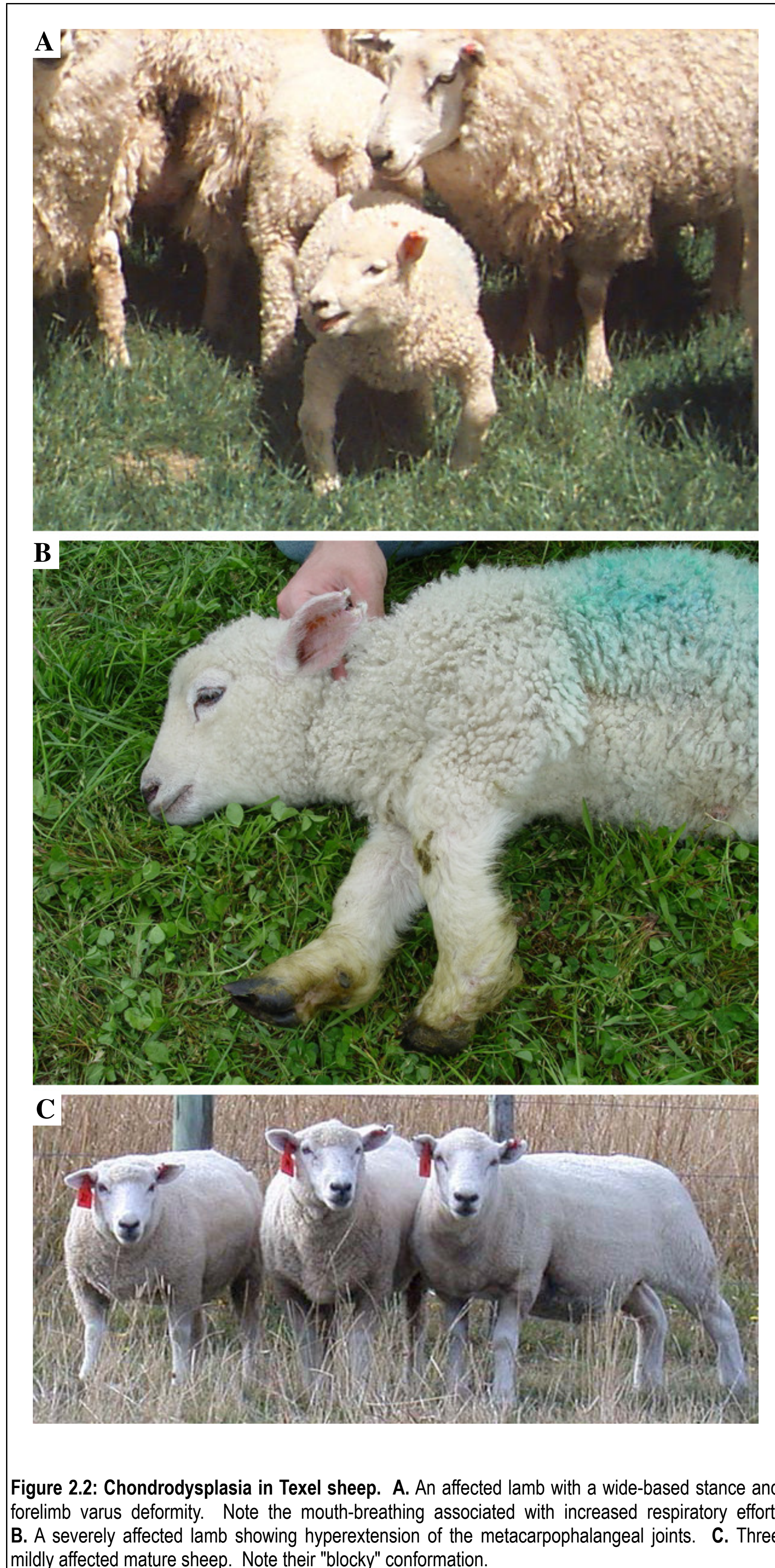


Figure 2.2: Chondrodysplasia in Texel sheep. A. An affected lamb with a wide-based stance and forelimb varus deformity. Note the mouth-breathing associated with increased respiratory effort. **B.** A severely affected lamb showing hyperextension of the metacarpophalangeal joints. **C.** Three mildly affected mature sheep. Note their "blocky" conformation.

2.3.2 Gross pathology

Post mortem examinations were carried out on 59 lambs ranging in age from 1-60 days, and 22 lambs ranging from 90-160 days. Three mature chondrodysplastic sheep were also examined. The youngest animal that demonstrated gross lesions of chondrodysplasia at post mortem examination was 2 weeks old.

The trachea of affected lambs was often twisted and possessed flattened and, in some cases, telescoping cartilage rings, resulting in an attenuated lumen (Figure 2.3). In cases of sudden death in severely affected lambs, marked narrowing of the tracheal lumen was visible radiographically and at necropsy. The tracheal lumen was consistently compressed to a triangle at the thoracic inlet, regardless of the degree of deformity elsewhere.

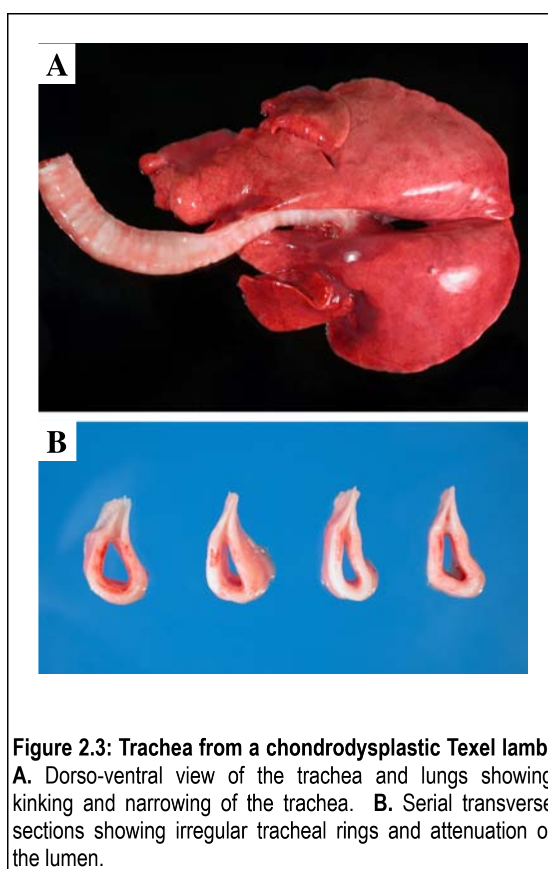


Figure 2.3: Trachea from a chondrodysplastic Texel lamb. **A.** Dorso-ventral view of the trachea and lungs showing kinking and narrowing of the trachea. **B.** Serial transverse sections showing irregular tracheal rings and attenuation of the lumen.

The thorax in chondrodysplastic lambs was abnormally shaped, with increased curvature and reduced length of ribs resulting in dorsoventral flattening of the thorax (Figure 2.4). In some cases, the caudal costal cartilages were undulating. The internal sterno-costal angle was typically reduced, and pectus excavatum was a feature in moderately- to severely-affected lambs. Costochondral junctions of all ribs in affected lambs were enlarged, creating sessile protrusions into the thoracic cavity (Figure 2.5). Vertebral bodies often appeared broad with bulging epiphyses.

Figure 2.4: Thoracic cavities from 3-4-month-old control and chondrodysplastic lambs. **A** and **C.** Control lamb. **B** and **D.** Chondrodysplastic lamb. Note the widths are similar between the two animals, yet the chondrodysplastic thorax is dorso-ventrally compressed with wider vertebral bodies. The chondrodysplastic thorax features shorter ribs, enlarged costochondral junctions, undulating caudal costal cartilages, and pectus excavatum.

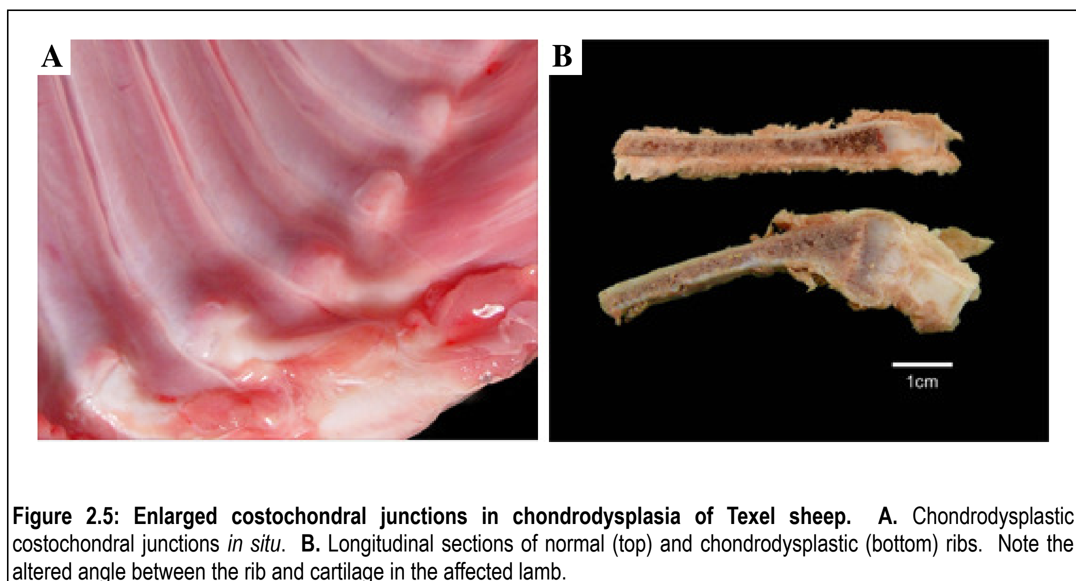


Figure 2.5: Enlarged costochondral junctions in chondrodysplasia of Texel sheep. **A.** Chondrodysplastic costochondral junctions *in situ*. **B.** Longitudinal sections of normal (top) and chondrodysplastic (bottom) ribs. Note the altered angle between the rib and cartilage in the affected lamb.

In severely affected lambs, erosion of articular cartilage from weight-bearing surfaces of major limb joints often occurred during the first 3 months of life and led to degenerative joint disease (Figure 2.6). Bone length appeared to be reduced and joints were enlarged in these animals (Figure 2.6B). In mildly affected animals that survived to adulthood, degenerative joint disease was largely confined to the shoulder and featured erosion of articular cartilage, osteophyte formation around chondro-osseous margins and development of loose bodies (Figure 2.6C). Articular and physeal cartilage of long bones was grossly thickened compared with age-matched controls, and was far easier to cut with a scalpel.

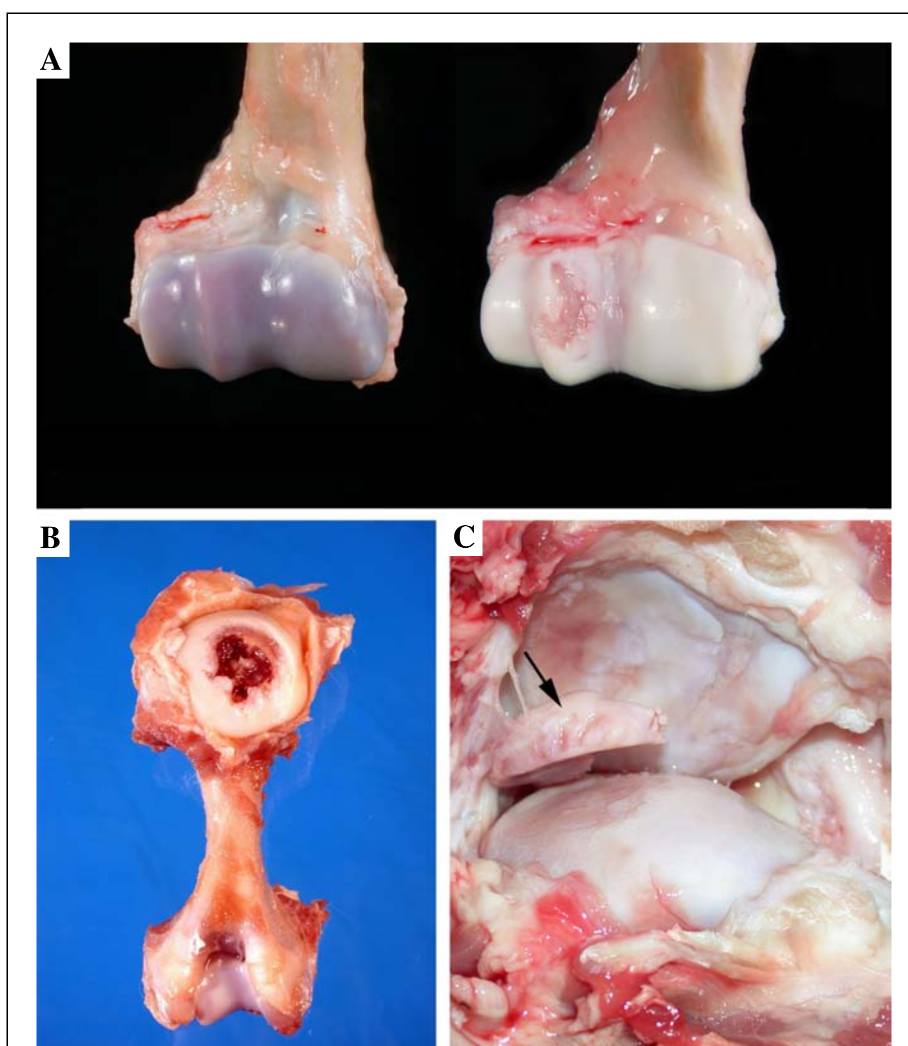


Figure 2.6: Joint lesions in chondrodysplasia of Texel sheep. A. Control (left) and chondrodysplastic distal humeri from 3-4-month-old lambs. Note the whiter colour of the chondrodysplastic cartilage, at least partly due to the increased cartilage thickness, and the erosion of the medial surface of the trochlear ridge. **B.** Extensive erosion of proximal humeral articular cartilage with exposure of subchondral bone in a severely affected 3-month-old lamb. Note the short appearance of the bone in conjunction with enlarged bone ends. **C.** Erosion of cartilage and osteophyte formation in the shoulder joint of a mature ram. Note the large loose body (arrow).

The only non-cartilaginous soft tissue lesion was bilateral hydronephrosis (Figure 2.7), identified in 2 month-old chondrodysplastic lambs and in 1 newborn lamb of unknown phenotype. In the latter, the hydronephrosis was accompanied by severe bladder distension and signs of dystocia. Hydronephrosis was not a constant finding in chondrodysplastic lambs, and may be unrelated.

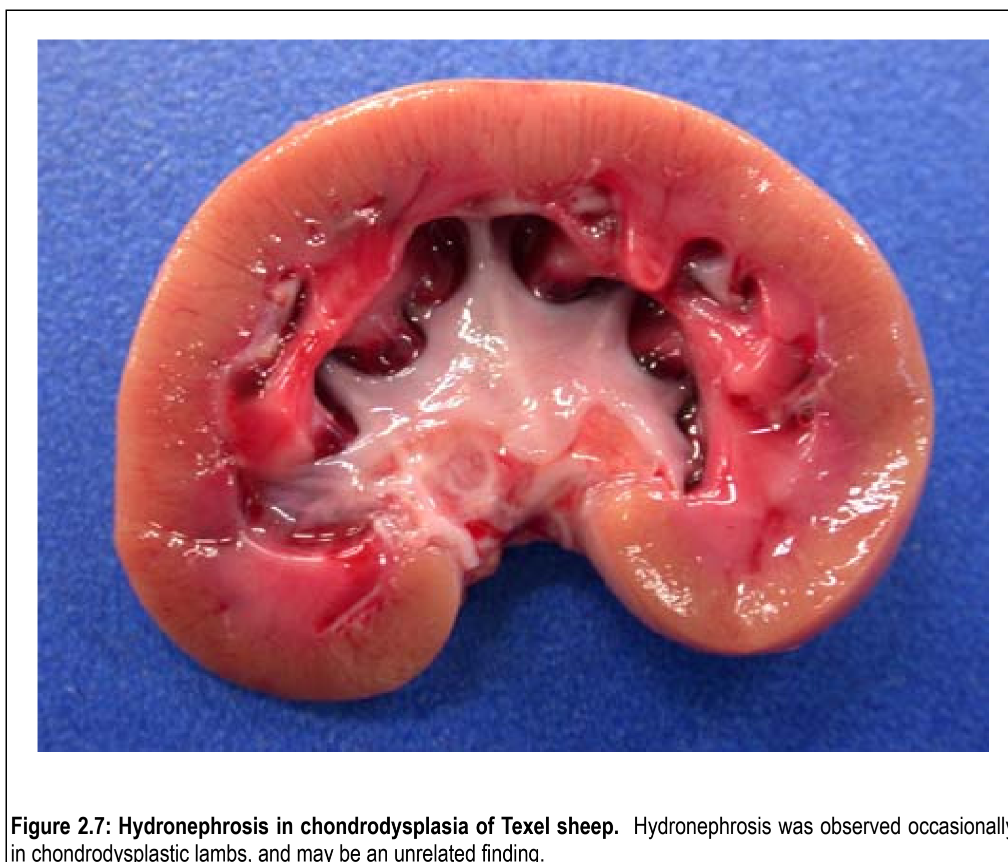


Figure 2.7: Hydronephrosis in chondrodysplasia of Texel sheep. Hydronephrosis was observed occasionally in chondrodysplastic lambs, and may be an unrelated finding.

2.3.3 Histological findings

The microscopic lesions of chondrodysplasia in Texel sheep were progressive and consistent in nature. They varied in severity depending on the age of affected lambs and tended to reflect the degree of gross deformity. No lesions were present in non-cartilaginous tissues other than bone, which frequently had abnormalities secondary to cartilaginous lesions.

Chondrocytes of hyaline cartilage throughout the articular-epiphyseal complex, deeper zones of articular and physeal cartilage, trachea and costochondral junction were surrounded by dense, concentric rings of abnormal, deeply basophilic fibrillar material (Figure 2.8). The interterritorial cartilage matrix often contained multiple, coalescing foci of rarefaction, merging in severe cases to form large areas of

chondrolysis traversed by coarse stands of denuded collagen. Chondrolysis was usually most severe in the transitional zone of articular cartilage and in the central area of tracheal cartilage, and in the most severe examples had progressed to regions of cystic degeneration devoid of matrix (Figure 2.9). Such lesions were also found adjacent to sites of articular cartilage erosion.

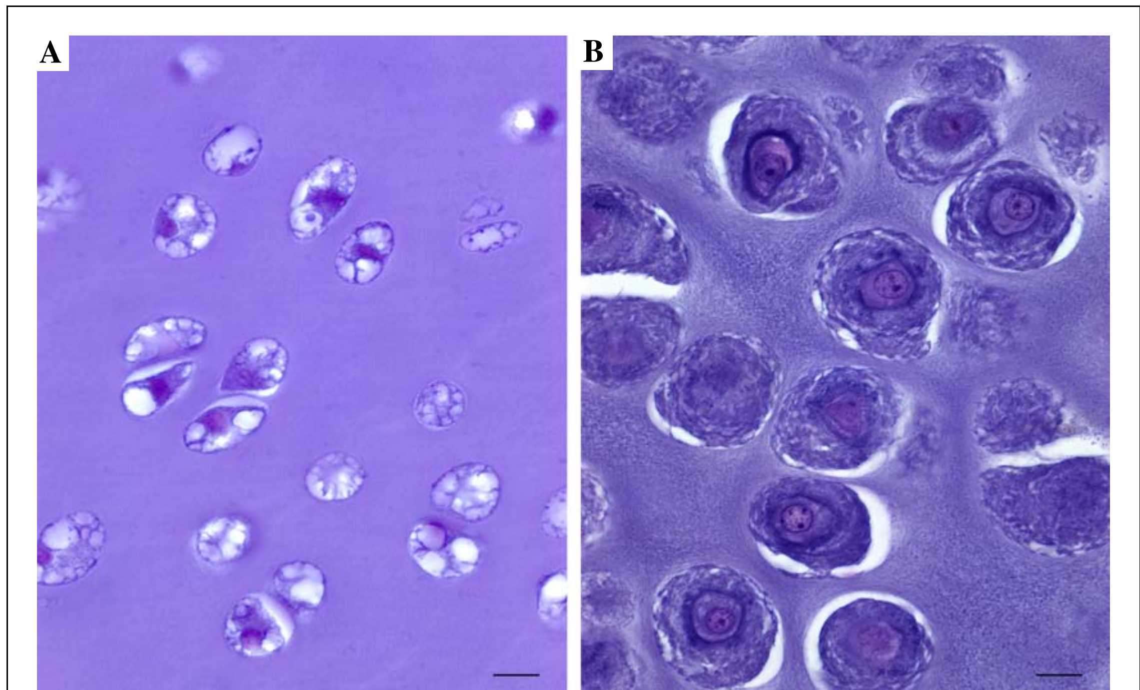


Figure 2.8: High-power view of control and chondrodysplastic hyaline cartilage. A. Cartilage from the trochanter of a 3-week-old control. **B.** Articular cartilage from a 3-week-old chondrodysplastic lamb. Note the characteristic perichondrocytic rings and coarse appearance of the chondrodysplastic cartilage matrix. TB stain, bar = 10μm.

Although approximately 50% of lambs in this study were expected to have chondrodysplasia, only 4 of the 38 necropsied earlier than 5 days of age had developed microscopic lesions of the disease. In these early cases, the lesions consisted of slight granularity of the articular and physeal cartilage matrix of the proximal humerus, and a narrow crescent of abnormal pericellular matrix associated with some chondrocytes in the deeper zones of articular cartilage. The youngest lamb demonstrating unequivocal microscopic lesions of chondrodysplasia was 9 days old.

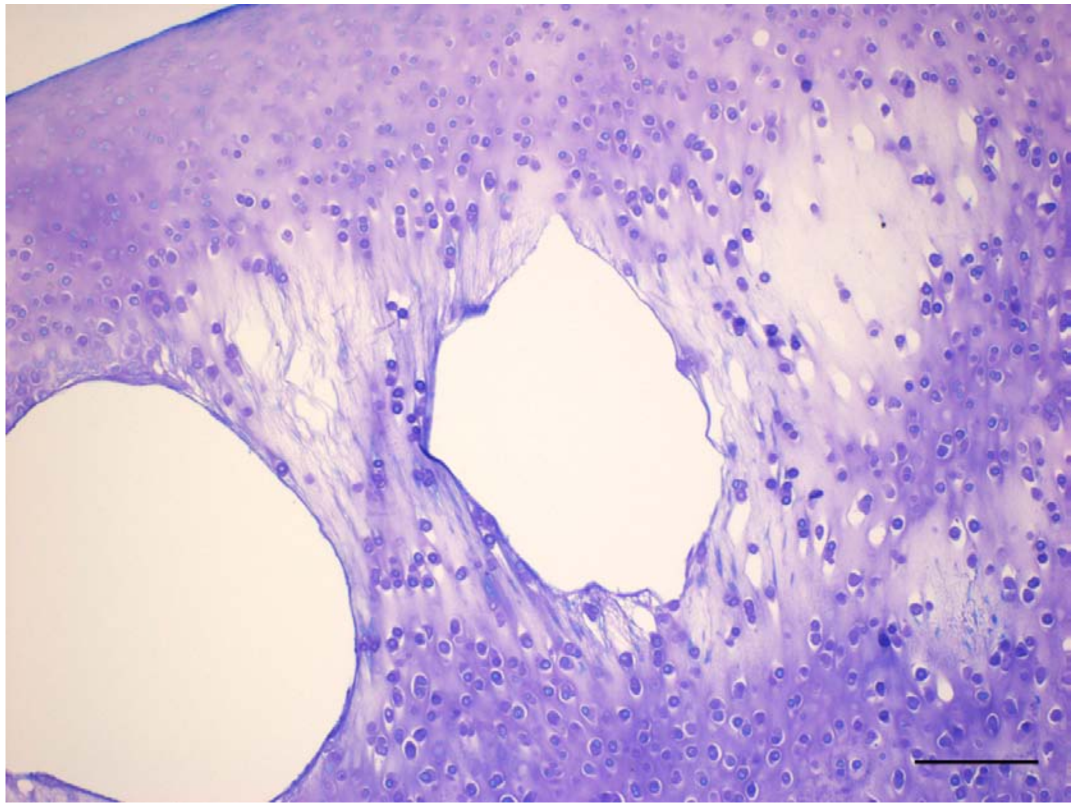


Figure 2.9: Articular cartilage from the proximal humerus of a 30-day-old chondrodysplastic lamb. Articular surface is at top-left. Note the severe chondrolysis and cystic degeneration of cartilage matrix. TB stain, bar = 200µm.

Microscopic lesions were present in all examined sections of hyaline cartilage from lambs showing gross lesions of chondrodysplasia. Although the severity of lesions varied between sites and between individual lambs, it did not appear to be related to the parental genotype. For example, one 22-day-old chondrodysplastic lamb with 2 chondrodysplastic parents had only mild lesions, including rarefaction of matrix in the transitional zone of the articular cartilage of the proximal humerus. In contrast, a 23-day-old chondrodysplastic lamb with only 1 affected parent showed far more pronounced lesions, including severe matrix rarefaction with fibrillar areas of denuded collagen.

The microscopic lesions in the cartilage of affected lambs surviving to 5 months of age were considerably milder than in severely chondrodysplastic lambs that died or were euthanased at 1 month of age. Pericellular rings of dense matrix were still prominent but there was little evidence of matrix rarefaction. The distal humeral

physes were closed in 5-month-old control lambs, but were still open in affected lambs at this age.

Physeal and metaphyseal involvement was variable. In some affected lambs, the physes and primary spongiosa were relatively well organised, while in others hypertrophic cell columns were disorganised, the primary spongiosa contained few normal trabeculae perpendicular to the physis, and the secondary spongiosa was comprised of sparse, thickened and sometimes transverse septae (Figures 2.10 and 2.11). The latter were probably growth-arrest lines, and therefore not directly related to the chondrodysplasia.

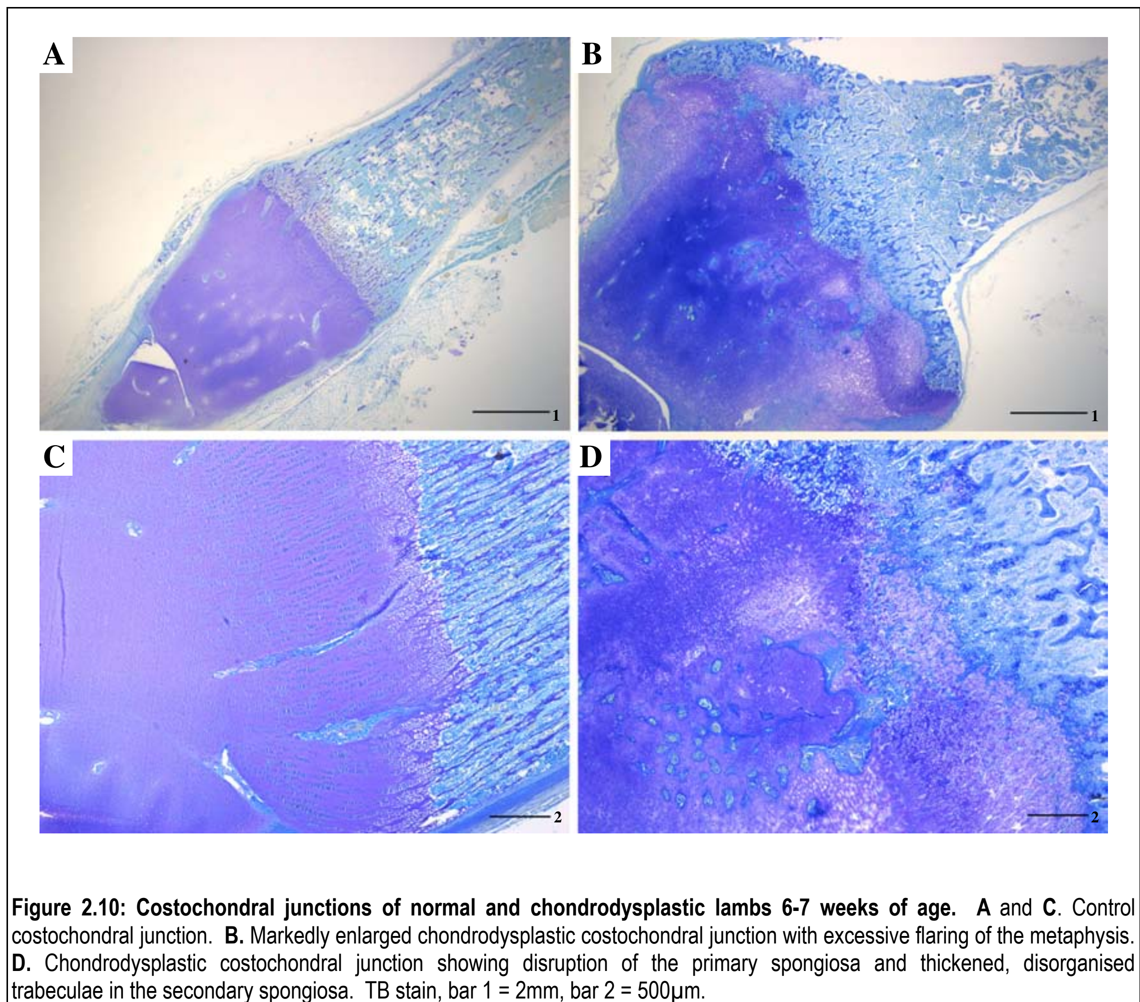


Figure 2.10: Costochondral junctions of normal and chondrodysplastic lambs 6-7 weeks of age. A and **C**. Control costochondral junction. **B.** Markedly enlarged chondrodysplastic costochondral junction with excessive flaring of the metaphysis. **D.** Chondrodysplastic costochondral junction showing disruption of the primary spongiosa and thickened, disorganised trabeculae in the secondary spongiosa. TB stain, bar 1 = 2mm, bar 2 = 500µm.

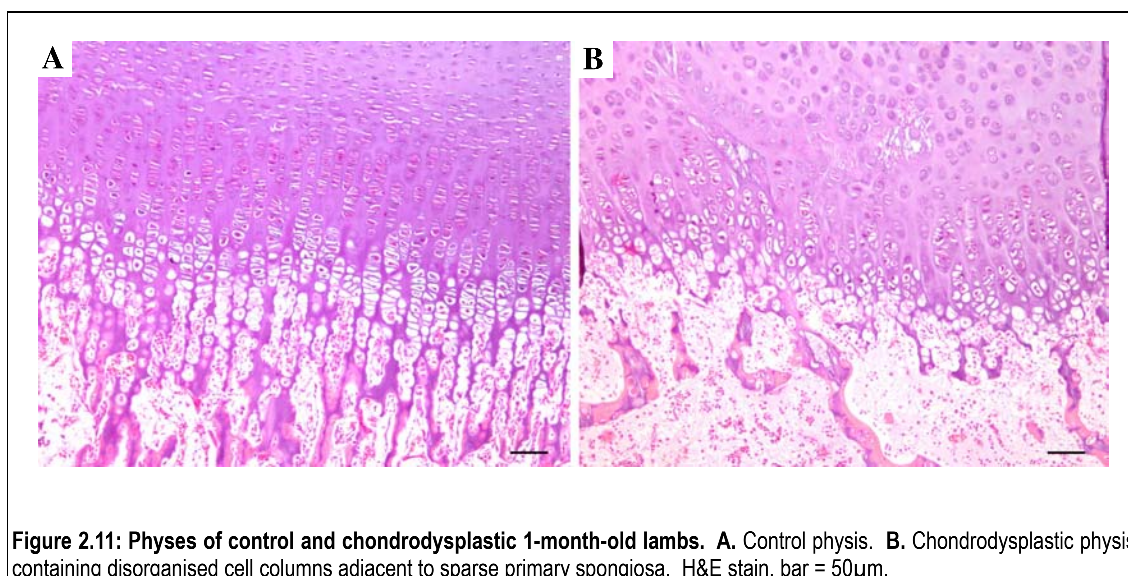


Figure 2.11: Physes of control and chondrodysplastic 1-month-old lambs. A. Control physis. **B.** Chondrodysplastic physis containing disorganised cell columns adjacent to sparse primary spongiosa. H&E stain, bar = 50µm.

The vertebral physes of chondrodysplastic lambs showed similar lesions to those in the appendicular skeleton. Vertebral bodies of young chondrodysplastic lambs (approximately 1 month of age) had thickened epiphyseal and physeal cartilages, and were markedly abnormal at 3-4 months of age (Figure 2.12). Vertebrae from some chondrodysplastic lambs contained islands of persistent cartilage in the metaphysis, aligned parallel to the physis. Similarly, excessive hyaline cartilage remained in epiphyses at the periphery of some vertebral bodies.

Although chondrocytes of the nucleus pulposus were sometimes surrounded by narrow collagen rings (usually <⅓ cell diameter), the matrix did not appear different from that of controls. In both affected and control lambs, the intensity of TB staining of cartilage matrix decreased with age, but cartilage from chondrodysplastic lambs consistently demonstrated less staining intensity than that of age-matched controls.

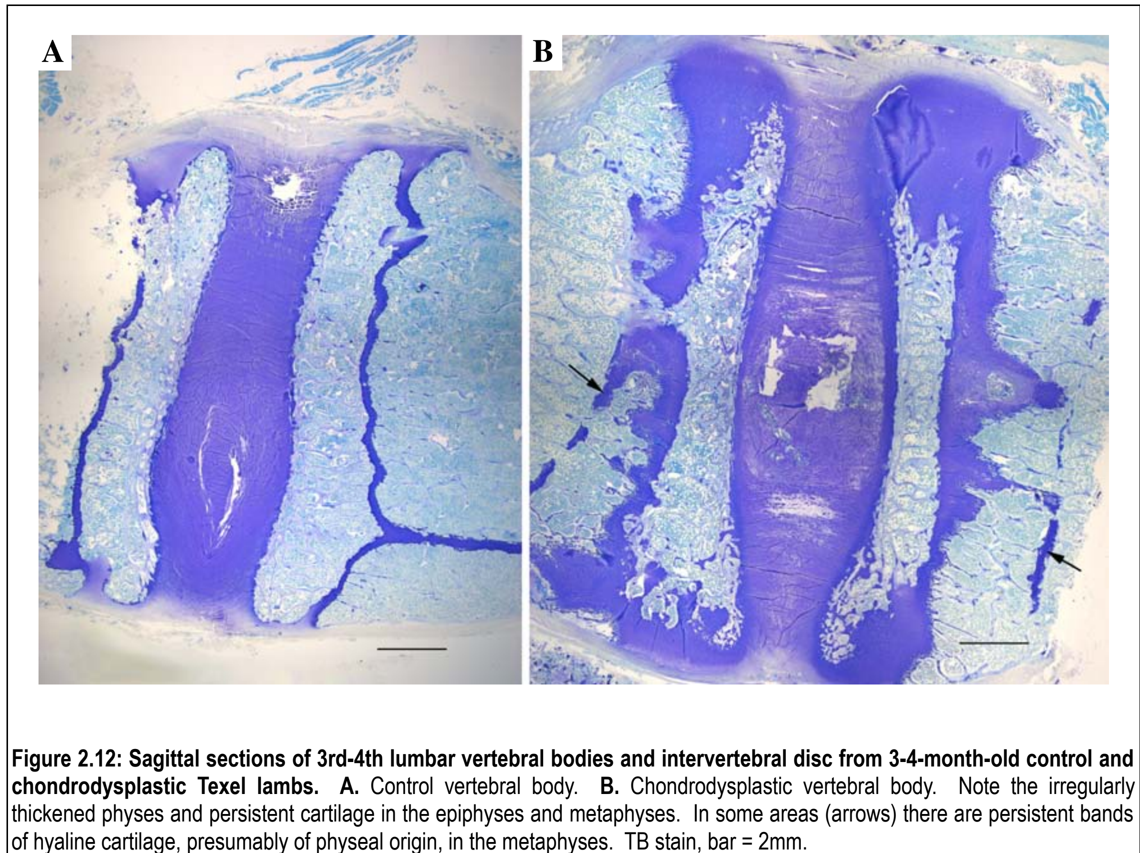


Figure 2.12: Sagittal sections of 3rd-4th lumbar vertebral bodies and intervertebral disc from 3-4-month-old control and chondrodysplastic Texel lambs. **A.** Control vertebral body. **B.** Chondrodysplastic vertebral body. Note the irregularly thickened physes and persistent cartilage in the epiphyses and metaphyses. In some areas (arrows) there are persistent bands of hyaline cartilage, presumably of physeal origin, in the metaphyses. TB stain, bar = 2mm.

Tracheal cartilage in severely affected lambs often had an asymmetric distribution of cells from the inner to outer surface of the tracheal ring (Figure 2.13). The superficial abluminal cartilage was irregularly thickened and disorganised, with perichondrocytic collagen rings often so extensive that little interterritorial matrix remained. Rarefaction of interterritorial matrix was only apparent in the trachea and bronchi. Perichondrocytic rings became smaller as airway size decreased, and in small bronchi existed only as narrow pericellular crescents of dense matrix.

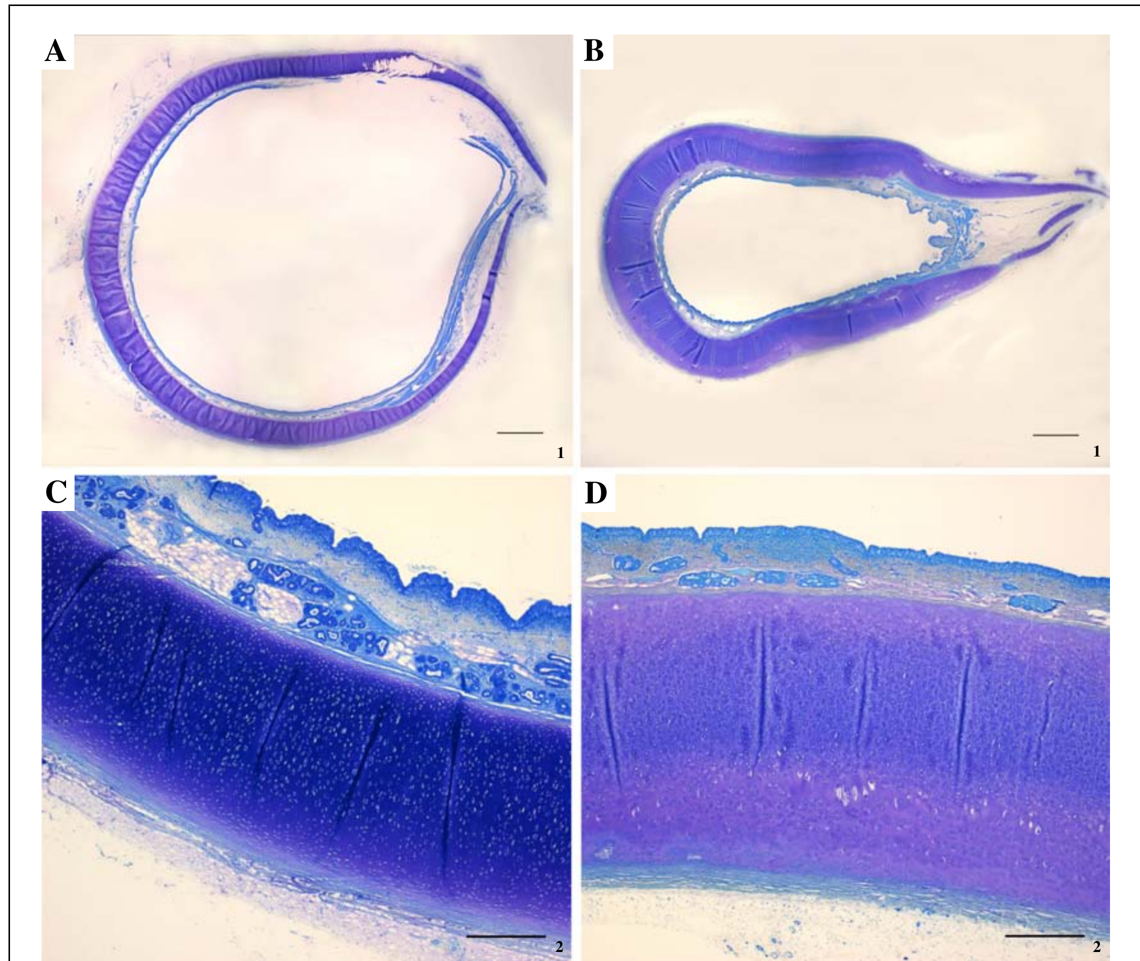


Figure 2.13: Tracheal cartilage from normal and chondrodysplastic lambs. A and **C.** Trachea from a normal 5-month-old lamb. **B.** Trachea from a chondrodysplastic 3-4-month-old lamb with thickened tracheal cartilage and attenuation of the tracheal lumen. **D.** Same trachea as B, showing thickened appearance and pale staining of the chondrodysplastic cartilage. TB stain, bar 1 = 2mm, bar 2 = 500µm.

2.3.4 Histomorphometry

The results of chondrocyte density estimation are summarised in Table 2.1. The tracheal, proximal humeral articular cartilage and articular-epiphyseal cartilage complex in chondrodysplastic lambs up to 2 months of age had a significantly lower cell density than that of controls. In lambs 3-5 months of age, only the articular cartilage from chondrodysplastic lambs was significantly hypocellular. Chondrocyte densities decreased with age in both normal and chondrodysplastic lambs, as would be expected (Mitrovic *et al.*, 1983).

Table 2.1: Chondrocyte density expressed as cells per 400X field in cartilage of chondrodysplastic and control Texel lambs. Cells were counted in the transitional zone of the proximal humeral articular cartilage, the articular-epiphyseal complex of the proximal humerus, and the central cartilage of the trachea.

Group	**2 weeks to 2 months of age**					
Location	Articular cartilage		Articular-epiphyseal complex		Tracheal cartilage	
Status	Control	Affected	Control	Affected	Control	Affected
Mean ± SEM	185 ± 29	115 ± 10	156 ± 15	102 ± 12	212 ± 32	140 ± 16
Difference between means	69 ± 24*		54 ± 22*		72 ± 32*	
Group	**3 months to 5 months of age**					
Location	Articular cartilage		Articular-epiphyseal complex		Tracheal cartilage	
Status	Control	Affected	Control	Affected	Control	Affected
Mean ±SEM	129 ± 11	82 ± 7	94 ± 10	74 ± 8	94 ± 5	76 ± 7
Difference between means	46 ± 14**		20 ± 14		18 ± 8	

* = P ≤ 0.05, ** = P ≤ 0.01 using the Student's t-test. Averaged results were rounded to the nearest whole number.

The results of cartilage thickness measurements are summarised in Table 2.2. Although some severely affected lambs had markedly thickened tracheal rings (see Figures 2.3 and 2.13), this was highly variable. In spite of their gross appearance, the mean thickness of the ventral tracheal rings was not significantly different from that of phenotypically normal carrier animals. The mean thickness of articular and physeal cartilage in chondrodysplastic lambs was significantly greater than that of controls at all ages. The articular cartilage of the proximal humerus was thicker by approximately 700µm in the younger group of chondrodysplastic lambs, and this difference declined to 317µm in older lambs. The mean thickness of the central physeal cartilage of the proximal humerus in chondrodysplastic lambs 2 weeks to 2 months of age was 360µm greater than in age-matched control lambs, the difference increasing to 563µm in the older age group.

Table 2.2: Cartilage thickness of chondrodysplastic and control Texel lambs (μm). Tracheal thickness was taken at the ventral area, articular and physeal measurements were taken in the central regions.

Group	**2 weeks to 2 months of age**					
Location	Tracheal cartilage		Articular cartilage		Physeal cartilage	
Status	Normal	Affected	Normal	Affected	Normal	Affected
Mean ± SEM	1476 ± 214	1464 ± 51	974 ± 83	1674 ± 87	699 ± 106	1059 ± 38
Difference between means	13 ± 155		700 ± 132***		360 ± 87**	
Group	**3 months to 5 months of age**					
Location	Tracheal cartilage		Articular cartilage		Physeal cartilage	
Status	Normal	Affected	Normal	Affected	Normal	Affected
Mean ± SEM	1594 ± 114	1849 ± 128	683 ± 38	1000 ± 57	492 ± 33	1055 ± 146
Difference between means	256 ± 172		317 ± 69***		563 ± 141**	

** = P ≤ 0.01, *** = P ≤ 0.001, using the Student's t-test. Averaged results were rounded to the nearest μm.

2.3.5 Immunohistochemistry

Immunohistochemical staining for types II and VI collagen in cartilage from chondrodysplastic and control Texel lambs is illustrated in Figure 2.14. In the control lamb, type II collagen was distributed throughout the matrix of articular-epiphyseal, physeal and tracheal cartilage, with increased epitope binding in territorial zones. Type VI collagen was mostly restricted to the immediate pericellular area, with some minor binding in interterritorial zones. In cartilage from the chondrodysplastic lamb, type II collagen was mostly absent from the condensed pericellular matrix, had a more granular appearance than in control cartilage, and showed strong epitope-binding in areas in which denuded collagen fibrils were visible using H&E and TB stains. Type VI collagen was arranged in large, dense, irregular rings about the chondrocytes. These rings had an identical distribution to the dense pericellular rings of matrix seen in H&E- or TB-stained sections of chondrodysplastic cartilage. The localised staining of type VI collagen and virtual absence of type II collagen in these areas indicates that the pericellular "collagen" rings that occur in the cartilage of chondrodysplastic Texel sheep contain type VI collagen.

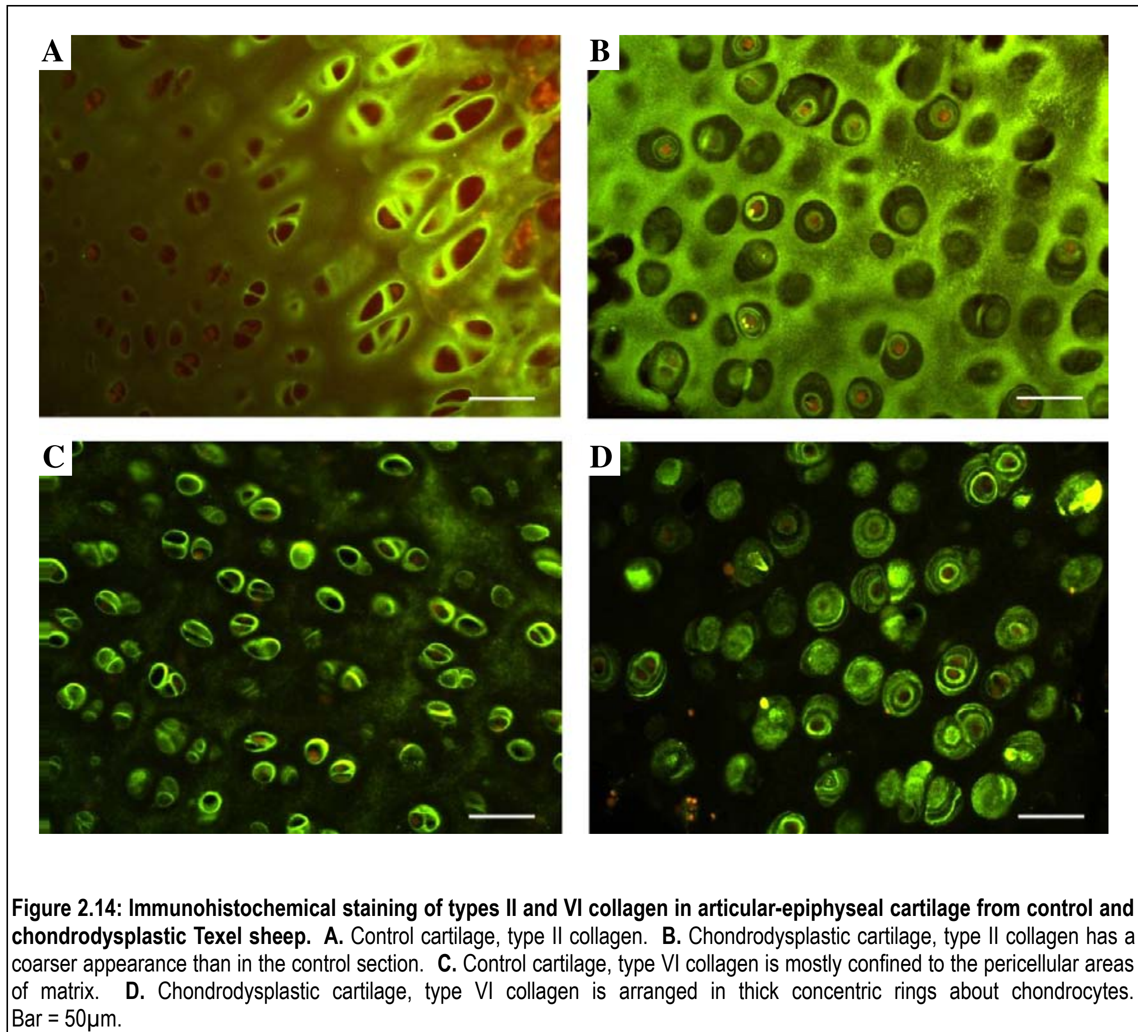


Figure 2.14: Immunohistochemical staining of types II and VI collagen in articular-epiphyseal cartilage from control and chondrodysplastic Texel sheep. **A.** Control cartilage, type II collagen. **B.** Chondrodysplastic cartilage, type II collagen has a coarser appearance than in the control section. **C.** Control cartilage, type VI collagen is mostly confined to the pericellular areas of matrix. **D.** Chondrodysplastic cartilage, type VI collagen is arranged in thick concentric rings about chondrocytes. Bar = 50µm.

2.3.6 Electron microscopy

Transmission electron microscopy of chondrocytes from control and chondrodysplastic Texel lambs is illustrated in Figures 2.15 and 2.16. The rings of dense matrix surrounding chondrocytes from chondrodysplastic lambs contained short, abnormally thick collagen fibrils. In contrast, pericellular collagen fibrils in cartilage from control animals were generally finer than those further from the cell (Figure 2.15). As suggested by light microscopy, the nature of the matrix changed abruptly at the junction of the pericellular collagen rings and the interterritorial matrix. Areas of chondrolysis within the interterritorial matrix of chondrodysplastic lambs contained dense clusters of parallel collagen fibrils, which were unmasked by the relative absence of proteoglycans. Other portions of the interterritorial matrix appeared normal (Figure 2.16). Neither the thickened pericellular collagen fibrils nor the areas of chondrolysis were present in control cartilage. No abnormalities were apparent in chondrocytes from affected lambs.

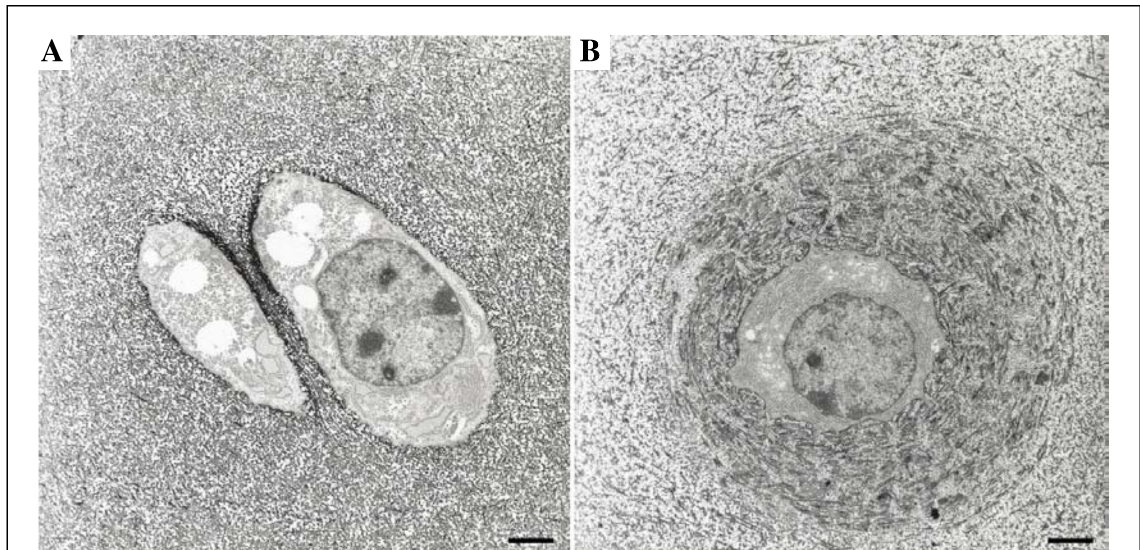


Figure 2.15: Articular cartilage chondrocytes and pericellular matrix from control and chondrodysplastic Texel lambs. **A.** Control chondrocytes. **B.** Chondrodysplastic chondrocyte. Note the concentric rings of dense, abnormal matrix surrounding the chondrocyte. RHT fixation, bar = 2μm.

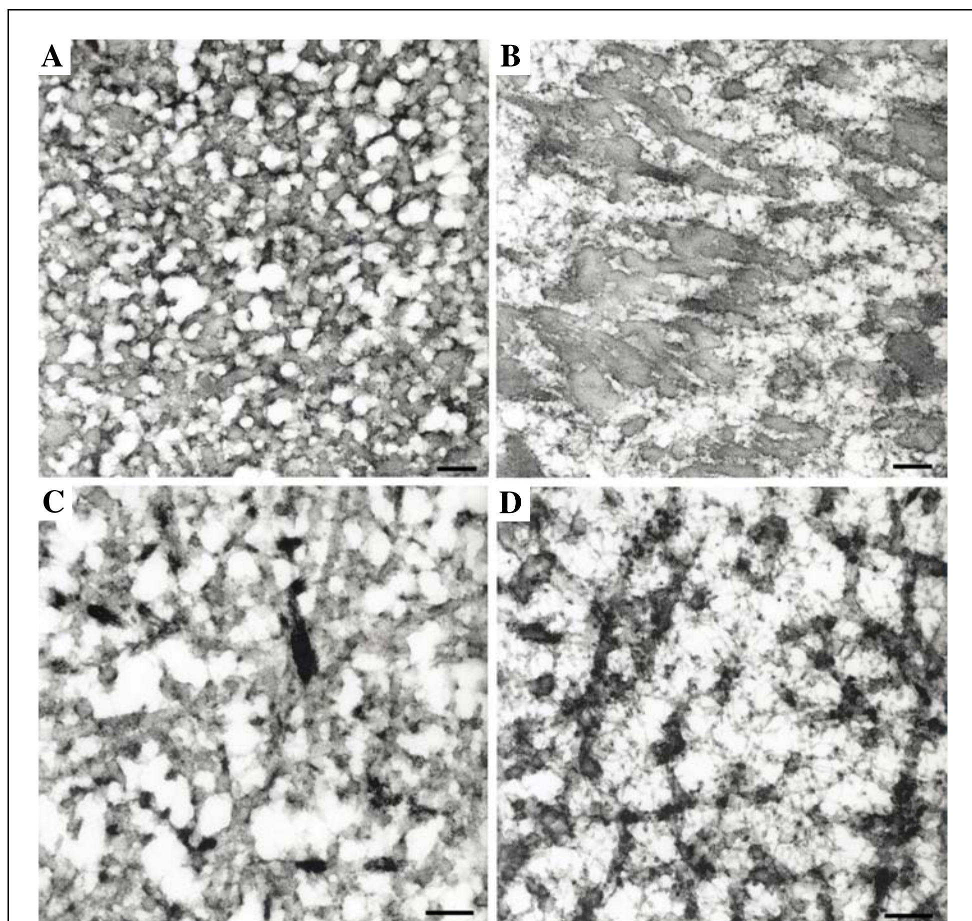


Figure 2.16: High power views of pericellular and interterritorial matrix of cartilage from control and chondrodysplastic Texel lambs. **A.** Control pericellular matrix. **B.** Chondrodysplastic pericellular matrix demonstrating irregular clumping of matrix components. **C.** Control interterritorial matrix. **D.** Interterritorial matrix from a chondrodysplastic lamb with similar appearance to control tissue, **D.** RHT fixation, bar = 0.2μm.

2.4 Discussion

Chondrodysplasia of Texel sheep is characterised grossly by disproportionate dwarfism, angular limb deformity, degenerative joint disease in severely affected animals, a barrel-shaped chest, twisting of the trachea with attenuation of the tracheal lumen and soft, thickened articular and physeal cartilage. Histologically, chondrocytes were surrounded by "collagen rings" containing type VI collagen, cartilage matrix contained areas of rarefaction and chondrolysis, and articular, physeal and tracheal cartilages were hypocellular compared with controls, at least in lambs younger than 2 months. The gross and microscopic lesions described here in Texel sheep confirm that this inherited chondrodysplasia is distinct from "spider lamb syndrome" (SLS) and other chondrodysplasias of sheep.

Disproportionate dwarfism, angular limb deformity and an abnormally shaped thorax are features of many chondrodysplasias in man and animals, and reflect the dependency of skeletal development on normal cartilage growth and maturation (Erlebacher *et al.*, 1995). The microscopic and ultrastructural lesions in hyaline cartilage matrix of affected Texel lambs are highly distinctive and closely match those seen in human achondrogenesis type 1b, atelosteogenesis type II and diastrophic dysplasia, all of which are recessively inherited disorders caused by mutations in the diastrophic dysplasia sulphate transporter, *SLC26A2* (van der Harten *et al.*, 1988; Shapiro, 1992; Sillence *et al.*, 1997; Superti-Furga, 2001). Neonatal death occurs in children with achondrogenesis type 1b and atelosteogenesis type II (van der Harten *et al.*, 1988; Superti-Furga, 1994; Hästbacka *et al.*, 1996b). Histologically, there may be failure of physeal organisation, degeneration and rarefaction of the interterritorial matrix, and condensation of matrix debris around chondrocytes, variably referred to as collagen rings, haloes, or pericellular debris (van der Harten *et al.*, 1988; Shapiro, 1992; Superti-Furga, 1994).

The decreased intensity of staining with TB in cartilage from chondrodysplastic Texel sheep compared with controls supports a reduction in sulphate ions in the matrix, which is consistent with the biochemical findings of Byrne (2005) (see Figure 1.7). An *SLC26A2* knock-out mutant mouse also demonstrated reduced staining with TB, and, although not discussed by the authors, histological images illustrate abnormal pericellular matrix (Forlino *et al.*, 2006).

Chondrodysplasia of Texel sheep progresses similarly to disorders of sulphate transport (Superti-Furga, 2001; Forlino *et al.*, 2005). Shapiro (1992) described the development of microscopic lesions in diastrophic dysplasia in two patients throughout childhood. The matrix rarefaction in these cases appeared in the earliest cartilage examined, while lesions corresponding to collagen rings (referred to as “target” cells) were not clearly evident until several years of age. A similar development of lesions was seen in Texel chondrodysplasia, where the earliest lesions involved matrix rarefaction, while collagen rings became more pronounced with age. An apparent deviation from this pattern occurred in affected lambs older than 1 month, in which microscopic lesions appeared to be less severe than in 1-month-old affected lambs. While this could be due to ‘regression’ of the lesions, it more likely reflects the death or euthanasia of the more severely affected lambs at a younger age. Severely affected lambs that either died following exercise or were euthanased due to respiratory distress had tracheal lesions that were consistent with trachal collapse. The soft, thickened cartilage of the tracheal rings was frequently deformed to a slim oval (see Figure 2.3B), and the increased respiratory effort clinically evident in severely affected Texel lambs probably generated enough negative pressure in the cervical airway to cause the lumen to collapse.

The lesions in chondrodysplasia of Texel sheep were highly variable in severity, corresponding to the variability of expression demonstrated in children with *SLC26A2* mutations (Superti-Furga, 2001). Although the nature of the mutation itself influences the phenotype through the residual level of function, other factors must be involved in determining the severity of disease, as first-degree relatives with the same *SLC26A2* mutation have demonstrated differing phenotypes (Superti-Furga, 2001). Variability in the capacity to utilise sulphate from other sources, such as intracellular sulphydral compounds (Rossi *et al.*, 2003), may explain some of the phenotypic irregularities in disorders caused by *SLC26A2* mutations.

Variability of the phenotype in diastrophic dysplasia may also reflect a complex pathogenesis, possibly involving both a cartilage matrix defect and secondary changes in the fibroblast growth factor (FGF) signalling pathway, which utilises heparan sulphate (Superti-Furga, 2001; Harmer, 2006). Undersulphation of heparan sulphate in disorders of sulphate transport has not been thoroughly investigated, but is considered likely to occur (Forlino *et al.*, 2005). This is supported by the undersulphation of heparan sulphate *in vitro* in the presence of

low-sulphate media (Tyree *et al.*, 1986). The reduced sulphation of cartilage demonstrated in Texel lambs with chondrodysplasia (Byrne, 2005) suggests that undersulphation of heparan sulphate may be a feature of this disease, potentially resulting in disturbed FGF signalling (van der Eerden *et al.*, 2003). Severe reduction of FGF signalling can result in ongoing chondrocyte proliferation, as occurs in SLS (Iwata *et al.*, 2000; Beever *et al.*, 2006). This mechanism could explain the delayed closure of physes and increased cartilage thickness, accompanied by reduced cell density, that is a feature of chondrodysplasia in Texel sheep. Logically, this action would result in elongated bones, as seen in SLS, and not the reduced bone lengths typical of chondrodysplasia in Texel sheep. Reduced FGF signalling secondary to another matrix defect, rather than a primary FGF receptor mutation, might produce a different phenotype to SLS. A potential outcome could be delayed maturation of chondrocytes without the stability of cartilage matrix and organisation of chondrocyte development required to develop normal endochondral bone, producing a combination of delayed physeal closure and reduced bone length.

The articular cartilage in chondrodysplastic lambs was consistently thicker than normal, but the difference lessened with age. This probably reflects death or euthanasia of the most severely affected animals prior to 100 days, and the survival of the less severely affected lambs meant that they were not included in the younger age group. Conversely, the physeal cartilage increased in relative thickness with age in chondrodysplastic lambs, perhaps due to the increased initial thickness of physes and their delayed closure. Conceivably, the increased articular and physeal cartilage thickness could be due to the influence of positive hydrostatic pressure on matrix proteins in conjunction with reduced structural integrity of the matrix. However, since a major factor in the generation of hydostatic pressure in cartilage is the presence of sulphate ions (Sandy *et al.*, 1997), it would be reasonable to anticipate reduced hydrostatic pressure in cartilage from chondrodysplastic Texel sheep, suggesting that a different mechanism is involved.

The abnormal arrangement of collagen fibrils in hyaline cartilage from chondrodysplastic Texel sheep is identical to that described in human disorders of sulphate metabolism. Collagen arrangement in diastrophic dysplasia is so deranged that investgators initially suspected a primary defect in type II collagen (Stanescu *et al.*, 1984). The mechanism for such a defect in cartilage matrix proteoglycan sulphation in association with these abnormalities is unclear, but

Superti-Furga (2001) suggests that the "unmasking" of collagen fibres in defects of sulphate metabolism is secondary to the deficiency of sulphated proteoglycans. This explanation seems likely, as sulphate is required for normal collagen fibrillogenesis (Eyre *et al.*, 1987; Eyre, 2002), and several disorders of cartilage sulphation feature similar abormalities of matrix collagen, including those caused by mutations in *SLC26A2* (van der Harten *et al.*, 1988; Shapiro, 1992; Sillence *et al.*, 1997), mutations in *CHST11* (Klüppel *et al.*, 2005) and congenital Mn deficiency (Valero *et al.*, 1990).

Immunohistochemistry of cartilage in Texel chondrodysplasia demonstrated that both types II and VI collagen are distributed abnormally in the matrix, suggesting that extracellular assembly of these collagen types is disturbed. Normally, type IX collagen is sulphated and is involved in the regulation of type II collagen fibril diameter (Eyre *et al.*, 1987; Eyre, 2002). Potentially, undersulphation of type IX collagen may play a part in the abnormal organisation of type II collagen, but type IX collagen in cartilage from Texel lambs with chondrodysplasia was reported to migrate normally on SDS-PAGE (Byrne, 2005). This suggests normal or near-normal charge density and conformation of this type of collagen, and does not support significant undersulphation of type IX collagen in affected lambs.

The close resemblance of chondrodysplasia in Texel sheep to the human chondrodysplasias caused by sulphate transporter defects suggested a similar mechanism of pathogenesis, making *SLC26A2* a prime candidate gene. A sequencing investigation virtually ruled out a mutation within the exonic code of this gene (Byrne, 2005), but did not explore regulatory sites. It is therefore possible that reduced expression of a normal sulphate transporter or a mutation in another component of the pathway whereby sulphate is transported within the cell and added to proteoglycans could be responsible for chondrodysplasia in Texel sheep. Even if the causative mutation in this disease does not involve *SLC26A2*, the microscopic, ultrastructural and biochemical features suggest that a similar mechanism is likely to be involved. This ovine disease may therefore be useful as a model to investigate ameliorative therapy and management in the milder (non-lethal) disorders of sulphate transport, such as diastrophic dysplasia, in human beings. Small laboratory animals are most commonly studied as models of human diseases due to the lower cost of management and the short generation interval. In diseases of the skeleton, however, the effect of gravity on the mass of the animal needs to be considered because of the potential influence of mechanical forces

during skeletal development (Carter, 1987). Because of this, larger animals such as dogs and sheep, which are closer in size to human beings, have been recognised as more suitable models for studies of the skeletal system (Pearce *et al.*, 2007). The delayed onset of lesions in chondrodysplasia of Texel sheep also creates an opportunity for intercessory treatment.

The development of chondrodysplasia in affected Texel lambs was rapid, with animals apparently normal at birth but usually displaying distinctive skeletal abnormalities by 2-3 weeks of age. In human beings, although many chondrodysplastic disorders are able to be diagnosed perinatally, some do not become apparent until childhood or later (Horton & Hecht, 2002b). Factors involved in the timing of disease onset are not clear. It would seem logical that because the majority of bones develop from embryonic cartilaginous anlagen, even the mildest of cartilage defects would cause gross lesions by the time of birth. The leakage of enzymes across the placenta during intra-uterine life can, in some disorders, explain the delay in development of lesions until postnatal life. In chondrodysplasia of Texel sheep this is unlikely to be the case, as even lambs born to affected ewes showed no overt signs of chondrodysplasia at birth. In fact, one lamb with 2 chondrodysplastic parents was less severely affected than other cohorts with only 1 chondrodysplastic parent (the sire).

The lack of microscopic abnormalities in very young lambs was consistent with the absence of clinical and gross lesions in these animals. While 50% of the lambs were expected to develop dwarfism, very few had any indication of the disease at birth, indicating that the histological lesions typical of Texel chondrodysplasia are usually inapparent in very young animals. The delayed development of lesions in this disease may indicate that the abnormal cartilage can function normally or near-normally until subjected to the stress of weight-bearing. This is supported by the relatively rapid development of severe lesions in lambs that initially appeared to be quick-growing. Lambs that did not thrive from birth, or had to compete with siblings, generally took 1-2 weeks longer to develop obvious signs of chondrodysplasia. The earlier appearance and increased severity of lesions in the forelimbs, both grossly and histologically, may reflect the greater weight-bearing of those limbs relative to the hind limbs, which are primarily used for propulsion in quadrupeds (Lee *et al.*, 2004). An alternative hypothesis for the progressive nature of the lesions in chondrodysplasia of Texel sheep considers interference by components of the pericellular matrix, some of which are involved in feedback and

interactions with cell surface ligands (Heinegård *et al.*, 2002). The gradual accumulation of abnormal matrix components in the pericellular region may inhibit the normal assembly of other molecules, either by steric interference or by secondary interaction with cell surface ligands.

In Texel lambs with chondrodysplasia, rarefaction of the cartilage matrix, leading to areas of chondrolysis in severe cases, consistantly began in the transitional zone of articular cartilage. This may be due to the orientation of collagen fibrils, which are less parallel in that area than in the tangential or radial zones, resulting in relative weakness of the transitional zone (Bellucci & Seedhom, 2001). The sulphated proteoglycans in cartilage required for normal collagen fibrillogenesis (Eyre *et al.*, 1987; Eyre, 2002) are at their lowest concentration in the interterritorial matrix (Ross *et al.*, 1995) and diminish with age (Oohira & Nogami, 1980). Perhaps in Texel chondrodysplasia the age-related decrease in proteoglycan concentration passes a threshold below which maintenance of interterritorial matrix structure is compromised.

Other heritable disorders of sulphate metabolism have been identified in human beings and animals. The brachymorphic mouse has decreased phospho-adenosine phosphosulphate (PAPS) synthesis and therefore reduced sulphate activation. This reduces the sulphate concentration of cartilage, but does not result in the distinctive lesions seen in DTDST-related disorders (Orkin *et al.*, 1977), suggesting that mechanisms other than simple undersulphation of matrix proteoglycans may be involved in the development of microscopic lesions in the sulphate transporter disorders. Another chondrodysplasia of mice described by Seegmiller *et al* (1971) featured a defective trachea, poor staining of cartilage matrix with TB accompanied by fibrillar degeneration of the matrix. In human beings, spondyloepiphyseal dysplasia Omani type, caused by loss of function of 6-*O*-sulphotransferase-1 (Thiele *et al.*, 2004), and spondyloepimetaphyseal dysplasia Pakistani type, caused by mutations in the PAPSS2 gene (orthologous to PAPS in mice) (ul Haque *et al.*, 1998), involve disturbances of sulphate metabolism, but are not well described histologically. Although not directly a disorder of sulphate metabolism, schneckenbecken dysplasia, caused by mutations in *SLC35D1* in human beings and in transgenic mice, is characterised by failure of chondroitin biosynthesis resulting in hypercellular cartilage with markedly diminished matrix (Hiraoka *et al.*, 2007). These lesions were not similar to the microscopic or histomorphometric findings of chondrodysplasia in Texel sheep.

English pointer enchondrodystrophy shares several gross and microscopic similarities with Texel chondrodysplasia. Pups appear normal at birth, but develop enlarged physes with metaphyseal flaring and angular limb deformities, erosion of cartilage in the shoulder joints, and dorso-ventral flattening of the thorax (Whitbread *et al.*, 1983; Lavelle, 1984). Histologically, the physes sometimes contain a thickened hypertrophic zone and a narrow, disorganised, proliferative zone. Degenerative changes in the transitional to radial zones in articular cartilage appear from 12 weeks of age, beginning as a reduction in staining density and progressing to the formation of cystic spaces (Lavelle, 1984). Unlike chondrodysplasia of Texel sheep, some dogs severely affected by with this disease have inferior prognathism and kyphosis.

Pseudoachondroplasia of miniature poodles develops similarly to chondrodysplasia of Texel sheep. Newborn pseudoachondroplastic pups appear phenotypically normal, but develop thickened tracheal rings, flared metaphyses, excessively curved ribs and a dorso-ventrally flattened thorax (Riser *et al.*, 1980). Proteoglycans are undersulphated and large "haloes" of dense matrix surround chondrocytes in these pups (Riser *et al.*, 1980; Bingel *et al.*, 1986), but the disease differs radiographically and microscopically from Texel chondrodysplasia.

Lesions similar to those of chondrodysplasia in Texel sheep are described in calves and lambs with congenital manganese (Mn) deficiency (Caskey *et al.*, 1944; Frost *et al.*, 1959; Valero *et al.*, 1990), and can also occur in young growing animals fed a deficient diet (Leach, 1968). Affected newborn calves typically have short limbs and excessive cartilage in long bone epiphyses, enlarged costochondral junctions and thickened tracheal rings, occasionally resulting in tracheal collapse (Valero *et al.*, 1990). Mn is required for the synthesis of chondroitin sulphate due to its function as a cofactor for polymerase enzyme which links UDP-N-acetyl-galactosamine and UDP-glucuronic acid to form the polysaccharide, and galactotransferase which incorporates galactose from UDP-galactose to the galactose-galactose-xylose trisaccharide and links the polysaccharide to the associated protein (Leach *et al.*, 1969). Deficiency of Mn has been shown to cause a reversible reduction in the incorporation of sulphate into cartilage with impaired glycosaminoglycan metabolism (Leach & Muenster, 1962; Bolze *et al.*, 1985).

The gross and microscopic similarities between chondrodysplasia of Texel sheep and certain chondrodysplasias in humans and other animals strongly supports a

defect in sulphate metabolism in affected Texel sheep. The lesions most specific to these disorders were the grossly thickened tracheal rings and the formation of microscopic perichondrocytic collagen rings with rarefaction of cartilage interterritorial matrix.

2.5 Summary

Chondrodysplasia of Texel sheep is a newly described heritable disorder which is distinct from other chondrodysplasias described in sheep. Phenotypically normal at birth, affected lambs develop microscopic lesions as early as 9 days of age, and usually show gross deformities by 2-3 weeks. The disorder is characterised by short stature, angular limb deformities, a barrel-shaped chest and wide-based stance. Gross pathological findings include tracheal narrowing and contortion, enlarged costochondral junctions, and erosion of articular cartilage in major limb joints. Primary microscopic lesions are confined to hyaline cartilage and are characterised by the presence of dense pericellular rings of matrix (predominantly type VI collagen) and degeneration of the interterritorial matrix. Articular and physeal cartilage is thickened in affected lambs, and hypocellular in lambs up to 2 months of age. Physeal closure is delayed. Severity of lesions is variable between chondrodysplastic lambs and between sites within lambs.

The microscopic lesions in chondrodysplasia of Texel sheep resemble those of sulphate transport disorders due to *SLC26A2* mutations in human beings. The variable expression and progressive nature of lesions in affected lambs is consistent with those seen in human diastrophic dysplasia. The similarities between chondrodysplasia of Texel sheep and certain disorders in human beings indicate that this disease of sheep has potential as an animal model.

3 Morphometric studies

3.1 Introduction

Skeletal growth and development is a complex event governed by many internal and external factors (Stokes, 2002; Cohen, 2006). Skeletal morphogenesis principally involves 4 distinct developmental processes: patterning, organogenesis, growth and homeostasis (Mundlos, 2001). Skeletal patterning includes functions influencing the size, shape and number of skeletal components. Regional defects in patterning alter specific parts of the skeleton, for example, the fusion and duplication of digits in synpolydactyly (Muragaki *et al.*, 1996). Skeletal organogenesis involves the differentiation of cell types and the subsequent formation of bone and cartilage tissue. An example of disruption of these processes is cleidocranial dysplasia, featuring persistently open skull sutures, due to failure of osteoblast differentiation (Mundlos *et al.*, 1997; Mundlos, 1999). Examples of defects in skeletal growth include the many disorders of growth plate function, which result in altered (usually decreased) growth rates and consequently dwarfism (Mundlos, 2001; Cohen, 2006) (refer to Chapter 1, Sections 1.3and 1.4). Skeletal homeostasis maintains bone mass, shape and strength, and is disrupted in Paget's disease, which is characterised by excessive resorption and formation of bone (Roodman, 1996; Mundlos, 2001). The lesions of chondrodysplasia in Texel sheep suggest that the disease is primarily associated with an abnormality of skeletal growth.

Chondrodysplasias in humans and animals may alter skeletal growth in a variety of ways (Rimoin *et al.*, 1998). Effects on function are varied, ranging from almost normal bone growth and shape to deformities so severe that they are incompatible with life (Vikkula *et al.*, 1995; Harper *et al.*, 1998). Depending on the disease, lesions may be evident at birth, as is typical of lethal disorders, or may manifest gradually, in some cases only becoming evident when skeletal growth is nearly complete (Superti-Furga, 1996; Superti-Furga *et al.*, 2000).

Chondrodysplastic Texel lambs develop the appearance of a wide, barrel-shaped chest and disproportionately shortened limbs, and show a decreased overall rate of growth (see Chapter 2, Figure 2.1). These changes usually become apparent in chondrodysplastic lambs at 2-3 weeks of age, but identifying the point of change and whether all skeletal components change at the same time is difficult or impossible through subjective means. The purpose of this investigation was to objectively describe the morphometric and allometric development of the skeleton

in Texel lambs affected by chondrodysplasia by comparing bone lengths, patterns of bone growth, and proportional sizes of anatomical features with control lambs. This information was used to test the hypothesis that bone lengths and growth rates in chondrodysplasia of Texel sheep are less than in controls. The findings were related to pathological features in lambs at different ages in order to characterise the development of the disease in more detail and contribute to a greater understanding of the pathogenesis of lesions.

3.2 Materials and methods

3.2.1 Animals used and measurements taken

An experimental flock of ewes either carrying or affected by Texel chondrodysplasia and mated to affected rams produced 109 lambs over a period of 1 month in the 2004 season. All lambs were either affected by chondrodysplasia or were phenotypically normal heterozygotes. The lambs were measured within 16 hours of birth, and at approximately 10-day intervals until 30 days of age, then at approximately 20-day intervals until 61-80 days of age, and once again at approximately 5 months of age. Some measurements were obtained post-mortem. All lambs were weighed at birth using a spring scale. Lambs were not weighed as they aged due to limited availability of equipment and the impracticality of driving them to yards.

The skeletal dimensions illustrated in Figure 3.1 were measured using a retractable metal tape with a small wire loop at the free end, adjusted so the thumbnail of the person taking measurements was at '0mm' on the tape when the thumb was inserted vertically through the loop and top of the nail positioned perpendicular to the tape. In this way, the free end of the tape measure was attached to the left thumb while the cassette holding the retractable tape was held in the right hand with the right thumb free. The exact points listed below were palpated with the thumbnails, which were facing each other and held perpendicular to the tape measure, and the reading in mm on the tape measure adjacent to the right thumbnail was recorded. Lengths measured were those of the humerus from the proximal border of the greater tubercle to the distal extent on the lateral condyle, ulna from the proximal tip of the olecranon to the distal point of the ulnar styloid process, metacarpus from the dorsal aspect of the carpometacarpal joint space to the most distal aspect of the convexity of the large metacarpal bone with the limb in

flexion, femur from the proximal border of the greater trochanter to the distal extent of the lateral condyle, tibia from the proximal ridge of the lateral condyle to the distal extent of the lateral malleolus, tarsus-metatarsus laterally from the proximal border of the calcaneus to the distal aspect of the convexity of the large metatarsal bone with the limb in flexion, and mandible from the caudal border of the ramus to the mentum. The thoracic width was defined as the widest part of the lamb at the level of ribs 6-7, estimated using a dorsal approach. Flat hands with their palms facing each other functioned as callipers. The left thumb pinched the loop on the free end of the tape measure against the straight index finger so that the '0mm' mark was level with the palm of the left hand, and the tape passed between the right thumb and straight right index finger. The thoracic depth was measured in an equivalent fashion, using a lateral approach to assess the distance between the highest point of the back and the lowest point of the sternum at the level of ribs 6-7. Crown-rump length was measured with the neck in flexion and taken from the crown to the tuber ischium, and shoulder-rump length was measured laterally from the cranial margin of the greater tubercle to the tuber ischium. Lambs were restrained in right lateral recumbency by a handler for all measurements other than thoracic width, which was taken with lambs held in sternal recumbency. Thoracic width measurements taken post-mortem were excluded due to the high influence of lung-fill on that dimension.

Initially, all measurements were performed in triplicate to assess repeatability. As triplicate measurements were found to vary by less than 3%, the subsequent measurements were taken only once to reduce penning time for lambs (which was a potential source of physiological stress during hot weather) and decrease potential measuring error due to operator fatigue. To minimise variability, all measurements in this study were taken by the same person (Burbidge & Pfeiffer, 1998). Lamb identification, age of lambs in days, disease status (control or affected), age of dams (ewe or hogget), sex, birthweight, and birth rank (single, twin or triplet) were recorded.

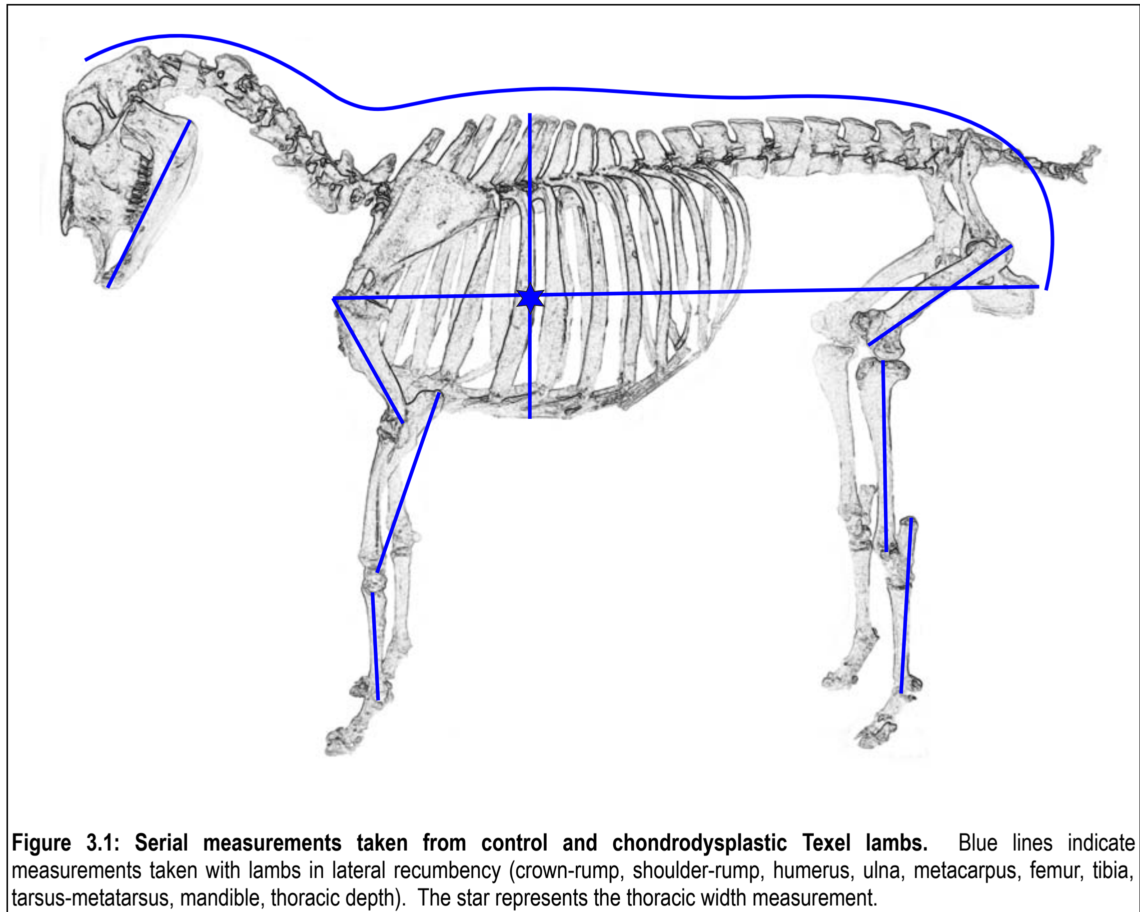


Figure 3.1: Serial measurements taken from control and chondrodysplastic Texel lambs. Blue lines indicate measurements taken with lambs in lateral recumbency (crown-rump, shoulder-rump, humerus, ulna, metacarpus, femur, tibia, tarsus-metatarsus, mandible, thoracic depth). The star represents the thoracic width measurement.

3.2.2 Analysis

Lamb measurement data were divided into the following age intervals: birth, 1-10 days, 11-20 days, 21-30 days, 31-40 days, 41-60 days, 61-80 days, and approximately 5 months. The closer chronological grouping of younger lambs was chosen to reflect the expected rapid growth for the earlier weeks of life. The increased range of ages in the older age intervals also serves to keep the number of animals in each age group greater than 20, as there were fewer lambs available for measurement in the later stages of the study.

Statistical tests were run using S-Plus 8.0 student edition (Insightful Corp., 2007). While repeated measures analysis is a valid statistical tool for this type of long-range study, the progressive loss of animals over time due to natural death, severity of disease, and the requirement for tissue samples made it inappropriate in this case. Analysis of variance (ANOVA) models were fitted to compare bone lengths: $\text{length}_{(\text{age interval})}$ ~ actual age + sex + dam age + rank + birthweight + disease status. "Actual age" was the age of the lamb in days when the measurement was taken, included to take into account the variation in age within age groups. The range of

ages within groups was particularly important to consider in the younger, faster growing lambs, where there were sometimes several days difference in age. Chi-squared tests were performed to test the relationship between disease status and sex, dam age or lamb birth-rank.

Growth rates were calculated by dividing the difference in length (mm) between time periods by the true difference in age (days) for that lamb. The true ages were used to allow for cases where the time elapsed between measurements was not the same for all animals. ANOVA models for growth rate were fitted in the same way as for bone length.

To investigate allometric differences between control and affected lambs, crown-rump lengths for each age group were used as a covariate in the following model: $\text{length}_{(\text{age interval})} \sim \text{crown-rump length}_{(\text{age interval})}$ +actual age + sex + dam age + rank + birthweight + disease status. To examine relative growth within limbs, metatarsal and metacarpal lengths were plotted against lengths of the other bones in the respective limbs. Comparison of the lengths of corresponding bones between the fore and hindlimbs was assessed to determine whether a gradient of severity of bone shortening existed within chondrodysplastic lambs. The ANOVA models listed in Table 3.1 were fitted for these analyses.

Table 3.1: ANOVA models fitted for the comparison of lengths of limb bones between control and chondrodysplastic Texel lambs.
Humeral $\text{length}_{(\text{age interval})} \sim$ ulnar $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Humeral $\text{length}_{(\text{age interval})} \sim$ metacarpal $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Ulnar $\text{length}_{(\text{age interval})} \sim$ metacarpal $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Femoral $\text{length}_{(\text{age interval})} \sim$ tibial $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Femoral $\text{length}_{(\text{age interval})} \sim$ tarsal-metatarsal $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Tibial $\text{length}_{(\text{age interval})} \sim$ tarsal-metatarsal $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Humeral $\text{length}_{(\text{age interval})} \sim$ femoral $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Ulnar $\text{length}_{(\text{age interval})} \sim$ tibial $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status
Metacarpal $\text{length}_{(\text{age interval})} \sim$ tarsal-metatarsal $\text{length}_{(\text{age interval})}$ + actual age + sex + dam age + rank + birthweight + disease status

3.3 Results

3.3.1 Overview

Subjectively, chondrodysplastic lambs were indistinguishable from controls at birth, but appeared to grow less rapidly and were noticeably smaller by 2-3 weeks of age. The thorax of affected animals acquired a distinctive barrel-shape, and the forelimbs often developed varus deformities. Affected lambs that appeared to grow quickly from an early age, compared with their cohorts, seemed to have a more rapid onset of the chondrodysplastic phenotype.

The variation in birthweight was influenced by sex, age of dam and birth rank. The mean birthweight of male lambs was approximately 0.6kg heavier than that of female lambs ($P \leq 0.005$), and ewes produced lambs approximately 0.7kg heavier than hoggets ($P \leq 0.0001$). Chi-squared tests demonstrated no significant relationship between disease status and sex or birth rank of lambs, or age of dam.

All dimensions other than thoracic width were significantly smaller in affected than normal lambs by 1 month of age, and this difference continued to be significant for the duration of the study. The mean percentage difference in length of bones in affected lambs relative to controls was greatest at 61-80 days for every dimension. The greatest difference was in thoracic depth, which was 20% less in chondrodysplastic lambs. The smallest difference was in thoracic width, which was 5% less in chondrodysplastic lambs. Growth rates of the crown-rump, shoulder-rump, humerus, femur, tibia and tarsus-metatarsus, mandibular length and thoracic depth were significantly reduced for part of the measured period but were not significantly different from controls in the last time period (5 months). Ulnar and metacarpal growth rates were less in chondrodysplastic lambs compared with controls at 5 months of age ($P \leq 0.05$). There was no significant difference in growth rate of thoracic width between control and affected lambs at any age. Details of these results are presented in the following sections and a digital copy of the raw data is included in the back of this thesis.

3.3.2 Whole body growth

The mean crown-rump length was less in affected than in control lambs from the 1-10 days age interval (P≤0.05) until the end of the study (P≤0.0001, Figure 3.2). From 21 days of age, the crown-rump length of affected lambs was 10-20% shorter than that of controls. For the age intervals from 11-60 days there was a significantly decreased rate of crown-rump growth in the affected lambs. The greatest difference in the rate of crown-rump growth was at 21-30 days, when the mean growth rate of affected lambs was 82% less than that of controls.

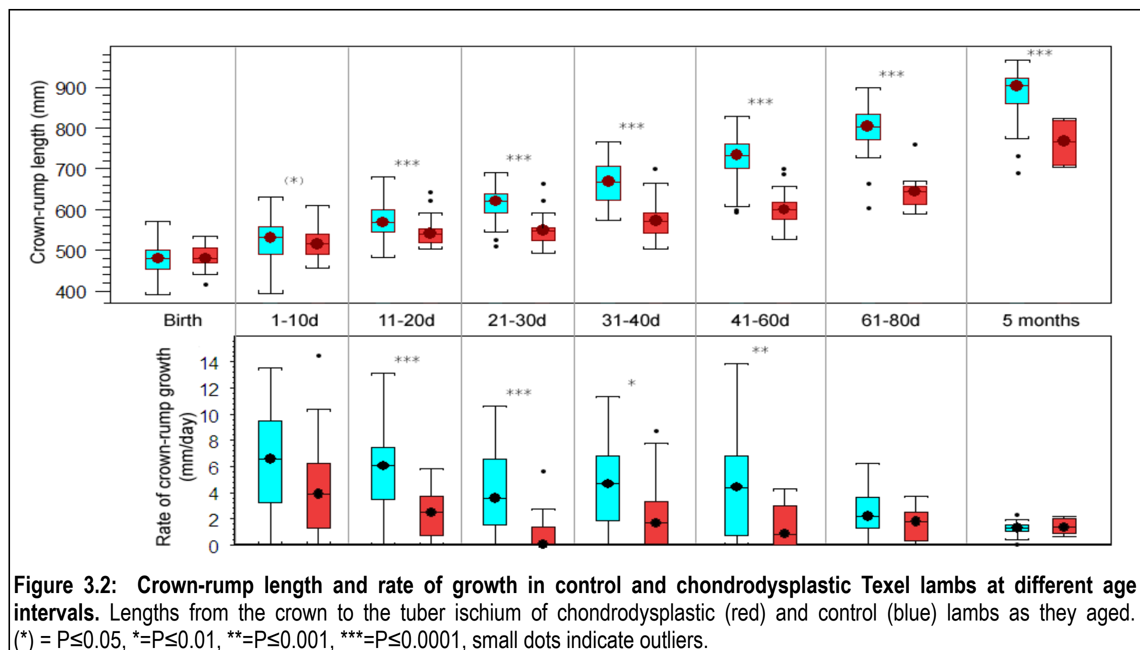


Figure 3.2: Crown-rump length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the crown to the tuber ischium of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Shoulder-rump measurements (Figure 3.3) followed a similar pattern to crown-rump measurements, but were less consistent and were not significantly less in chondrodysplastic lambs in the 1-10 days time period. As with crown-rump length, the greatest difference in mean shoulder-rump length between affected and control animals was at 61-80 days, when the mean length of chondrodysplastic lambs was 18% less than that of controls. This time period also had the greatest difference in rate of shoulder-rump growth, with the mean growth rate of affected lambs 71% less than that of control lambs. The rate of shoulder-rump growth in chondrodysplastic lambs was also significantly less than that of controls at the 11-20 and 21-30 day age intervals.

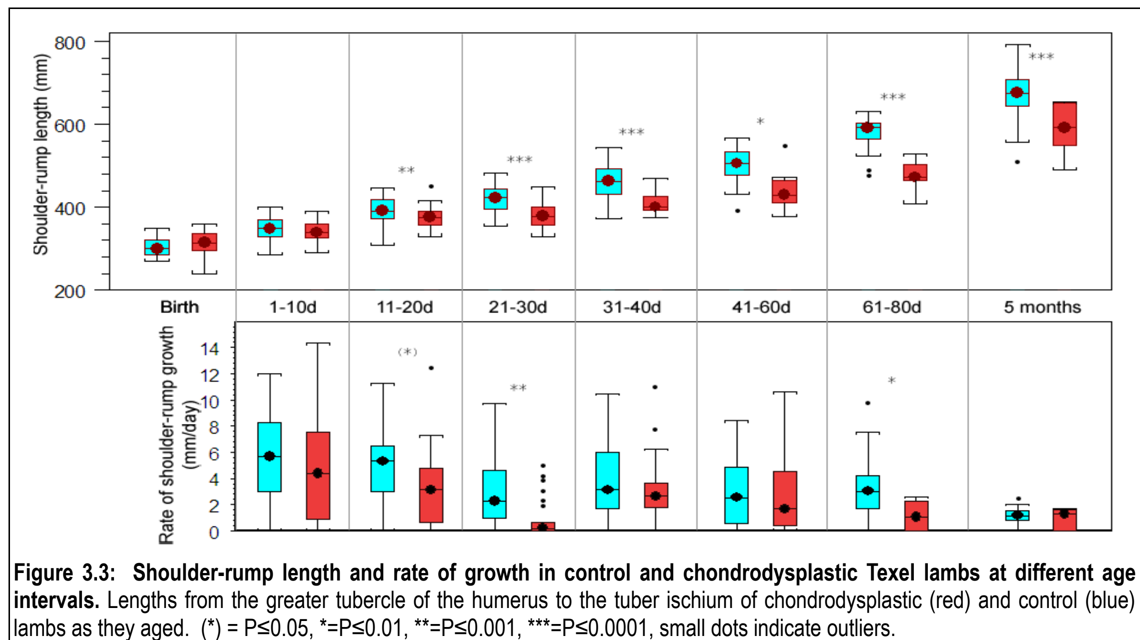


Figure 3.3: Shoulder-rump length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the greater tubercle of the humerus to the tuber ischium of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Mean crown-rump and shoulder-rump lengths at birth were influenced by several factors (Table 3.2). Within the group of lambs for which the disease status could be identified, males had a crown-rump length on average 18mm longer at birth than females (P≤0.05), and ewes produced lambs approximately 31mm longer at birth than those from hogget dams (P≤0.0001). Age of dam continued to exert a strong influence on crown-rump length until 41-60 days of age, with lambs from ewes persistently longer than lambs from hoggets. Sex, dam experience, birth rank and birth weight of lambs did not significantly influence growth rates.

Table 3.2: Influences other than chondrodysplasia on body length. BW = Birthweight, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age interval	Crown-rump length	Shoulder-rump length
Birth	Sex* Dam*** Rank* BW***	Dam*** Rank(*) BW***
1-10 days	Dam*** BW***	Dam(*) Rank* BW***
11-20 days	Dam*** Rank*	BW*
21-30 days	Dam* BW(*)	Dam(*)
31-40 days	Dam*** Rank*	Rank(*)
41-60 days	Dam(*) Rank(*) BW(*)	Rank(*)
61-80 days	BW*	BW(*)
5 months	-	Sex(*) BW(*)

3.3.3 Thoracic growth

Thoracic width (Figure 3.4) was less in chondrodysplastic lambs than in controls at 1-10, 21-30, 31-40, 41-60 and 61-80 days, but no significant difference in the rate of increase in thoracic width was detected at any age interval.

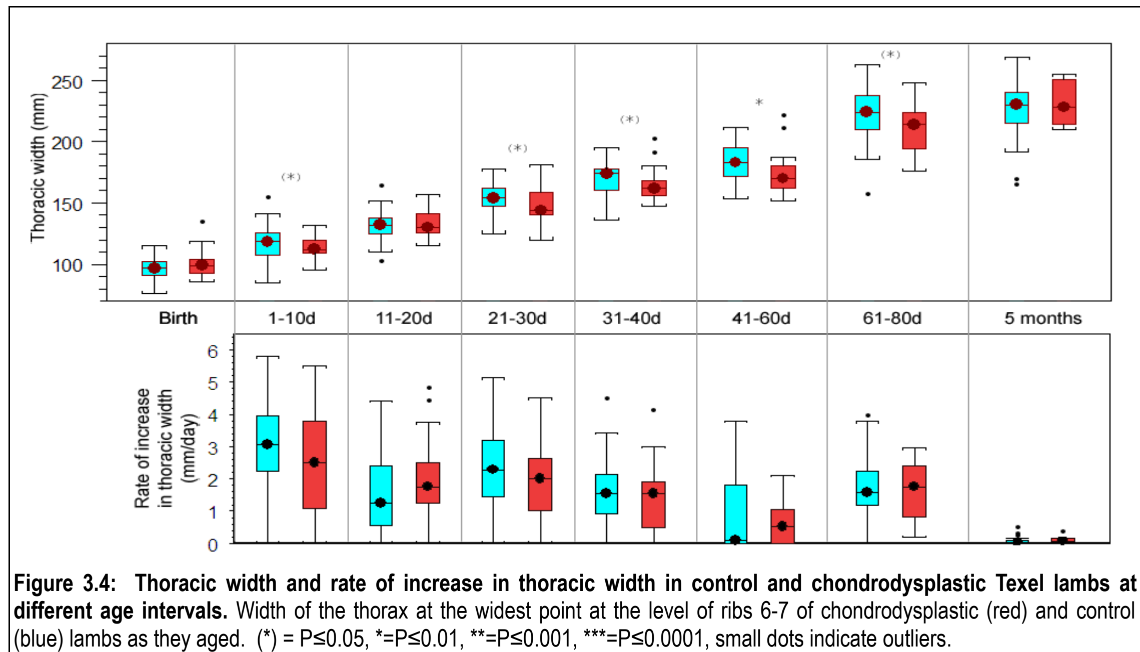


Figure 3.4: Thoracic width and rate of increase in thoracic width in control and chondrodysplastic Texel lambs at different age intervals. Width of the thorax at the widest point at the level of ribs 6-7 of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Thoracic depth (Figure 3.5) in chondrodysplastic lambs was less than in controls at birth (P≤0.05), and from 11-20 days onwards (P≤0.001). The rate of increase in thoracic depth was less in affected lambs compared with controls in the 21-30, 31-40 and 41-60 day age intervals. The greatest difference in the rate of increase in thoracic depth was at 21-30 days, at which age interval the rate was 67% less in chondrodysplastic lambs than in controls.

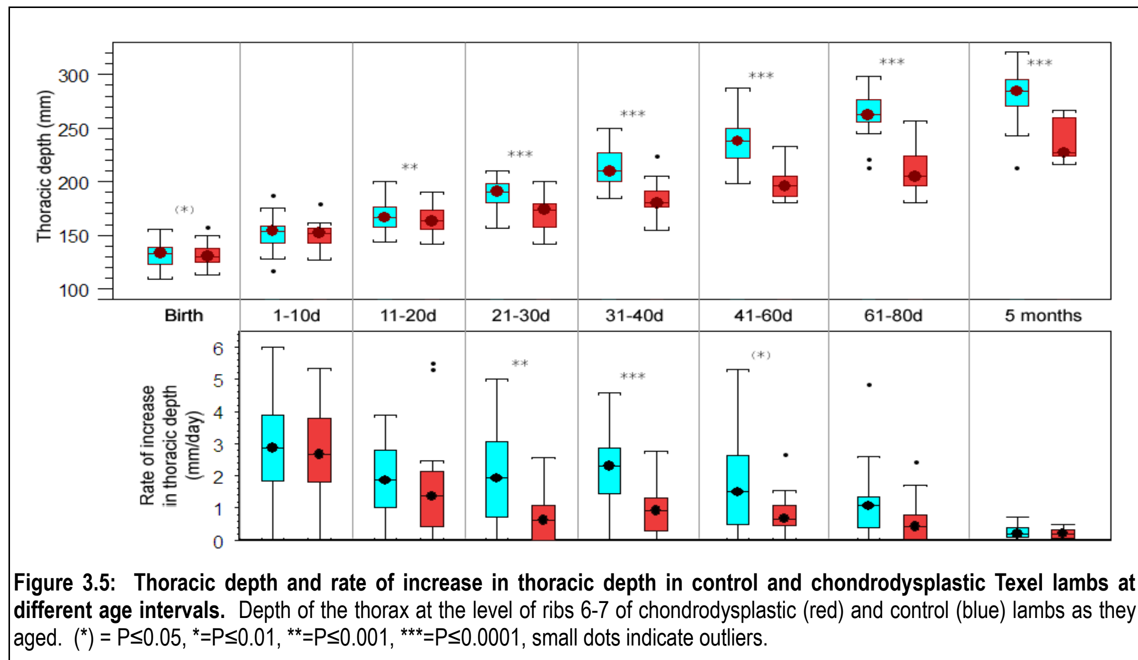


Figure 3.5: Thoracic depth and rate of increase in thoracic depth in control and chondrodysplastic Texel lambs at different age intervals. Depth of the thorax at the level of ribs 6-7 of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Thoracic width was positively correlated with birth weight up to and including the 41-60 days age interval (Table 3.3). Dam experience influenced thoracic depth up to 41-60 days, with lambs from ewes having a deeper thorax than lambs from hoggets. At birth, thoracic depth was 4.6mm deeper in male than in female lambs (P≤0.01). Birth weight was positively correlated with thoracic depth until the 11-20 days age interval, and birth rank had a sporadic influence, lambs from multiple births tending to have deeper thoraxes than singleton lambs.

Table 3.3: Influences other than chondrodysplasia on thoracic dimensions. BW = Birthweight, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age	Thoracic width	Thoracic depth
Birth	Dam(*) Rank(*) BW***	Sex* Dam*** Rank** BW***
1-10 days	Dam(*)	Dam** BW**
11-20 days	Rank* BW**	Sex* Dam* BW***
21-30 days	Rank* BW(*)	Dam*
31-40 days	Dam(*) Rank(*)	Dam** Rank*
41-60 days	Rank(*) BW(*)	Dam*
61-80 days	BW(*)	BW(*)
5 months	-	-

3.3.4 Mandibular growth

Although there was no clear difference in mean mandibular length between chondrodysplastic and control lambs at the 11-20 and 31-40 day age intervals (Figure 3.6), when the other variables in the ANOVA model were accounted for, a

difference was present (P≤0.05). The length of the mandible was significantly less in chondrodysplastic lambs compared with controls from 31 days of age. The growth rate of the mandible was 47% less in affected lambs than in controls in the 11-20 days age interval (P≤0.01).

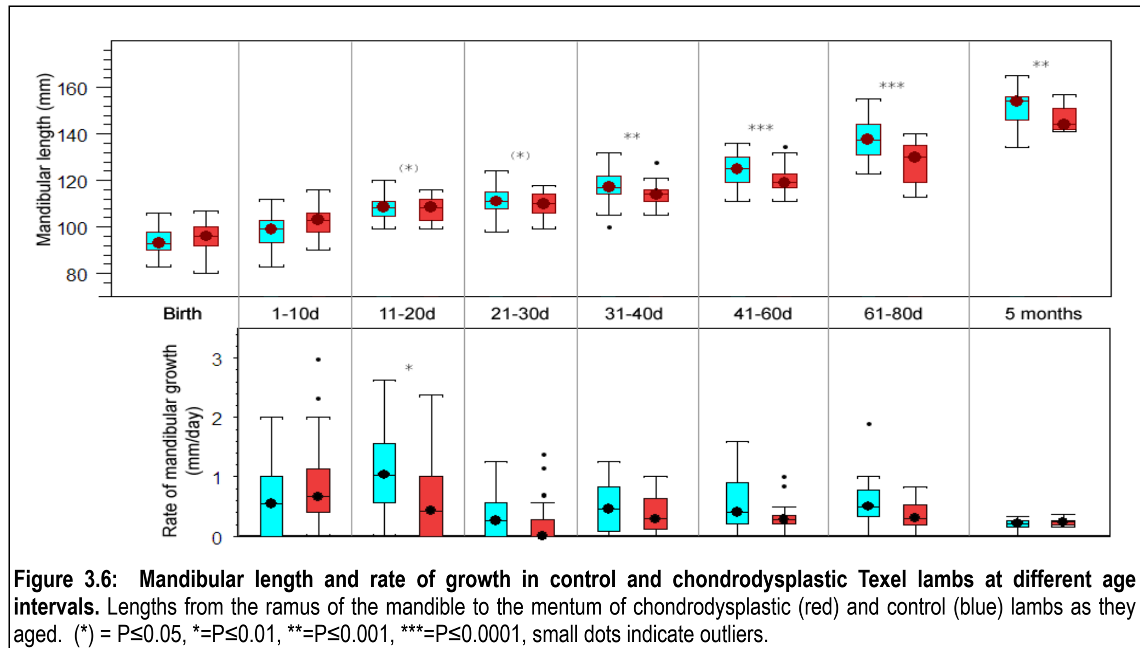


Figure 3.6: Mandibular length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the ramus of the mandible to the mentum of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Mandibular length was positively correlated with birth weight throughout this study, and was influenced by dam experience up to the 41-60 day interval, with ewes producing lambs with longer mandibles than those from hoggets (P≤0.01, Table 3.4).

Table 3.4: Influences other than chondrodysplasia on mandibular length. BW = Birthweight, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age interval	Mandibular length
Birth	Dam** BW***
1-10 days	Dam** BW***
11-20 days	BW***
21-30 days	Dam* BW***
31-40 days	Dam(*) BW**
41-60 days	Dam* BW***
61-80 days	BW(*)
5 months	BW*

3.3.5 Forelimb bone growth

Although there was no difference in mean humeral length between control and chondrodysplastic lambs in the 1-10 days age interval, when the other variables in the ANOVA model were accounted for, a difference was present (P≤0.05, Figure 3.7). In all later age groups, humeral length was significantly less in chondrodysplastic lambs than in controls, corresponding with a significantly slower humeral growth rate in the age intervals between 21 and 80 days. At 61-80 days, the mean humeral length of affected lambs was 16% less than that of controls, and the mean growth rate of the humerus was 82% less.

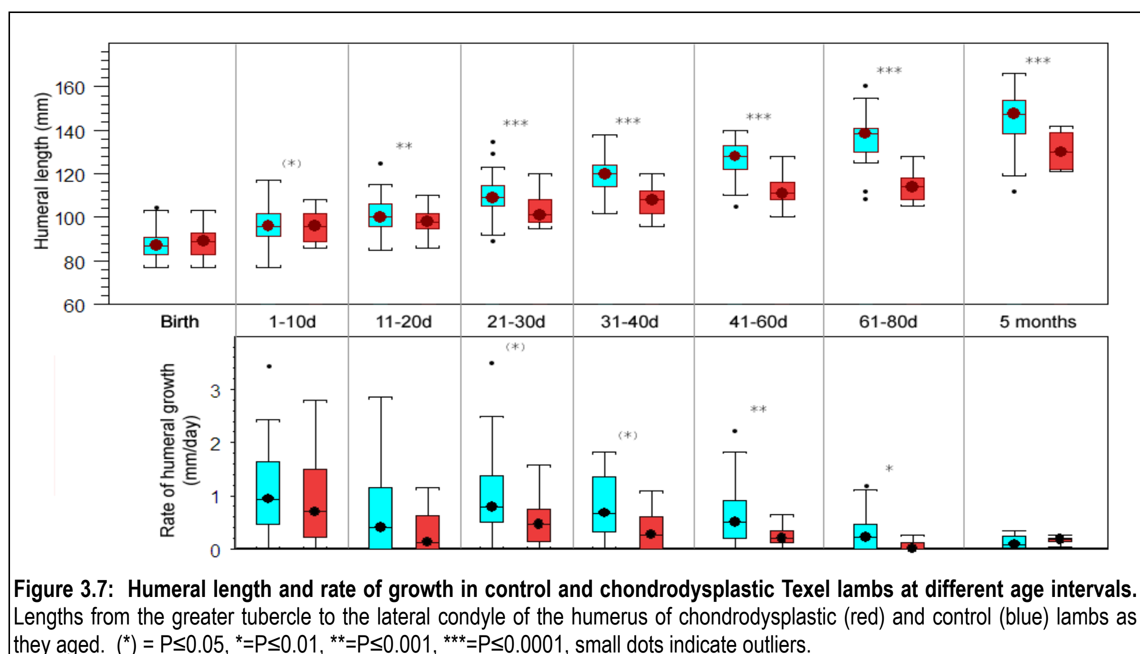


Figure 3.7: Humeral length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the greater tubercle to the lateral condyle of the humerus of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

The length and growth rate of the ulna (Figure 3.8) were significantly less in chondrodysplastic lambs than in controls after the 11-20 day interval. Mean ulnar length was 14% less than that of controls at 61-80 days, and the growth rate was 79% less in the 21-30 days age interval.

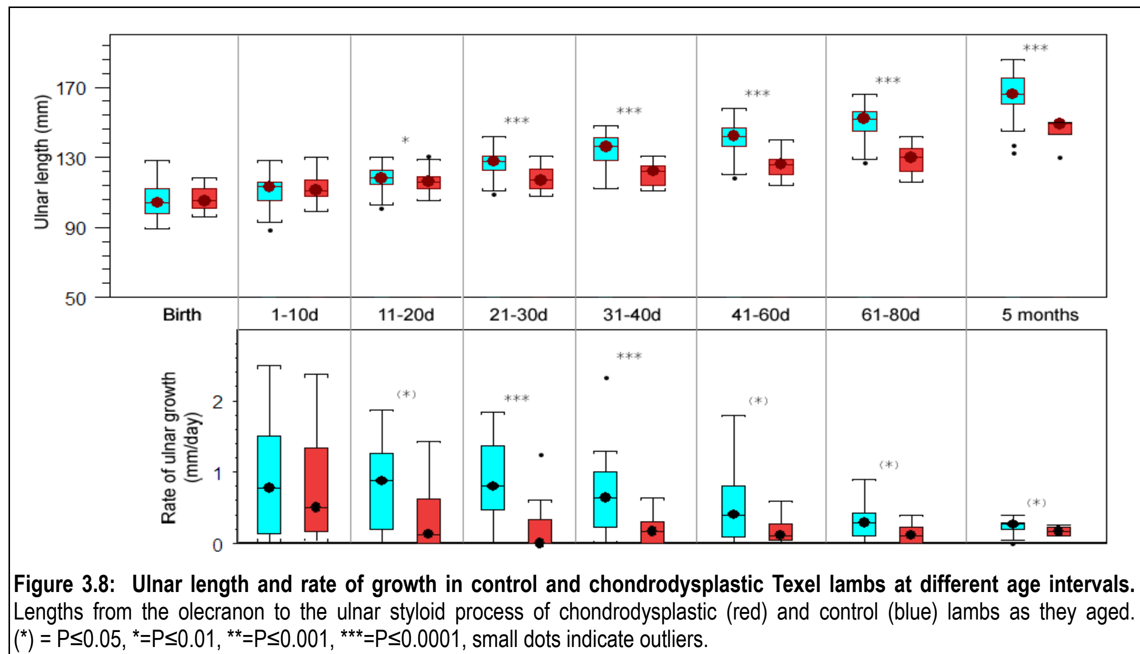


Figure 3.8: Ulnar length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the olecranon to the ulnar styloid process of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Metacarpal length of chondrodysplastic lambs (Figure 3.9) was significantly less than that of controls in the 1-10 days and 11-20 days intervals, and the difference became greater as lambs aged. The greatest difference was at 5 months, when metacarpal length of affected lambs was 9% less than that of controls. At 31-40 days, the mean rate of metacarpal growth in affected lambs was 66% less than that of controls. Metacarpal growth rates were also less in chondrodysplastic lambs than in controls at 21-30 days and 5 months.

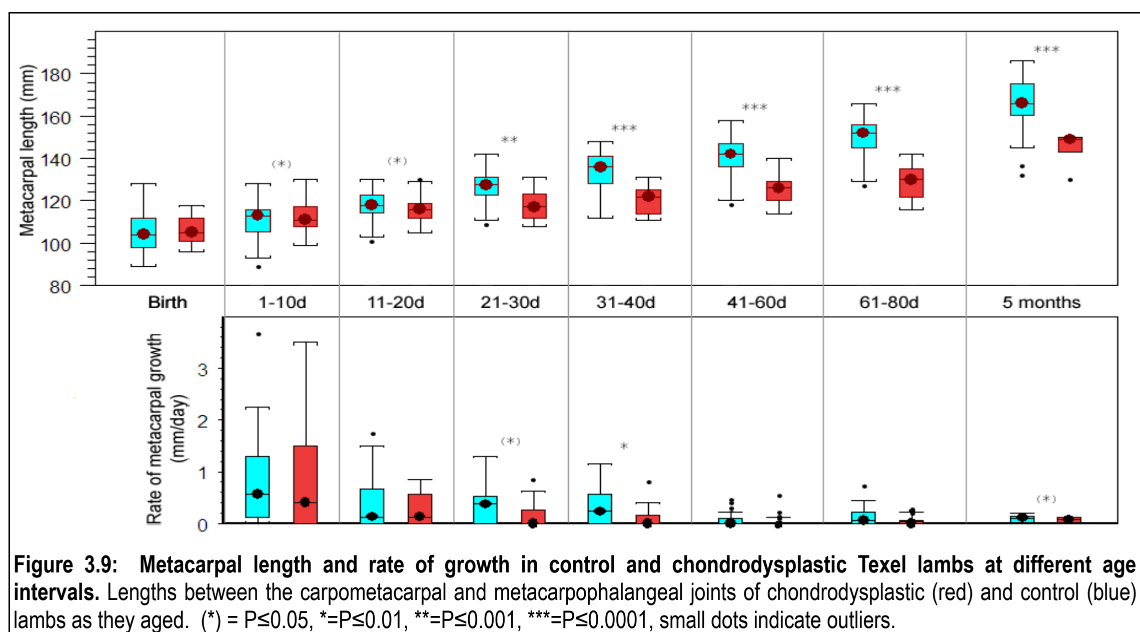


Figure 3.9: Metacarpal length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths between the carpometacarpal and metacarpophalangeal joints of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Maternal age had a strong influence on humeral and ulnar length until 41-60 days of age, adult ewes producing lambs with longer bones than those from hoggets (Table 3.5). The metacarpal bone appeared to be influenced by different factors to the other forelimb bones. In the age intervals for which sex had a significant influence of metacarpal length, male lambs had a greater mean length than did female lambs. This difference was significant at birth, with a mean difference of 4.2mm (P≤0.05), 1-10 days with a mean difference of 4.2mm (P≤0.01), 11-20 days with a mean difference of 4.4mm (P≤0.001), 31-40 days with a mean difference of 3.5mm (P≤0.01), and at 41-60 days with a mean difference of 3.6mm (P≤0.001). Dam experience had a significant effect until 61-80 days, adult ewes having lambs with longer metacarpi than those from hoggets. Rank had a significant effect on metacarpal length, with lambs from multiple births having longer metacarpi than singletons by 2.9mm at 61-80 days (P≤0.001) and 4.8mm at 5 months (P≤0.05).

Table 3.5: Influences other than chondrodysplasia on forelimb bone length. BW = Birthweight, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age interval	Humeral length	Ulnar length	Metacarpal length
Birth	Dam** Rank* BW***	Dam*** Rank(*) BW***	Sex(*) Dam*** BW**
1-10 days	Sex* Dam** BW***	Dam*** BW***	Sex* Dam** BW***
11-20 days	Dam*** Rank(*) BW***	Dam*** Rank(*) BW***	Sex* Dam* BW**
21-30 days	Dam*** BW**	Dam*** Rank(*) BW**	Dam*
31-40 days	Dam* BW(*)	Sex(*) Dam*** Rank(*) BW*	Sex* Dam**
41-60 days	-	Dam(*) BW(*)	Sex* Dam* BW(*)
61-80 days	BW*	BW*	Rank(*) BW***
5 months	BW(*)	Sex(*) BW*	Dam(*) BW**

3.3.6 Hindlimb bone growth

Femoral length (Figure 3.10) was significantly different in control and chondrodysplastic lambs at 1-10 days and the differences increased with age, becoming most marked at 61-80 days, when mean femoral length of affected lambs was 16% less than that of controls. Mean femoral growth rate was significantly less in chondrodysplastic lambs than in controls at 1-10 and at 41-60 days, when the mean femoral growth rate was 77% slower than that of controls.

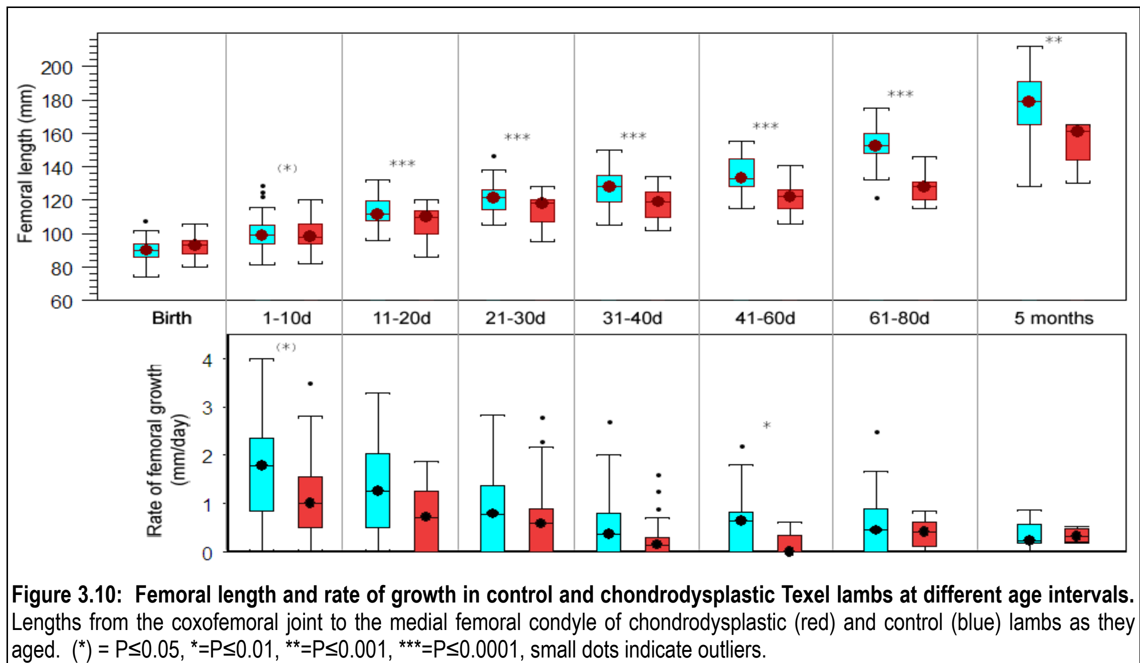


Figure 3.10: Femoral length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the coxofemoral joint to the medial femoral condyle of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Tibial length (Figure 3.11) was significantly less in chondrodysplastic lambs than in controls from 21 days of age. The greatest difference was at 61-80 days, with mean tibial length 17% shorter in affected lambs than in controls. Tibial growth rate of chondrodysplastic lambs was significantly less than that of controls in the age intervals between 21 and 60 days, and as with the femur, the difference was greatest at the 41-60 days age interval.

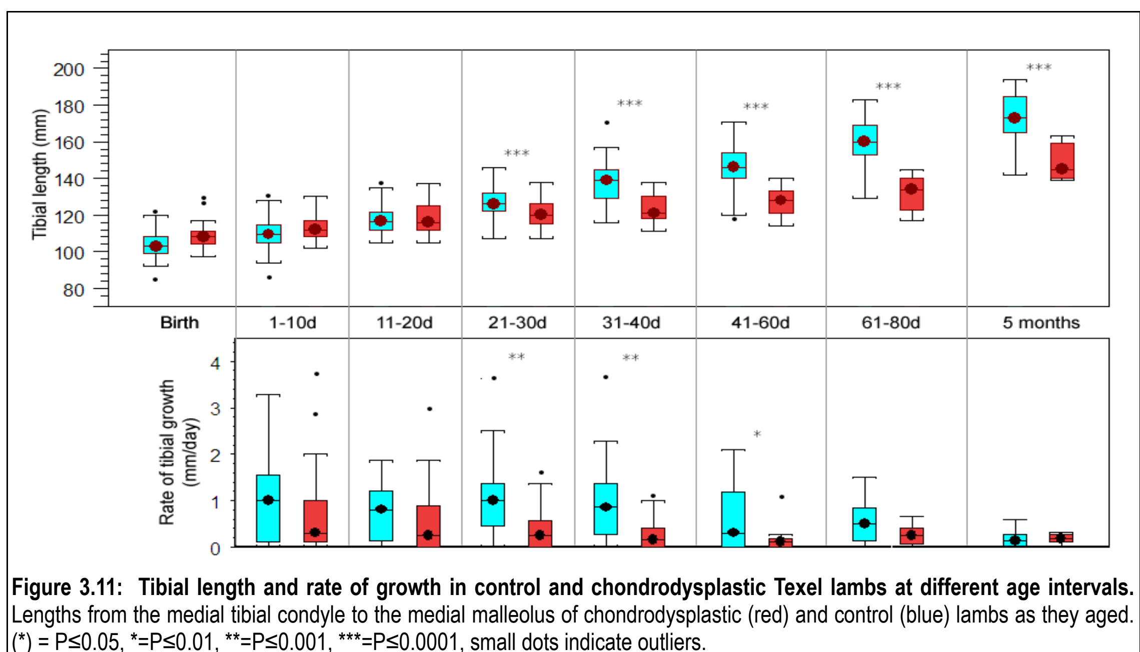


Figure 3.11: Tibial length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the medial tibial condyle to the medial malleolus of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

Tarsal-metatarsal length (Figure 3.12) was less in chondrodysplastic lambs than in controls at 1-10 days, and the difference became more pronounced with age. The disparity peaked at 61-80 days, when tarsal-metatarsal length in affected lambs was 11% less than in controls. Tarsal-metatarsal growth rate was less in chondrodysplastic lambs than in controls at the 11-20, 21-30, 31-40 and 41-60 days age intervals. The greatest difference was at 31-40 days, when the mean growth rate in affected lambs was 73% less than in controls.

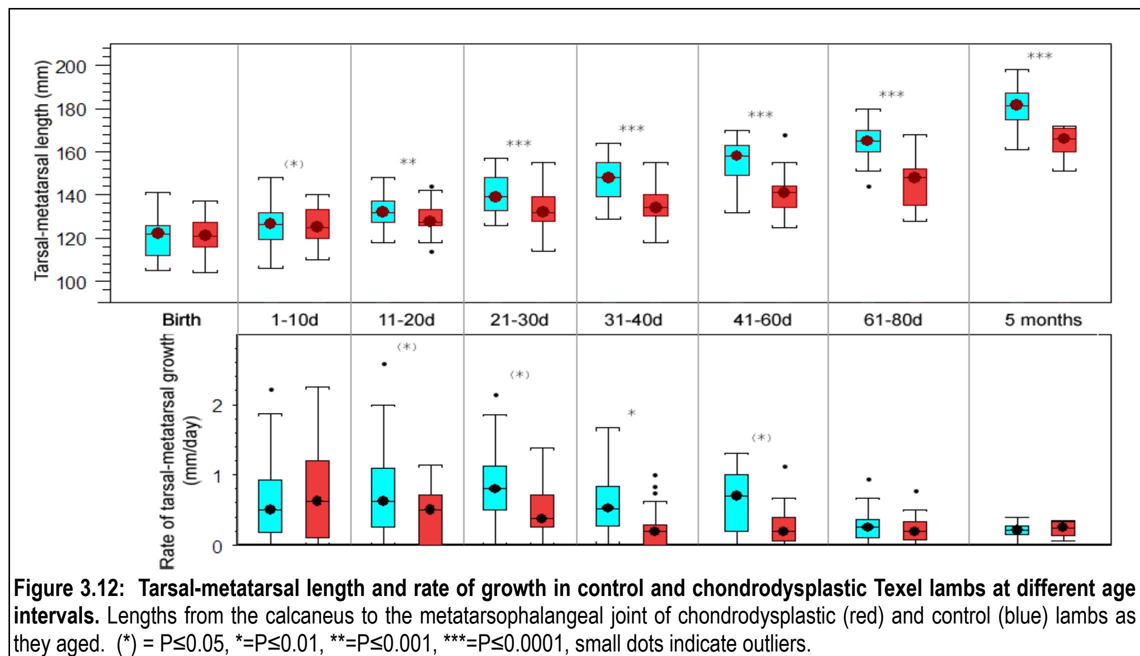


Figure 3.12: Tarsal-metatarsal length and rate of growth in control and chondrodysplastic Texel lambs at different age intervals. Lengths from the calcaneus to the metatarsophalangeal joint of chondrodysplastic (red) and control (blue) lambs as they aged. (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001, small dots indicate outliers.

As with the metacarpus, rank had some influence on femoral length (Table 3.6), although in contrast with the findings for metacarpal length, singleton lambs had longer femurs than lambs from multiple births by 0.8mm at 1-10 days (P≤0.01), 3mm at 11-20 days (P≤0.05), 3.4mm at 21-30 days (P≤0.01), 2.2mm at 31-40 days (P≤0.05), and 3.2mm at 41-60 days (P≤0.05). Birthweight was positively correlated with femoral length until the 21-30 days age interval, and dam age was only a significant factor until 1-10 days, with lambs from adult ewes having longer femurs than those from hoggets. There was a significant interaction of dam age and birthweight up to 61-80 days of age, with adult ewes producing lambs with greater tarsal-metatarsal lengths than lambs from hoggets. Tibial length was positively correlated with birth weight up to 31-40 days of age, and was influenced by dam age up to 61-80 days of age, with adult ewes producing lambs with longer tibias than did hoggets.

Table 3.6: Influences other than chondrodysplasia on hindlimb bone length. BW = Birthweight, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age interval	Femoral length	Tibial length	Tarsal-metatarsal length
Birth	Sex(*) Dam*** BW***	Sex(*) Dam* BW***	Dam*** Rank(*) BW***
1-10 days	Rank(*) BW***	Dam* BW***	Dam*** Rank* BW***
11-20 days	Rank(*) BW**	Sex(*) Dam* Rank** BW***	Sex(*) Dam** Rank(*) BW***
21-30 days	Rank* BW**	Dam* Rank* BW**	Dam*** BW*
31-40 days	Rank(*) BW(*)	Sex(*) Dam* Rank(*) BW(*)	Dam* BW*
41-60 days	Rank(*) BW(*)	Sex(*) Dam(*)	Dam(*) BW*
61-80 days	BW(*)	-	Dam* BW**
5 months	-	-	BW(*)

3.3.7 Allometry

Bone lengths in relation to crown-rump length

When crown-rump length was used as a covariate for other measurements within each age group, birthweight was responsible for most variation in the majority of measurements until 21-30 days (Table 3.7). Sex influenced metacarpal length in most age groups other than newborn, with male lambs having longer metacarpi relative to crown-rump length than female lambs. Tibial length was influenced by sex at the 11-20, 31-40 and 41-60 day age intervals, with male lambs having longer tibias relative to crown-rump length compared with female lambs. Other body parts were inconsistently affected by rank, sex and dam age. Measurements in which chondrodysplastic lambs showed a disproportionate shortening relative to crown-rump length when compared with control lambs were shoulder-rump length at 61-80 days (P≤0.05), ulna at 21-30 days (P≤0.01) and 31-40 days (P≤0.05), metacarpus at 61-80 days (P≤0.05), femur at 1-10 and 11-20 days (P≤0.05), tibia at 41-60 days (P≤0.01), and thoracic depth at birth (P≤0.05), 21-30 days (P≤0.01), 61-80 days (P≤0.05) and 5 months (P≤0.001). The tarsal-metatarsal length at 21-30 days was proportionally longer relative to crown-rump length in chondrodysplastic lambs when compared with control lambs (P≤0.05).

Table 3.7: Bone length and body dimensions influenced by sex, dam, rank, birthweight or status when crown-rump length is a covariate. SR = shoulder-rump length, MC = metacarpal length, MT = tarsal-metatarsal length, TW = thoracic width, TD = thoracic depth, (*) = P≤0.05, *=P≤0.01, **=P≤0.001, ***=P≤0.0001

Age interval	Sex	Dam	Rank	Birthweight	Status
Birth	-	MC(*)	-	Humerus** Ulna*** MC* Femur*** Tibia** MT*** SR*** Mandible** TW* TD***	TD(*)
1-10 days	MC(*)	Ulna(*) MT(*)	-	Humerus* Ulna*** Femur** Tibia** MT* Mandible***	Femur(*)
11-20 days	MC* Tibia(*) MT(*) TD(*)	Ulna(*)	Tibia(*) TW(*)	Humerus* Ulna** Tibia* MT(*) Mandible*** TW* TD*	Femur(*) MT(*)
21-30 days	-	Humerus* Ulna(*) MT*	Femur(*) Tibia(*) TW(*)	Humerus(*) Ulna(*) Femur(*) Tibia* MT(*) Mandible**	Ulna* TD*
31-40 days	Ulna(*) MC* Tibia(*)	-	MT(*)	MT(*)	Ulna(*) TD(*)
41-60 days	MC* Tibia(*) TW(*)	MC(*)	MT(*)	-	Tibia*
61-80 days	-	MT(*)	MC*	MC* MT(*)	MC(*) SR(*) TD(*)
5 months	MC(*) MT(*) SR*	-	MC(*)	MC* Mandible(*)	TD**

Relative limb bone lengths

The humerus and ulna, while shorter in chondrodysplastic than in control lambs, were in proportion with each other at 21-30 days of age (Figure.3.13), and were disproportionately shorter than the metacarpus in chondrodysplastic lambs compared with controls (P≤0.0001).

At 31-40 days of age, the femur and tibia of chondrodysplastic lambs shared the same proportionate relationship as that of control lambs (Figure 3.14). Tibial length was significantly less when compared with tarsal-metatarsal length in chondrodysplastic lambs relative to controls (P≤0.05), but femoral length was not.

At birth, tibial length was greater relative to tarsal-metatarsal length in chondrodysplastic lambs compared with controls (P≤0.05). In the 21-30 and 41-60 day age intervals, this pattern was reversed (P≤0.01) (Figure 3.15). A comparison of lengths of equivalent bones between the fore and hind limbs of chondrodysplastic and control lambs in the 31-40 days age interval is shown in Figure 3.16. In chondrodysplastic lambs, there was a more marked reduction in length of the humerus, ulna and metacarpus than of the femur, tibia and tarsus-metatarsus, respectively (P≤0.0001).

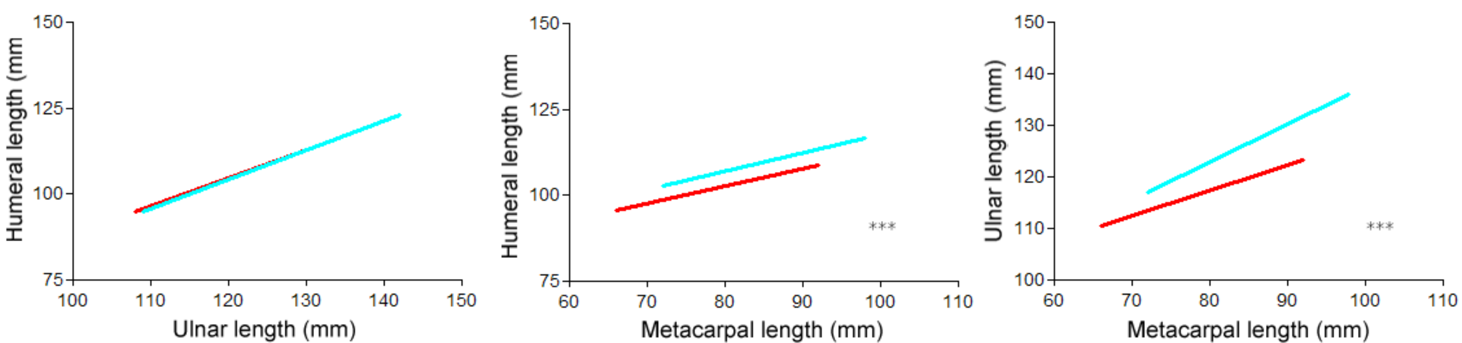


Figure 3.13: Relative bone lengths in the forelimb of chondrodysplastic and control Texel lambs at 21-30 days of age. Blue lines represent controls, red lines represent chondrodysplastic lambs. *** = $P \leq 0.0001$.

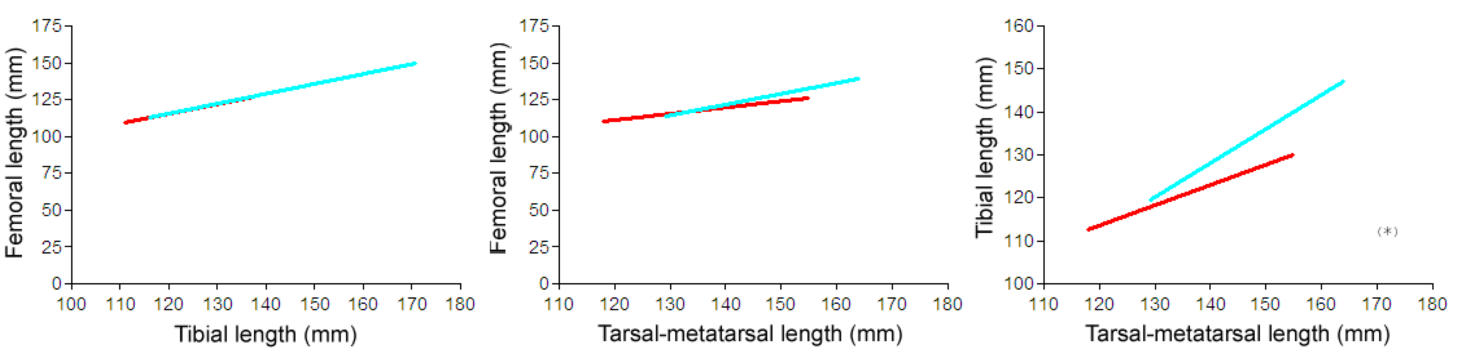


Figure 3.14: Relative bone lengths in the hindlimb of chondrodysplastic and control Texel lambs at 31-40 days of age. Blue lines represent controls, red lines represent chondrodysplastic lambs. (*) = $P \leq 0.05$.

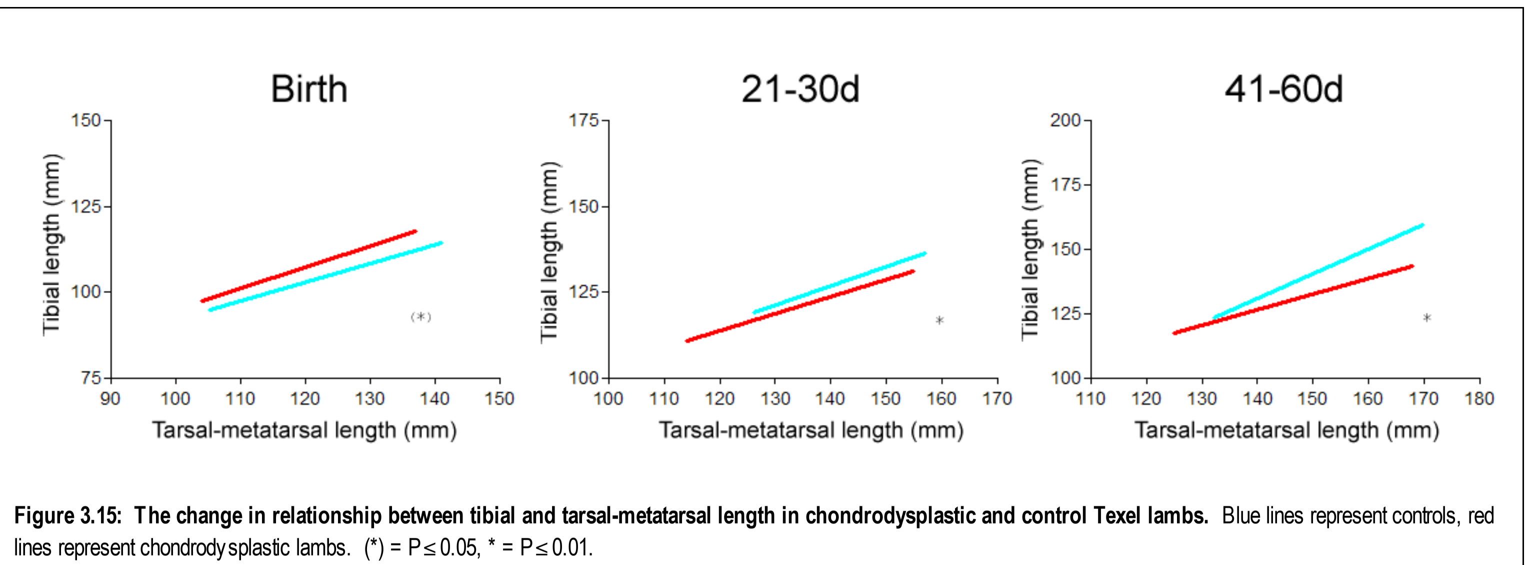


Figure 3.15: The change in relationship between tibial and tarsal-metatarsal length in chondrodysplastic and control Texel lambs. Blue lines represent controls, red lines represent chondrodysplastic lambs. (*) = $P \leq 0.05$, * = $P \leq 0.01$.

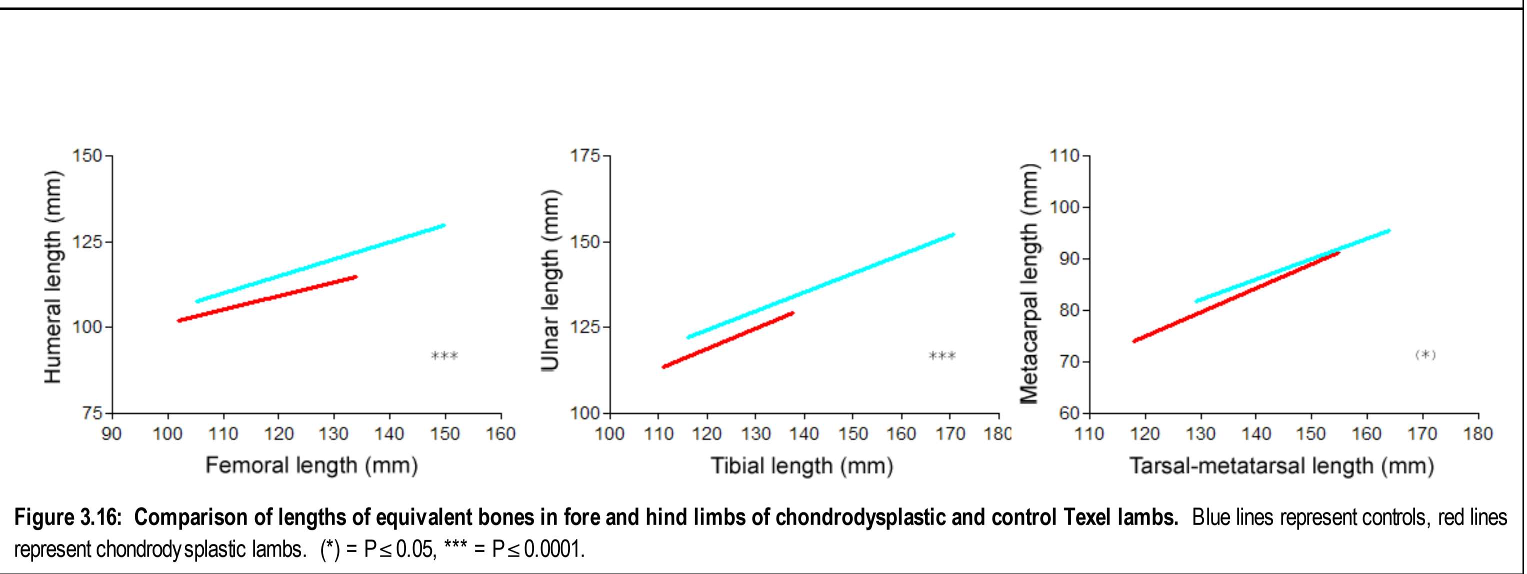


Figure 3.16: Comparison of lengths of equivalent bones in fore and hind limbs of chondrodysplastic and control Texel lambs. Blue lines represent controls, red lines represent chondrodysplastic lambs. (*) = $P \leq 0.05$, *** = $P \leq 0.0001$.

3.4 Discussion

The results of this study support the hypothesis that bone lengths and growth rates in chondrodysplastic Texel lambs are less than in controls. The findings also confirm the clinical impression of gradual progression of the phenotype in chondrodysplasia of Texel sheep. Chondrodysplastic Texel sheep have an overall disproportionately reduced stature, which becomes apparent over the first 2 weeks of life. Chondrodysplastic lambs were indistinguishable from control lambs at birth. Although statistically significant differences in thoracic depth and in the relationship between tibial and tarsal-metatarsal lengths could be identified when comparing a number of animals, the overlap in values between affected and controls lambs at birth was too great to confidently diagnose chondrodysplasia in any one lamb using these criteria.

The greatest differences in mean bone lengths and body dimensions between chondrodysplastic and control lambs were generally in the 61-80 days age interval, with somewhat less marked differences in mean length at 5 months. The latter is most likely explained by the removal of the most severely affected chondrodysplastic lambs from the flock prior to 5 months, rather than any "catch-up" in growth at a later stage.

The results for rate of increase in many of the measured dimensions indicated that bone growth in chondrodysplastic lambs was initially the same as in controls, slowed down during the first two weeks of life, then apparently returned to normal by 5 months of age. This may have been due to the removal of the more severely affected lambs earlier on leaving only mildly affected lambs that do not have such a reduced rate of growth. However, if this was the case, the actual measurements of each bone would not be expected to be as short as they were, relative to controls at that point. An alternative explanation may be that by 5 months of age long bone growth in control lambs was slowing and some physes were beginning to ossify, while those in chondrodysplastic lambs remained open. This was supported histologically, where there was complete bony union across the whole physis of the distal humerus in control animals by this time. In chondrodysplastic lambs, no physes were fused at 5 months (see Chapter 2), and long bone physes were significantly thicker than in control lambs (see Chapter 2, Table 2.2). This does not necessarily mean that all bones were still increasing in length at this age, as the cartilaginous physis persists for some time after effective longitudinal growth has

ceased (Roach *et al.*, 2003). Nonetheless, it is possible that the bones of chondrodysplastic lambs surviving to 5 months of age continued to grow slowly due to delayed physeal closure, while those of control lambs grew quicker earlier on but slowed or ceased in growth by 5 months of age.

The allometric comparisons of bone lengths in chondrodysplastic and control Texel lambs demonstrated that shortening of limb bones was more marked in the proximal limb than in the distal limb (rhizomelia), and that the forelimbs were more severely affected than the hindlimbs. This pattern of development reflects to some degree the spectrum of severity of histological lesions within affected animals (see Chapter 2). The mechanism behind this uneven distribution of the effects of chondrodysplasia in Texel sheep may be related to relative growth rate gradients within young lambs. In normal lambs, the proximal limb bones and caudal end of the skeleton are growing faster than the distal limb bones and cranial end of the skeleton (Davies *et al.*, 1984). A similar cranio-caudal gradient of increasing relative growth rate has been described in young pigs (Liu *et al.*, 1999). Therefore, within limbs, the bones with the fastest expected rate of growth relative to their potential final length were more severely shortened in chondrodysplastic lambs. Physeal cartilage from faster-growing bones has an increased rate of chondrocyte cell division and sulphate incorporation into matrix per wet weight than physeal cartilage from slower-growing bones, indicating the former has a higher level of metabolic activity than the latter (Seinsheimer & Sledge, 1981). It is likely that cells with a higher metabolic rate would be more affected by disorders involving insufficiency of a metabolic product or enzymatic cofactor. If such a mechanism is involved in chondrodysplasia of Texel sheep, it would help to explain the pattern of lesions, both gross and microscopic, that develop as affected lambs grow. The idea of a cartilage defect which is dependent to some degree upon the local metabolic rate is also supported by the subjective observation that the lambs that were initially faster-growing appeared to have a more sudden onset of lesions of chondrodysplasia. The data collected in this study was not able to provide great enough resolution to verify this hypothesis.

While the pattern of limb growth in chondrodysplastic lambs appeared to be inversely related to expected relative growth rate, the converse was seen when comparing relative reduction of bone length between fore and hindlimbs. If the degree of reduction in bone length was determined only by the expected rate of growth for that bone, the hindlimbs should have been more severely affected than

the forelimbs, as is seen in Hyena disease (Yamamoto *et al.*, 2003). An explanation for the increased effects of chondrodysplasia in the forelimb may simply be that more weight is carried on the forelimbs in quadrupeds, due to the position of the centre of mass immediately posterior to the point of the elbow (Jayes & Alexander, 1978). Mechanical compression of growth plates can constrain longitudinal growth (Stokes, 2002), and perhaps the defective cartilage, which is soft compared with control cartilage (see Chapter 2), may be less able to resist compressive forces, resulting in disturbance of normal physeal growth (Horton & Hecht, 2002a; Remes *et al.*, 2002).

As with the reduction in bone lengths, angular limb deformities were also more severe in the forelimbs than in the hindlimbs. This may be because abnormal loading can exacerbate the severity of angular limb deformities, as mechanical compression slows longitudinal bone growth, while sustained traction causes a degree of increased growth (Frost, 1997; Stokes, 2002; Stokes *et al.*, 2006).

The data on the relationship between thoracic width and depth supports the appearance of a broad, barrel-shaped chest, even though both thoracic width and depth measurements were less in chondrodysplastic lambs than in controls. This broadening of the thorax could be due to the relatively soft chondrodysplastic cartilage (see Chapter 2): on inspiration, the bony and cartilaginous portions of the rib cage are moving against the negative pressure within the thoracic cavity. The soft cartilage would deform towards the core of the animal on each inspiration, creating an abnormally acute internal costochondral angle (see Chapter 2, Figures 2.4 and 2.5). As the animal grows, this deformity would develop until the sternocostal angle is nearly flat or inverted, leading to pectus excavatum, a gross lesion present in severely affected lambs. The greater inspiratory effort seen with partial obstruction of the cervical trachea due to collapse of tracheal rings (see Chapter 2, Figure 2.3) may add to these pressures on the costochondral cartilage. In children, even minor respiratory obstruction is considered to be able to induce retraction of the sternum (Goretsky *et al.*, 2004).

The differences in bone lengths and body dimensions between chondrodysplastic and control lambs were often significant, but were contradicted by the lack of significant differences in the rate of bone growth. This can be explained by the relative variability of the measurements. While variability of the measurements was relatively low, <3% of the total length of the dimension measured, it became a

much larger proportion of the difference between consecutive measurements, from which the growth rates were calculated. For this reason, while the growth rate graphs often showed an apparent difference between control and chondrodysplastic lambs, the difference was not statistically significant.

Shoulder-rump measurements were more variable than other dimensions measured, and were influenced by rumen-fill and thoracic shape to some extent due to the measurement method. This greater variability could account for some of the differences between shoulder-rump and crown-rump results. By 61-80 days of age, subjective assessment indicated that some of the more severely affected lambs were losing body condition. This could explain the significant difference in the rate of increase in shoulder-rump length between affected and control lambs in this age group, as the difference in muscle mass, fat deposits and gut-fill may have distorted the shoulder-rump measurements more than other dimensions. By 5 months, a difference in the rate of increase in shoulder-rump length was no longer identifiable, most likely because the most severely affected lambs with poor body condition had either died or been euthanased on humane grounds by this time.

3.5 Summary

Chondrodysplasia of Texel sheep is a gradual-onset disorder of skeletal growth, resulting in disproporitonate shortening of all body dimensions. Overt signs of the disease became evident in the first two to three weeks of life. Growth rates of all measured dimensions in affected and control lambs were initially similar, but decreased in chondrodyslastic lambs between 11 and 80 days of age. The difference in body dimensions between controls and affected lambs became more marked with age, although because the most severely affected animals were removed from the study population before the end of the study, differences between chondrodysplastic and control lambs at the final age interval were usually less marked than in the previous age interval. Thoracic depth and crown-rump length showed the greatest difference in affected lambs compared with controls, while thoracic width and mandibular length were least affected by the chondrodysplastic phenotype.

Within the limbs, a gradient of severity of bone shortening was identified in affected lambs. The proximal bones of the limbs were more severely shortened than the metapodial bones, and the forelimb bones were more severely shortened than the

corresponding hindlimb bones. This pattern may in part reflect the relative rates of postnatal bone growth in lambs, as the greatest shortening within limbs occurred in the bones with the fastest expected growth rates for normal lambs.

4 Sulphate uptake by dermal fibroblasts

4.1 Introduction

Chondrodysplasia of Texel sheep is an inherited disease characterised by dwarfism, limb deformities, distinctive microscopic lesions and undersulphation of hyaline cartilage (see Figure 1.7) (Byrne, 2005; Thompson *et al.*, 2005). Reduced sulphation of cartilage matrix proteoglycans occurs with age (Bayliss *et al.*, 1999), in osteoarthritis (Plaas *et al.*, 1998) and in some genetic disorders (Superti-Fuga *et al.*, 2001). Undersulphation of cartilage can be caused by decreased function of transporters or enzymes involved in the metabolic pathway whereby sulphate groups are added to cartilage matrix proteoglycans (see Chapter, 1, Figure 1.3). Of the genetic disorders that result in undersulphated cartilage, those caused by mutations in *SLC26A2*, which encodes the diastrophic dysplasia sulphate transporter (DTDST), are the best described. The mutations in *SLC26A2* that are associated with chondrodysplasia result in varying degrees of dysfunction of the DTDST (Satoh *et al.*, 1998; Superti-Furga, 2001). This results in reduced uptake of sulphate by chondrocytes, and consequently in undersulphation of proteoglycans in hyaline cartilage (Rossi *et al.*, 1996a).

The activity of the DTDST in human beings, mice and cattle has been assessed using radiolabelled sulphate uptake assays performed *in vitro* on chondrocytes (Rossi *et al.*, 1996a; Rossi *et al.*, 1997) and fibroblasts (Rossi *et al.*, 1996b; Cetta *et al.*, 1997; Brenig *et al.*, 2003). While chondrocytes demonstrate a greater uptake of sulphate than do fibroblasts *in vitro* (Rossi *et al.*, 1996a), the latter are easier to obtain and grow in culture (Grinnel, 1992).

The microscopic and clinical characteristics of chondrodysplasia in Texel sheep resemble those that occur in heritable disorders caused by mutations in *SLC26A2* in human beings. Mutations in *SLC26A2* have been identified in achondrogenesis type 1b, atelosteogenesis type II, diastrophic dysplasia and recessive multiple epiphyseal dysplasia (rMED) (Hästbacka *et al.*, 1994; Hästbacka *et al.*, 1996b; Superti-Furga *et al.*, 1996b; Cai *et al.*, 1998; Ballhausen *et al.*, 2003). Achondrogenesis type 1b and atelosteogenesis type II are both lethal in the perinatal period (van der Harten *et al.*, 1988; Rossi *et al.*, 1996b). Diastrophic dysplasia is not lethal, but results in severe dwarfism with multiple joint contractures (Langer, 1965), while rMED is a relatively mild disorder characterised by short to normal stature and early-onset degenerative joint disease (Superti-Furga *et al.*, 1999). Microscopic lesions have not been assessed in rMED, but the

more severe chondrodysplasias caused by *SLC26A2* mutations have distinctive lesions in hyaline cartilage, characterised by rarefaction of interterritorial matrix and perichondrocytic collagen rings (van der Harten *et al.*, 1988; Shapiro, 1992; Superti-Furga, 1994). These lesions are identical to those of chondrodysplasia in Texel sheep (see Chapter 2, Figures 2.8 and 2.9). The gross skeletal changes of chondrodysplasia of Texel sheep are less severe than those of diastrophic dysplasia, but more severe than those of rMED. Gross lesions of diastrophic dysplasia and rMED become more pronounced throughout childhood (Superti-Furga, 2001; Mäkitie *et al.*, 2003), with microscopic lesions of diastrophic dysplasia initially limited to hyaline cartilage matrix rarefaction, and followed by the development of pericellular collagen rings (Shapiro, 1992). Gross and microscopic lesions developed similarly in chondrodysplasia of Texel sheep (see Chapter 2).

The resemblance of chondrodysplasia in Texel sheep to the chondrodysplasias caused by mutations in *SLC26A2* in human beings suggests a similar mechanism, possibly involving defective cellular uptake or intracellular processing of sulphate. The aim of this investigation was to test the hypothesis that undersulphation of cartilage matrix in chondrodysplasia of Texel sheep is due to reduced cellular uptake of sulphate.

4.2 Materials and methods

4.2.1 Cell culture

Dermal fibroblasts were harvested from 2 chondrodysplastic Texel lambs and 2 control lambs 1-2 months of age, in addition to a mature chondrodysplastic ram and a mature control ewe. Full-thickness skin biopsies were collected from the dorsolateral neck of either living animals or dead animals immediately after euthanasia. The live animals were sedated with a slow intravenous injection of diazepam into the jugular vein (5mg/ml at a rate of 1ml per 10kg estimated bodyweight, Pamlin, Parnell Laboratories Ltd., NZ), given local anaesthesia via a cutaneous ring-block with lignocaine hydrochloride (20mg/ml at a rate of 10ml per animal, Bomacaine, Bomac Laboratories Ltd., NZ), and received amoxycillin trihydrate injected subcutaneously in the dorsal thorax for antibiotic prophylaxis (150mg/ml at a rate of 1ml per 10kg estimated bodyweight, Betamox LA, Norbrook NZ Ltd., NZ). The protocol for isolation and culture of fibroblasts was adapted from a previously described method (Takashima, 1998). Briefly, skin samples were

immediately placed in phosphate-buffered saline (PBS) consisting of 137mM NaCl, 10mM phosphate and 2.7mM KCl at pH 7.4 in distilled water. Samples were washed in PBS, and incubated in 0.3% trypsin in PBS overnight at 4°C. The epidermis was mechanically removed from each sample using forceps, and the remaining tissue was diced to $2mm^3$ and air-dried for 30min in 6-well culture plates (Raylab, NZ) before the addition of culture media: Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 1:1 with penicillin/streptomycin at 100U/ml and 100µg/ml respectively, and Fungizone at 2.5 µg/ml (Gibco, Invitrogen). Cells were passaged once confluence was reached. At the 3rd-6th passage the cells were seeded into type I collagen cellcoat 24-well plates (Raylab, NZ) at 10,000 cells per well and cultured to confluence.

4.2.2 Radiolabelled sulphate uptake

The protocol for radiolabelled sulphate uptake was adapted from a method described by Satoh *et al* (1998). Radiolabelled sulphate was purchased from GE Healthcare Biosciences. Cell cultures were washed 3 times in 37ºC low ionic strength washing solution (300mM sucrose, 1mM $MgCl_2$, 10mM N - 2 -hydroxyethylpiperazine - N' - 2 - ethanesulphonic acid/ tris - (hydroxymethyl) - aminomethane (HEPES/Tris), pH 7.5), and preincubated for 2min at 37°C. Cells were then incubated in 500µL of uptake solution (300mM sucrose, 1mM $MgCl_2$, 10mM HEPES/Tris, pH 7.5, 40µCi/ml [35S]sulphate) with non-radiolabelled Na_2SO_4 at 2µM, 25µM, 50µM, 100µM, for 5min at 37ºC. Cells were washed 4 times with ice-cold washing solution containing 100mM sucrose, 100mM $NaNO_3$, 1mM $MgCl_2$, 10mM HEPES/Tris, pH 7.5, then lysed in 0.3mL 10% sodium dodecyl sulphate (SDS). Cell lysate aliquots of 150µl were mixed with 3ml scintillation fluid (toluene, 2,5-diphenyloxazole, 2-p-phenylenebis 5-phenyloxazole) and allowed to settle overnight. Each reaction was performed in triplicate, and evaluated for 5min using a Tri-Carb 2900TR liquid scintillation analyser (Perkin-Elmer).

4.2.3 Protein determination

The cell lysate protein concentrations were determined in 96-well microtitre plates (Raylab, NZ) using the modified Lowry procedure (Lowry *et al.*, 1951; Hartree, 1972; Oostra *et al.*, 1978; Caprette, 1995). Lowry concentrate was prepared by mixing 3 parts copper reagent (0.63M Na_2CO_3, 5mM $CuSO_4.5H_2O$, 2mM sodium potassium tartrate in water), 1 part 1% SDS and 1 part 1M NaOH. Folin reagent was

prepared by dilution of 2N Folin reagent (Sigma-Aldrich Corp., St. Louis, MO, USA) to 0.2N with water.

To dilute the cell lysate, 20µl was mixed with 60µl distilled water, followed by 80µl of Lowry concentrate. The solution was pipette-mixed and incubated at room temperature. After 10min, 40µl Folin reagent was added and the solution was pipette-mixed and incubated at room temperature for 30min. Cell lysate samples were assayed in triplicate and absorbance was read at 490nm. A standard curve was constructed using bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA).

4.2.4 Analysis

Protein concentration in µg per 20µl was multiplied by 7.5 to find the total µg protein per 150µl. Scintillation counts per minute (cpm) were corrected for quenching by dividing by 0.01 multiplied by the SIS number generated by the Tri-Carb analyser for each sample. The counts were then corrected for protein concentration by dividing by total protein per 150µl, giving cpm per µg protein. Scintillation cpm corrected for quenching and protein concentration were analysed using S-Plus 8.0 student edition (Insightful Corp., 2007). The ANOVA model fitted was: cpm ~ age + sex + disease status, and the Student's t-test was used to compare individual animals of interest.

4.3 Results

The radiolabelled sulphate uptake by fibroblasts from chondrodysplastic and control Texel sheep is illustrated in Figure 4.1. One animal, the mature control ewe, was an outlier, with 3-5 times the sulphate uptake of other animals. For this animal, radiolabelled sulphate uptake decreased with increasing non-radiolabelled sulphate concentration. This was the expected result, as non-radiolabelled sulphate should compete with the radiolabelled sulphate for transport into cells. When this animal was removed from analysis and the results were fitted for age, sex and disease status, there was no difference in radiolabelled sulphate uptake between the different concentrations of non-radiolabelled sulphate concentrations. Because of this outcome, the results for each animal were pooled.

The Student's t-test demonstrated that the control female lamb had a greater mean uptake of radiolabelled sulphate than the affected female by 249.3 ± 36.84cpm

(P≤0.0005), but there was no significant difference between the affected and control male lambs. The control female lamb had a greater mean uptake of radiolabelled sulphate than the control male lamb by 261.3 ± 38.77cpm (P≤0.0005). There was no difference in mean or variance between affected female and male lambs and between the affected lambs and the control male. Sulphate uptake from female lambs was far more variable than from males.

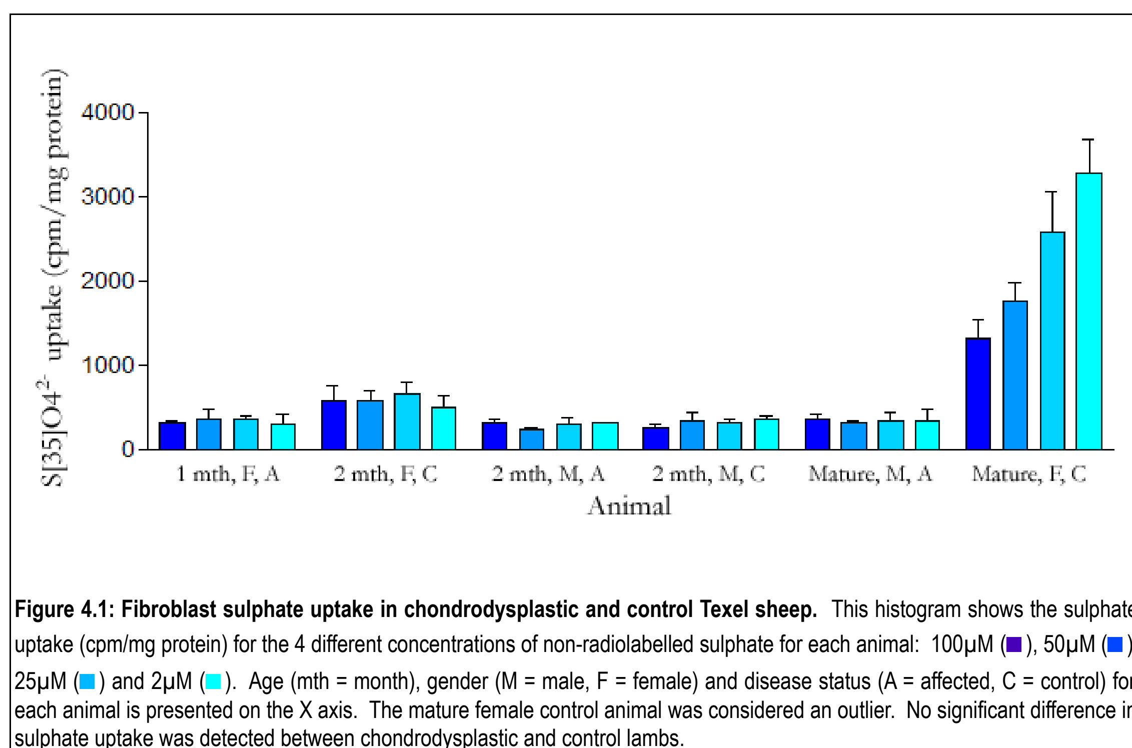


Figure 4.1: Fibroblast sulphate uptake in chondrodysplastic and control Texel sheep. This histogram shows the sulphate uptake (cpm/mg protein) for the 4 different concentrations of non-radiolabelled sulphate for each animal: 100µM (■), 50µM (■), 25µM (■) and 2µM (■). Age (mth = month), gender (M = male, F = female) and disease status (A = affected, C = control) for each animal is presented on the X axis. The mature female control animal was considered an outlier. No significant difference in sulphate uptake was detected between chondrodysplastic and control lambs.

4.4 Discussion

The results of this study did not support the hypothesis that undersulphation cartilage matrix in chondrodysplasia of Texel sheep is the result of reduced cellular uptake of sulphate. While there was a difference in sulphate uptake between the chondrodysplastic and control female lambs, there was a slightly greater difference between the female and male control lambs. This outcome was not consistent with a relationship between disease status and sulphate uptake, although it cannot be ruled out. These findings in chondrodysplasia of Texel sheep were consistent with those of a previous study in which the examined exonic sequence of the *SLC26A2* gene in an affected sheep was found to be mutation-free (Byrne, 2005). While it has been suggested that the recessive nature of diseases caused by mutations in the *SLC26A2* gene may mean that 50% functionality of the DTDST is adequate for normal skeletal development (Cohn, 2001), there have been reports of studies *in*

vitro in which no difference in activity between some affected and wild-type transporters could be detected (Karniski, 2001). It is also possible that a very mild reduction in DTDST activity may not have been able to be detected using fibroblast cultures. Since chondrocytes have a greater potential for sulphate uptake than fibroblasts (Rossi *et al.*, 1996a), chondrocyte cultures may be better able to expose small differences in sulphate uptake. Unfortunately, initial efforts to conduct the trial with cultured chondrocytes were unsuccessful. At the later stage, fewer affected sheep were available, and dermal fibroblasts were used instead in order to avoid euthanasia of potentially valuable experimental animals. A trial using more sheep and higher concentrations of radiolabelled sulphate may have been able to detect slight differences in sulphate uptake, but laboratory constraints on the use of radioactive substances prevented this, even had the animals been available.

Chondrodysplasia of Texel sheep appears to be due to a disorder of sulphate metabolism. Evidence for this is provided by reduced staining of extracted cartilage proteoglycans by toluidine blue, as described by Byrne (2005) (see Figure 1.7), by the reduced staining of cartilage in histological sections, and by the distinctive histological lesions, which strongly resemble disorders of sulphate metabolism in human beings (see Chapter 2). Even if sulphate is able to enter cells normally, there are several other points at which the pathway involved in the sulphation of proteoglycans could be interrupted (see Chapter 1, Figure 1.3). Reduced activity of phosphoadenosine-phosphosulphate synthase 2 (PAPSS2) would decrease the amount of phosphoadenosine-phosphosulphate (PAPS) available, as occurs in murine brachymorphism (Orkin *et al.*, 1976; Kurima *et al.*, 1998) and spondyloepimetaphyseal dysplasia, Pakistani type (ul Haque *et al.*, 1998). Normal quantities of PAPS must then be translocated across the golgi membrane and sulphate must be transferred to the recipient proteoglycan via sulphotransferases, such as chondroitin 6-sulphotransferase and chondroitin 4-sulphotransferase 1 (Fukuta *et al.*, 1998; Yamauchi *et al.*, 2000; Hiraoka *et al.*, 2001). Defects in any of these components, or their regulation, could result in chondrodysplasia with undersulphated cartilage matrix (Thiele *et al.*, 2004; Klüppel *et al.*, 2005).

4.5 Summary

Chondrodysplasia of Texel sheep is associated with reduced sulphation of cartilage proteoglycans, suggesting a defect in sulphate metabolism, but no reduction in

radiolabelled sulphate uptake by cultured dermal fibroblasts was demonstrated in this study. Although the number of animals used was small and the technique may have lacked sensitivity, the results suggest that the defect may involve intracellular sulphate activation, transport or incorporation into glycosaminoglycans, rather than cellular uptake.

5 Genetics of Texel chondrodysplasia

5.1 Introduction

Chondrodysplasia of Texel sheep is a genetic disorder with a simple autosomal recessive mode of inheritance (Byrne, 2005) and with striking microscopic and biochemical similarities to human achondrogenesis 1b and diastrophic dysplasia (see Chapter 2, Figures 2.8, 2.9 & 2.15). Achondrogenesis 1b and diastrophic dysplasia are two examples of a group of diseases caused by mutations in *SLC26A2*, which encodes the diastrophic dysplasia sulphate transporter (DTDST) (Cai *et al.*, 1998; Satoh *et al.*, 1998; Superti-Furga *et al.*, 1999). These mutations result in decreased transport of sulphate into chondrocytes and fibroblasts (Karniski, 2004) and *SLC26A2* was therefore considered a prime candidate gene for chondrodysplasia of Texel sheep, although no mutation was identified in the sequenced 85.4% of exonic DNA from *SLC26A2* in chondrodysplastic Texel sheep (Byrne, 2005). This result did not preclude the possibility of a mutation in the remaining 14.6%, or of reduced expression of a normal transporter due to a mutation in a non-transcribed regulatory region. Another candidate gene involved in the pathway for activation and transfer of sulphate to cartilage proteoglycans is *PAPSS2* (see Chapter 1, Figure 1.3). Although the precise locus of this gene had not been determined at the time of this investigation, comparative maps indicated that it may be located on ovine chromosome 22 (Australian Sheep Gene Mapping Web Site, 2004; Entrez Gene, 2007). Identification of the gene responsible for chondrodysplasia in Texel sheep would enhance understanding of the pathogenesis of the disease, focus the study of metabolic features, potentially extend current knowledge about cartilage matrix synthesis, and may lead to the development of a genetic test able to identify animals carrying the defective gene.

Approaches to the identification of disease-causing mutations may include direct examination of individual genes which are considered to be candidates because of their biological function, scanning of chromosomes and scanning the entire genome (Gut, 2004; Zhu & Zhao, 2007). While it is usually cheaper and less resource-intensive to examine individual candidate genes than to perform chromosome or genome scans, it is frequently impossible due to the poor level of existing knowledge about the biology of the phenotype under investigation. Furthermore, it is of limited use for polygenic traits (Tabor *et al.*, 2002; Zhu & Zhao, 2007), and in species for which large areas of the genome is unmapped, such as the ovine genome (Australian Sheep Gene Mapping Web Site, 2004). Scanning techniques, however,

will generally identify a relatively large chromosomal region which may still contain many candidates, depending on the density of markers available. Because in the case of chondrodysplasia of Texel sheep the initial candidate gene, *SLC26A2*, did not contain a mutation (Byrne, 2005), the next step in the genetic investigation became a scan of several chromosomes.

An opportunity for collaborative research on the genetics of chondrodysplasia in Texel sheep arose with the Centre for Integrated Animal Genomics in the Department of Animal Science at Iowa State University. Previous studies within this group had successfully employed microsatellite marker linkage disequilibrium (LD) mapping to locate the causative mutation for a form of dwarfism in American Angus cattle (Mishra *et al.*, 2004). The following study was performed with the assistance of Dr J. Reecy and Dr J. Koltes.

Microsatellites, or "short tandem repeats", are short arrays of repeated sequences which tend to be highly polymorphic due to their high rate of mutation (Lynch & Walsch, 1998). Different alleles are identified by the number of repeats in an array. Because microsatellite alleles are scored by array length, they are codominant, allowing heterozygotes to be differentiated from homozygotes. A potential drawback of microsatellite markers is the presence of null alleles, which fail to amplify to detectable levels in PCR assays (Dakin & Avise, 2004).

Linkage disequilibrium is the non-random association of alleles, and can be used with several markers on a chromosome as a tool to identify the location of a gene of interest, even if a marker does not lie at that exact locus (Lynch & Walsch, 1998; Gaut & Long, 2003). This process requires DNA from a group of animals for which the pedigree and phenotypes (affected and normal) are known. Inbreeding, cross-breeding and selection are examples of influences that tend to increase LD. If the LD is extensive, it can mask the true chromosomal region of interest. In this way LD is both necessary in order to refine the region of interest, and a hindrance when present in large amounts (Lynch & Walsch, 1998).

The aim of this study was to identify genetic linkage of the chondrodysplastic phenotype in Texel sheep to a chromosome and refine an area of interest on that chromosome, thus assisting with the identification of likely candidate genes, and to rule out others.

5.2 Materials and methods

5.2.1 Animals used

Only DNA from animals for which the phenotypes were known was used in this study. DNA samples were available from 2 of the 3 sires (all affected) of the lambs from the original back-cross breeding trial, 18 chondrodysplastic lambs and 9 carrier lambs. Lambing took place in a common paddock, all animals were checked twice daily, and new lambs were tagged at these times. The ewe that was attentive to a lamb at the time of tagging was recorded as the dam.

5.2.2 DNA extraction

Samples of spleen were collected during necropsy and stored at -20°C until DNA extraction. While most samples were extracted using a Wizard genomic DNA extraction kit (Promega, Madison, WI, USA), samples from 03-04 and 70-04 were extracted using a GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich Corp., St. Louis, MO, USA), and 59-04 using a high pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany). Briefly, the Promega protocol involved freezing 10mg samples of spleen in liquid nitrogen and grinding the tissue to powder, lysing the cells and nuclei, and incubating the lysate with a proteinase. The proteins were precipitated and the samples centrifuged at 13,000g for 4min to separate protein from the solution. The supernatant containing the DNA was transferred to isopropanol and gently inverted until a mass of DNA was visible, then centrifuged at 13,000g for 1min to obtain a DNA pellet. This pellet was then washed in ethanol and air-dried.

5.2.3 Microsatellite selection

Microsatellite markers were selected from ovine chromosomes 1, 5, 6, 13 and 22 using information from Sheep Predicted Map version 1.4 (Australian Sheep Gene Mapping Web Site, 2004). These chromosomes were selected due to either the known location of candidate genes or their linkage to a high number of chondrodysplastic diseases in cattle and human beings. Ideally the microsatellite markers would be spaced less than 20cM apart, but their limited availability in the ovine genome prevented this in some areas. Markers used in this study are listed in Table 5.1, microsatellite primer sequences are listed in Appendix 13. Primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA).

Table 5.1: List of microsatellite markers chosen for amplification, together with nearby genes related to cartilage or skeletal development. Genes in parentheses are only speculated positions based on comparative gene maps.				
Chr.	**Marker**	**Locus (cM)**	**Nearby genes of interest**	
1			(*MATN1*)	Matrilin-1, cartilage matrix protein
	BMS2833	14.2	*BMP8A*	Bone morphogenic protein 8a
			BMP8B	Bone morphogenic protein 8b
			COL9A2	Collagen type IX, α2
	BMS835	45.3	(*COL16A1*)	Collagen type XVI, α1
	MCMA41	68.1	*SLC35D1*	UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter
	CSRD108	86.1	*LEPR*	Leptin receptor
			COL11A1	Collagen type XI, α1
			CSF1	Colony-stimulating factor 1
	MCM58	112.1	*CHI3L2*	Chondrocyte protein YKL39
	BMS482	131.2	*ITGA10*	Integrin, α10
			HAPLN2	Hyaluronan and proteoglycan link protein 2
			ADAMTS4	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 4
			S100A4	S100 calcium binding protein A4
			CTSK	Cathepsin K
			ADAMTS5	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5
	MAF64	164	(*HSPG2*)	Heparan sulphate proteoglycan 2
	INRA11	210.6		
	BM8246	241.1		
	BM864	263.6		
	BM3205	300		
	EPCDV13	348.4		
5	TGLA303	40.8	*P4HA2*	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II
	BM741	52.2	*SLC26A2*	Solute carrier family 26 (sulphate transporter), member 2
	BM7247	64.3		
	JAZ	66.7	*HRH2*	Histamine receptor H 2
			FBN2	Fibrillin 2
6	CP125	2.6	(*FGF2*)	Fibroblast growth factor 2
	MCM53	29.7	*PAPSS1*	3'-phosphoadenosine 5'-phosphosulphate synthase 1
	HH55	54.6		
	JMP1	76.1	*BMPR1B*	Bone morphogenetic protein receptor, type IB
	BM4311	111.6	*BMP3*	Bone morphogenetic protein 3
	JMP8	135.5	*FGFR3*	Fibroblast growth factor receptor 3
13	BMC1222	12.3	(BMP2)	Bone morphogenic protein 2
	SCYAMS	37.3	*OTOR*	Otoraplin
	MCMA2	58		
	BL42	74.4	*CDMP1*	Cartilage-derived morphogenic protein-1
	BL1071	106.6	*MATN4*	Matrilin 4
			MMP9	Matrix metallopeptidase 9
	BMS995	125.9		
22	BMS651	0	(*PAPSS2*)	3'-phosphoadenosine 5'-phosphosulphate synthase 2
	BMS907	13.8		
	FASMC2	15.4		
			CRTAC1	Cartilage acidic protein 1
	HEL11	30	*TLL2*	Tolloid-like 2
	BM6041	57.7		
	BM7237	71.6		

5.2.4 PCR protocol

In order to fluorescently label PCR products, a FAM-tailed protocol was used in which a FAM-labelled sequence, 5'-TGAAAAACGACGGCCAGT-3', was added to the 5' end of the forward primer for each microsatellite marker (Schuelke, 2000). The concept behind this method is illustrated in Figure 5.1.

All consumables used in microsatellite amplification, other than primers and template DNA, were obtained from Promega, Madison, WI, USA. The original PCR recipe contained 1µl 10x buffer solution, 0.8µl 25mM $MgCl_2$, 0.4µl 10mM dNTPs, 0.4µl 25nM FAM-tail, 0.4µl 25nM reverse primer, 0.1µl 25nM forward primer, 0.1µl Taq, 1µl DMSO, 4.3µl distilled water and 0.5µl template DNA. Owing to changes in supply, the PCR recipe was altered partway through the experiment to 2µl Green GoBuffer, 1.5µl $MgCl_2$, 0.4µl 10mM dNTPs, 0.4µl FAM tail, 0.4µl 25nM reverse primer, 0.1µl 25nM forward primer, 0.1µl GoTaq, 4.5µl distilled water and 0.5µl template DNA. The differences in protocols were not expected to affect the results, as markers were amplified in complete batches containing all animals of interest.

The standard thermocycler protocol was 94°C for 5min, (94°C for 45s, first annealing temperature for 45s, 72°C for 45s) 29 times, (94°C for 30s, 53°C for 45s, 72°C for 45s) 7 times, and 72°C for 10min. Samples were then held at 4°C until collection. These cycles were performed by the MJ Research PTC-200 Peltior Thermal Cycler DNA Engine. Initially, to optimise primer binding the protocol was run with the first annealing temperature set to a gradient of 50-66°C. The first annealing temperatures used are listed in Table 5.2.

Table 5.2: Optimal annealing temperatures for microsatellite primers.

50°C	51°C	52°C	53°C	54°C	56°C	57°C	59°C	60°C	62°C
BM4311	BMS482	HH55	BM8246	BL42	CP125	BMS835	CSRD108	MAF64	JMP8
JMP1	MCMA2	HEL11	BL1071	BMS907	BM7247	BMS2833	BM741	INRA11	
BMC1222	BMS995		TGLA303	BM7237	BMS651	MCM58	JAZ		
			BM6041		FASMC2				
					MCM53				

To test for PCR product, 4µl of primer was mixed with yellow loading dye and run in 4% agarose gel with ethidium bromide. Gels were run at 100-120V. When satisfactory PCR product was identified, sample dilutions were prepared in a range

from full strength to ten-times dilution based on the brightness of the bands, and submitted for genotyping at the Iowa State University DNA facility (IA, USA). Genotyping was performed using an Applied Biosystems Prism 3100 Genetic Analyser and a 6-carboxyl-X-rhodamine (ROX)-labelled size standard (500-ROX, GeneScan, Applied Biosystems, Foster City, CA, USA).

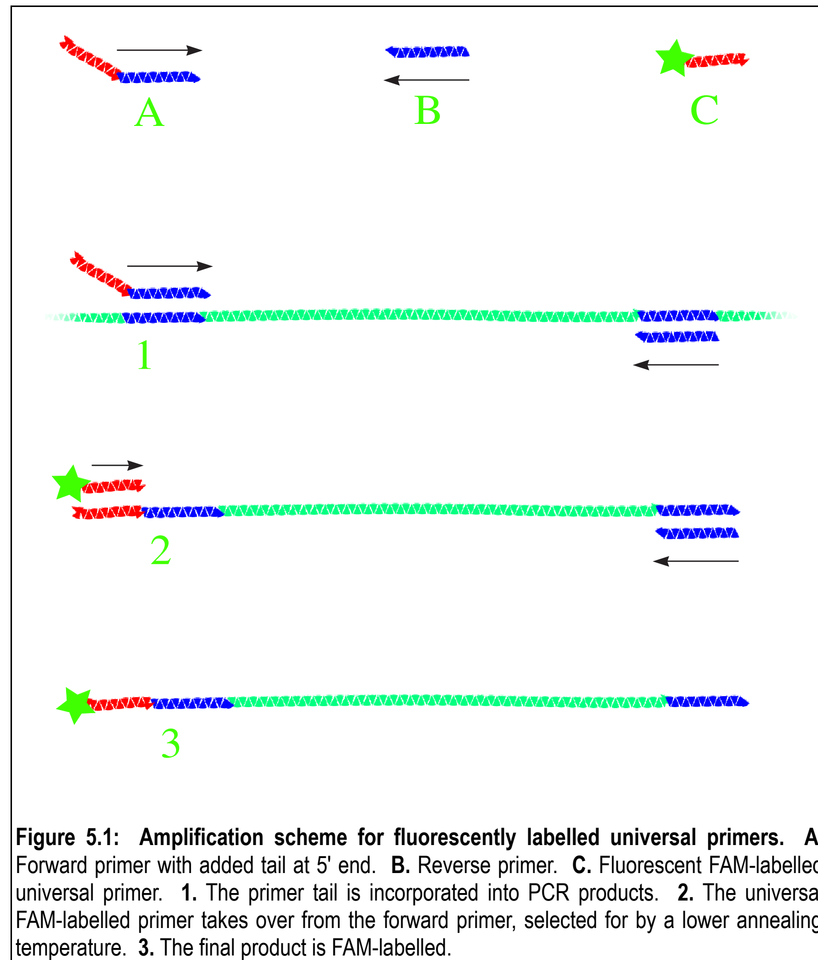


Figure 5.1: Amplification scheme for fluorescently labelled universal primers. A. Forward primer with added tail at 5' end. **B.** Reverse primer. **C.** Fluorescent FAM-labelled universal primer. **1.** The primer tail is incorporated into PCR products. **2.** The universal FAM-labelled primer takes over from the forward primer, selected for by a lower annealing temperature. **3.** The final product is FAM-labelled.

5.2.5 Analysis

Microsatellite amplification was scored using Genoprofiler version 2.0.2 (UC Davis, CA, USA) and Chromas version 2.31 (Technelysium Pty. Ltd., Helensville, QLD, Australia). Chi-squared tests were performed to assess changes in heterozygosity, allele frequency and genotype in association with the chondrodysplastic phenotype. A pedigree was then constructed using the pooled microsatellite data, and the phenotype for each animal was indicated by "0" in phenotypically normal animals and "1" in chondrodysplastic animals. Half-sib regression interval mapping was performed using QTL express (University of Edinburgh, qtl.cap.ed.ac.uk) using Haseman-Elston regression, with bootstrap resampling set to 2000 iterations in

order to generate a 95% confidence interval for the locus of interest. To test for significance, the analysis was run with chromosome-wide permutations set to 2000 iterations. Haseman-Elston regression evaluates pairs of relations and the number of alleles identical by descent. This technique regresses the squared difference in trait score of each pair of relations on the fraction of alleles identical by descent at a given marker locus. A significant negative slope indicates genetic linkage (Haseman & Elston, 1972; Lynch & Walsch, 1998).

5.3 Results

5.3.1 Pedigree information

The sires of all but two of the lambs were able to be identified using microsatellite data. The pedigree information is illustrated in Appendix 14. Because genetic information was only available for Rams 1 and 2, only their combined 18 offspring were able to be used for half-sib regression interval mapping.

5.3.2 Microsatellite scoring

Not all markers amplified clearly and time constraints prevented further reaction attempts or substitution with other markers where available. Markers that either failed to amplify or were unable to be reliably scored were MCMA41, BM864, BM3205, EPCDV13, BM741, BM7247 and SCYAMS. Although there was no previous record of a null allele for BM741 in sheep (Australian Sheep Gene Mapping Web Site *et al.*, 2006), repeated amplification attempts failed to demonstrate any alleles in several animals, one of which was a sire. In other animals, the same technique resulted in amplification of a single allele 200 base pairs in length. Because the genotype of animals could not be established for BM741 (homozygosity for the amplified allele could not be distinguished from heterozygosity for the allele and a null allele), this marker could not be included in the analysis.

Alleles were assigned arbitrary identification numbers based on the size of the amplified microsatellite, with higher numbers indicating a longer amplified sequence (Figure 5.2). Full genotyping results are available in Appendix 15.

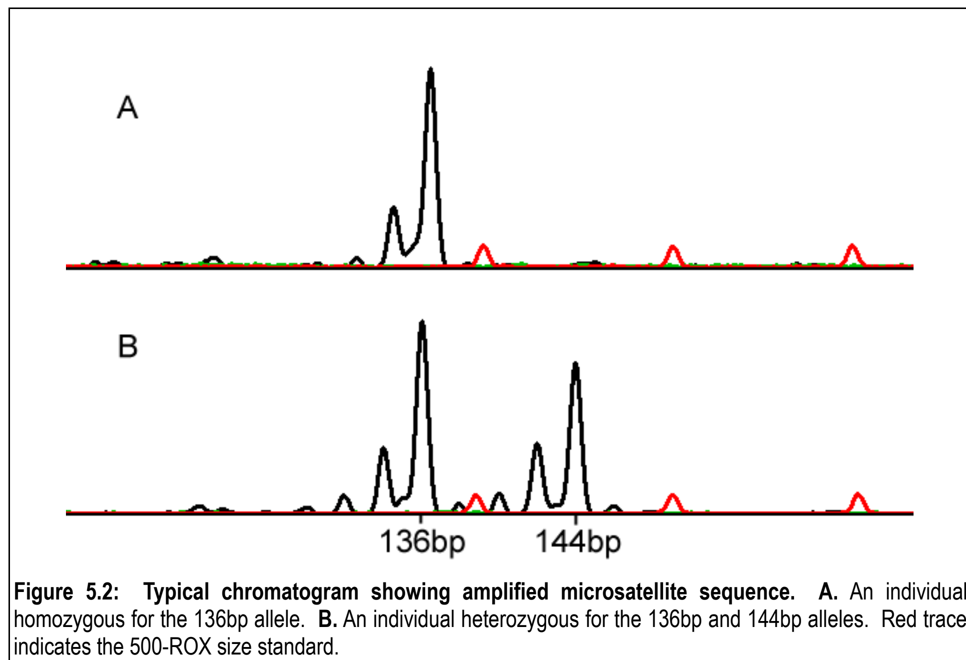


Figure 5.2: Typical chromatogram showing amplified microsatellite sequence. A. An individual homozygous for the 136bp allele. **B.** An individual heterozygous for the 136bp and 144bp alleles. Red trace indicates the 500-ROX size standard.

5.3.3 Chi-squared analysis

The only significant result of chi-squared testing was an increased level of heterozygosity in affected sheep relative to phenotypically normal carrier animals at marker BMS2833, chromosome 1 ($P \leq 0.05$) (Figure 5.3). Some differences in allele frequency or genotype between affected and carrier sheep appeared at markers SCYAMS and BMS995 on chromosome 13 and FASMC2 on chromosome 22, but did not reach significance. Chi-squared scores low enough to support no loss of heterozygosity occurred at MAF64 on chromosome 1, BM4311 on chromosome 6, SCYAMS on chromosome 13, and both BMS651 and FASMC2 on chromosome 22.

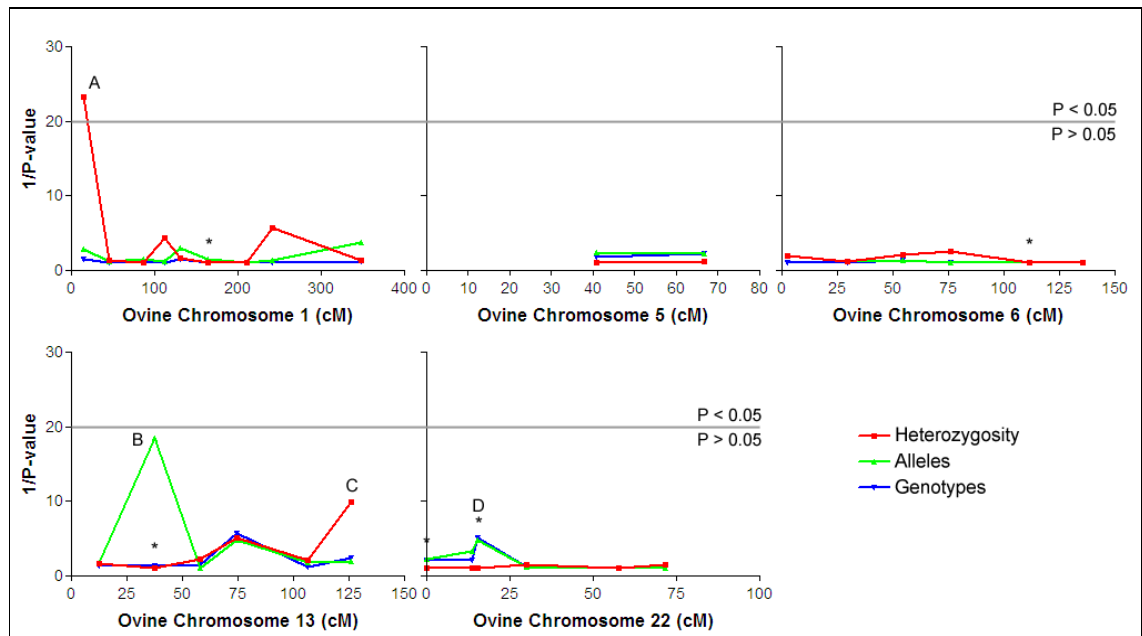


Figure 5.3: Chi-squared test results for differences between control and chondrodysplastic Texel sheep in heterozygosity, allele frequency and genotype of microsatellite markers. A. A significant difference in the level of heterozygosity in the marker BMS2833 was due increased heterozygosity in chondrodysplastic sheep (P≤0.05). **B.** A difference in allele frequency between affected and control sheep at the marker SCYAMS failed to reach significance. **C. M**arker BMS995 demonstrated an increase in heterozygosity in chondrodysplastic sheep, but this was not significant. **D.** The marker FASMC2 demonstrated a slight, but non-significant, difference in allele frequency and genotype between affected and control sheep. * = markers at which chi-squared testing supported no loss of heterozygosity in affected animals relative to carriers.

5.3.4 Half-sib regression interval mapping

The initial results for the half-sib regression interval mapping are listed in Table 5.3. Because 2 of the 4 markers on chromosome 5 amplified poorly and another marker demonstrated nearly total homozygosity, half-sib regression interval mapping was unable to be performed on this chromosome.

Table 5.3: Half-sib regression interval mapping results for microsatellite data from chondrodysplastic and control Texel sheep. The peak loci listed here are the points at which linkage disequilibrium was most evident for ovine chromosomes 1, 6, 13 and 22. Significance was not reached at any point.

	Chromosome 1	Chromosome 6	Chromosome 13	Chromosome 22
Peak Locus	128cM	0cM	94cM	0cM
95% CI	2-228cM	0-131cM	2-112cM	0-71cM
Likelihood ratio	4.6	3.37	2.75	3.58
Significance	none	none	none	none

5.4 Discussion

The results of linkage disequilibrium testing for chondrodysplasia of Texel sheep in this study failed to identify a likely candidate gene. Although chromosome 1 demonstrated a significant difference in the level of heterozygosity between

chondrodysplastic and control sheep ($P \leq 0.05$), given the number of chi-squared tests performed on the microsatellite data, this one-off outcome may have been coincidental (Moore & McCabe, 1993). The difference consisted of an increase in heterozygosity in chondrodysplastic lambs, which is not consistent with linkage to a recessively inherited disease (Futuyma, 1998), suggesting that the difference between genotypes of affected and control lambs at this locus was not linked to disease status.

The main difficulties experienced in this study were the due to the limited number of animals for which the phenotype was known, and poor amplification of several of the markers. Had it been possible to verify the phenotype of newborn lambs, the number of samples available would have increased from 26 to 64. Such an increase in sample size may have generated sufficient statistical power to produce a more definitive result (Moore & McCabe, 1993). Another potential limitation to this study was the practice of lambing in a common paddock, allowing the opportunity for lamb-stealing by ewes to occur (Alexander *et al.*, 1983). Although all animals were checked twice-daily, lamb-stealing could not be absolutely ruled out, affecting the integrity of the constructed pedigree. Ideally, the dams would have been genotyped along with the lambs, enabling the detection of cases of lamb-stealing. Unfortunately, tissue samples were not routinely collected from the dams as the genetic study described in this chapter had not been anticipated at the time of the breeding trial, which was originally established for a different research purpose.

Because the animals available for use in this study were closely related and originated from a population where the disease frequency was artificially increased (through back-crossing), there was a risk of spurious association of the disease to any marker allele in high frequency (Pritchard *et al.*, 2000). Transmission disequilibrium testing (TDT) is a statistical tool able to overcome this difficulty by comparing the transmission of a disease-linked marker allele from a heterozygous parent to its affected offspring (Spielman *et al.*, 1993). Unfortunately, in this study DNA was only available from affected parents. This prevented the use of TDT, as chondrodysplasia of Texel sheep is a recessive disorder and heterozygosity of sires does not support linkage of the marker to the disease, while homozygosity may be due to high allele frequencies in the inbred population (Lynch & Walsch, 1998).

The relatively recent discovery of chondrodysplasia in Texel sheep (Thompson *et al.*, 2005) indicates that this disease is due to either a reasonably new mutation in

the population, or an old mutation that has gone unrecognised or unreported for many generations. If the former is the case, markers some distance from the causative mutation would be expected to show linkage because there has been less opportunity for recombination. This effect is useful for detection, but would hinder fine mapping using LD methods (Devlin & Risch, 1995; Lynch & Walsch, 1998).

Further genetic research on chondrodysplasia of Texel sheep will require an increased number of samples, preferably not sourced from a back-cross trial. An increased number of unaffected relations to the chondrodysplastic sheep would make the analysis more powerful, as would an increase in marker density. An alternative to undertaking another large-scale breeding trial would be to approach Texel breeders. If a farm was found to regularly produce chondrodysplastic lambs, samples from a related group of affected and non-affected animals and their parents could be used to perform another genome-scanning experiment. As more markers, both microsatellites and single nucleotide polymorphisms (SNPs), are identified for the ovine genome (Cockett, 2006; Pariset *et al.*, 2006), LD mapping will likely become an increasingly useful tool for this type of study. Because chondrodysplasia of Texel sheep is believed to have a simple mendelian mode of inheritance (Byrne, 2005), a candidate gene approach in further genetic research may be appropriate (Zhu & Zhao, 2007), especially if further classification of the disease phenotype reveals candidate genes that have not already been investigated. An alternative type of investigation would be to examine differential expression of genes in chondrodysplastic Texel sheep, although studies of this kind often identify a large number of differently expressed genes, and additional supporting evidence should be considered before using this technique to identify candidate genes (Okuda *et al.*, 2002; Zhu & Zhao, 2007).

5.5 Summary

This study did not find evidence for genetic linkage of chondrodysplasia of Texel sheep to ovine chromosomes 1, 5, 6, 13 or 22. The effectiveness of the study was hampered by the low number of samples, the level of inbreeding involved in the sample animals, and the poor amplification of several microsatellite markers. A new breeding trial to generate samples, involving multiple affected and unaffected parents and minimised inbreeding would be advisable before repeating this style of genetic investigation.

6 Sulphation of chondroitin disaccharides in cartilage

6.1 Introduction

Chondroitin sulphate is the major sulphated polysaccharide attached to the core protein, aggrecan, in hyaline cartilage matrix (Krueger *et al.*, 1990; Sandy *et al.*, 1997) (see Chapter 1, Figure 1.2). Sulphation of cartilage matrix polysaccharide is vital for normal matrix integrity and compressive stiffness of the tissue, and is therefore crucial to normal cartilage formation, and consequently to endochondral ossification (Sandy *et al.*, 1997). Several forms of chondrodysplasia characterised by reduced levels of sulphate in cartilage have been identified in both human beings and animals (Rossi *et al.*, 1996a; Sandy *et al.*, 1997; Rossi & Superti-Furga, 2001; Forlino *et al.*, 2005). These can occur as a result of mutations reducing the ability of chondrocytes to extract sulphate from the extracellular fluid (Rossi & Superti-Furga, 2001), mutations affecting the activation and addition of sulphate to proteoglycans within the chondrocyte (ul Haque *et al.*, 1998), or fetal deficiency of manganese, which is required for normal function of enzyme systems involved in sulphate activation and transfer to proteoglycans (Leach & Muenster, 1962; Bolze *et al.*, 1985).

Chondrodysplasia of Texel sheep shares microscopic lesions, and certain developmental characteristics, with several diseases resulting from defective sulphate metabolism in human beings and other animals (van der Harten *et al.*, 1988; Valero *et al.*, 1990; Shapiro, 1992; Superti-Furga, 1994). This finding suggests a metabolic defect in sulphate metabolism is the likely underlying defect in affected sheep. Support for this hypothesis was provided by a previous study where hyaline cartilage from chondrodysplastic Texel lambs was shown to contain undersulphated matrix proteoglycans (see Figure 1.7) (Byrne, 2005). Undersulphation of hyaline cartilage matrix was also apparent in histological sections stained with toluidine blue (see Chapter 2, Figure 2.13). A more sensitive study of the cartilage proteoglycan sulphation profile in chondrodysplasia of Texel sheep would provide additional information about the pathogenesis of this disease, and could help to relate biochemical changes to the development of cartilage lesions. An investigation of cartilage sulphation in control and chondrodysplastic Texel sheep of varying ages would also help to determine whether the undersulphation of cartilage proteoglycans is progressive or remains constant. Furthermore, if the biochemical defect is detectable in newborn lambs, it would provide a means for neonatal diagnosis of this chondrodysplasia, which otherwise

cannot be unequivocally recognised until 2-3 weeks of age. The inability to diagnose the chondrodysplastic phenotype in newborn lambs has previously limited the number of samples available for genetic analysis (see Chapter 5).

Historically, studies to compare chondroitin disaccharides have typically used high performance liquid chromatography (HPLC) (Plaas *et al.*, 1986; Rossi *et al.*, 1996a). More recently, capillary electrophoresis (CE) has been used for some of these investigations (Theocharis & Theocharis, 2002; Zamfir *et al.*, 2003). CE allows separation of ionic species by their charge and frictional forces (Gordon *et al.*, 1988) and is able to provide greater resolution of ionic species while requiring a smaller sample size than HPLC (Karamanos *et al.*, 1995).

This pilot study aimed to use CE to quantify chondroitin 4-sulphate and chondroitin 6-sulphate disaccharide concentrations in articular cartilage of chondrodysplastic Texel and control sheep, investigate age-related differences in concentrations of these disaccharides, and to test the hypothesis that a difference in chondroitin sulphate disaccharides is detectable in affected newborn lambs prior to the development of lesions.

6.2 Materials and methods

6.2.1 Animals used and samples collected

An entire humerus was collected from all animals that underwent post mortem examination, and was stored at -20°C for 6-24 months. Articular cartilage shavings (10-15mg) were harvested from the central humeral heads of 8 animals of various ages. Samples 1 and 2 were from obligate affected newborn lambs (for which both parents were chondrodysplastic), samples 3 and 4 were from control newborn lambs, sample 5 was from a 1-month-old lamb severely affected by chondrodysplasia, sample 6 was from a 1-month-old control lamb, sample 7 was from a mature chondrodysplastic ram, sample 8 was from a mature control sheep. Because newborn lambs from the flock carrying the defective gene could not be positively identified as chondrodysplastic by gross or microscopic examination, control samples were collected from mixed-breed newborn lambs that did not have ancestors in common with the affected Texel flock or its contributing members.

6.2.2 Chondroitin disaccharide extraction

The method for extraction of chondroitin disaccharides from articular cartilage was adapted from similar previous studies (Price *et al.*, 1996; Bayliss *et al.*, 1999). The β-*o*-glycosidic bond linking sulphated glycosaminoglycans to aggrecan was cleaved by incubation with 100μl of 0.5M NaOH per mg of sample at 4°C, with agitation overnight. The NaOH was then removed by minidialysis against distilled water in 3.5kDa molecular weight cut-off dialysis cassettes (Slide-A-Lyzer, Pierce Biotechnology, Rockford, IL, USA) for 48 hours at 4°C. Samples were then centrifuged at 13,000g for 3min, and the supernatant freeze-dried then rehydrated in 25μl deionised water. In order to digest the extracted polysaccharides into disaccharides, 10μl of resuspended supernatant was incubated with 10μl chondroitinase ABC (from *Proteus vulgaris*, Sigma-Aldrich Corp., St. Louis, MO, USA) (0.05mU/μl in 200mM Tris base at pH 7.3) for 1 hour at 37°C. Digested samples were filtered through 3kDa nominal molecular weight limit centrifugal filter units (Microcon ultracel YM-3, Amicon Bioseparations, Millipore, Billerica, MA, USA) at 13,000g for 90min, and the filtrate containing the disaccharides was further diluted with 20μl distilled water.

6.2.3 Capillary electrophoresis

Experiments were performed using an automated CE system (HP 3D), equipped with a diode array detector, based on a previously reported method (Karamanos *et al.*, 1995; Theocharis & Theocharis, 2002). Electrophoresis was carried out in a fused silica capillary of internal diameter 50μm and a total length of 46.5cm (40cm from inlet to detector). The capillary incorporated an extended light-path detection window (150μm) and was thermostatically controlled at 25°C. Phosphate buffer (15mM) at pH 3.0 was used as a CE background electrolyte. Between runs, the capillary was washed for 1min with 0.1M NaOH and for 4min with background electrolyte. Detection was carried out using ultraviolet light, with the unsaturated bond resulting from enzymatic digestion absorbing at 232nm. Samples were loaded hydrodynamically at 5000Pa for 10s, giving injection volumes of approximately 10nL, and typically electrophoresed across a potential difference of 20kV. All experiments were carried out at reverse polarity (outlet anodic). Standard samples of 2-acetamido-2-deoxy-3-O- (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) -4-O-sulpho-D-galactose (known as chondroitin 4-sulphate disaccharide, chondroitin sulphate A disaccharide or Δdi-mono4S) and 2-

acetamido-2-deoxy-3-O- (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) -6-O-sulpho-D-galactose (known as chondroitin 6-sulphate disaccharide, chondroitin sulphate C or Δdi-mono6S) (Sigma-Aldrich Corp., St. Louis, MO, USA) were used to identify ionic species and to construct standard curves. Standard preparations of unsulphated chondroitin disaccharide were unavailable commercially.

All samples were run at least 4 times and the peaks integrated using HP Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Where a particular disaccharide exhibited a double peak, both anomeric forms were pooled. The peak areas were subsequently normalised according to their migration times, in order to take into account the differing amount of time that compounds with different electrophoretic velocities spend in the detector window (Goodall *et al.*, 1991). The normalised peak areas were subsequently used, via a calibration of absorptivity using the standard samples, to produce concentrations in mg/L.

6.2.4 Analysis

Disaccharide concentrations were corrected for the original cartilage sample size by multiplying the raw concentration by 10 and dividing by the wet weight in mg of the original cartilage sample. This gave the concentration of disaccharide in mg/L per 10mg of cartilage. In order to compensate for slight differences in injection volume, the concentration of Δdi-mono4S was divided by the concentration of Δdi-mono6S for each sample. This number is referred to as the ratio of Δdi-mono4S to Δdi-mono6S. Statistical analyses were performed using S-Plus 8.0 student edition (Insightful Corp., 2007). The ANOVA model was: ratio of Δdi-mono4S to Δdi-mono6S ~ Age + Status, where "Age" was the age of the animal from which cartilage was taken, and "Status" was the presence or absence of chondrodysplasia. The Student's T-test was used to compare individual animals.

6.3 Results

Although some run to run variation was observed for the absolute amounts of the differently sulphated disaccharides, the ratios of Δdi-mono4S to Δdi-mono6S varied little for each cartilage sample. A typical electropherogram is shown in Figure 6.1, demonstrating peaks caused by Δdi-mono4S, Δdi-mono6S and a peak at the expected location for unsulphated chondroitin disaccharide, as demonstrated by Karamanos *et al.* (1995). Double peaks were routinely observed, owing to the different anomeric forms of the hexosamine present at the reducing terminal of the

disaccharide. The degree of resolution of these anomeric forms was not significantly affected by the voltage applied, but was found to vary with the age of the background electrolyte, presumably due to slight variations in pH.

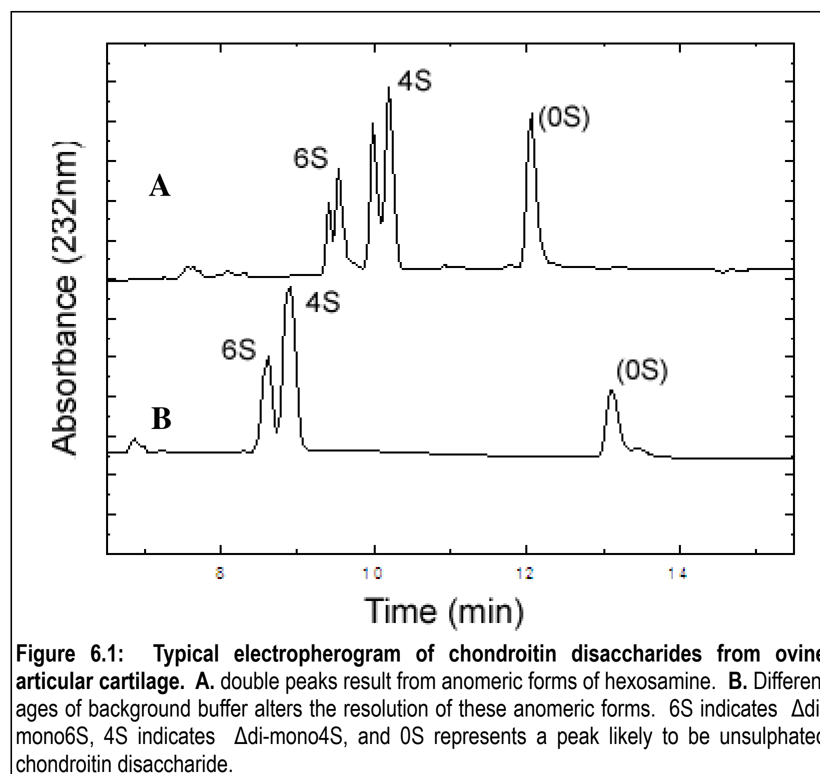


Figure 6.1: Typical electropherogram of chondroitin disaccharides from ovine articular cartilage. A. double peaks result from anomeric forms of hexosamine. **B.** Different ages of background buffer alters the resolution of these anomeric forms. 6S indicates Δdi-mono6S, 4S indicates Δdi-mono4S, and 0S represents a peak likely to be unsulphated chondroitin disaccharide.

Mean concentrations of disaccharide for each animal are listed in Table 6.1, expressed as mg/L per 10mg of sample. Concentrations of Δdi-mono4S were markedly lower in cartilage from chondrodysplastic sheep of all ages than in controls, but there was no such difference in Δdi-mono6S concentrations. The ratios of Δdi-mono4S to Δdi-mono6S were also lower in chondrodysplastic sheep than in unrelated control animals in all age groups ($P \leq 0.0001$, Figure 6.2). The mean ratio of Δdi-mono4S to Δdi-mono6S was markedly lower in the mature control than in the younger controls and in the mature affected ram than in the younger affected lambs ($P \leq 0.0001$). The mean ratio of Δdi-mono4S to Δdi-mono6S in affected newborn lambs and the mature affected ram was 25% less than in age-matched controls, and the same ratio in the 1-month old affected lamb was 40% less than the 1-month old control. Although not detailed here, the area under the peak believed to represent unsulphated chondroitin disaccharide did not vary significantly with disease status in these animals.

Table 6.1: Concentrations of chondroitin sulphate disaccharides from chondrodysplastic and control Texel sheep of varying ages. Concentrations are expressed as mg/L per 10mg of sample.

Animal	Status	Age	Δdi-mono6S	Δdi-mono4S
1	Affected	Newborn	24.2	48.6
2	Affected	Newborn	28.8	55.2
3	Control	Newborn	26.1	74.3
4	Control	Newborn	38.7	91.1
5	Affected	1 month	41.2	61.9
6	Control	1 month	28.6	71.4
7	Affected	Mature	38.3	36.1
8	Control	Mature	44.5	56.3

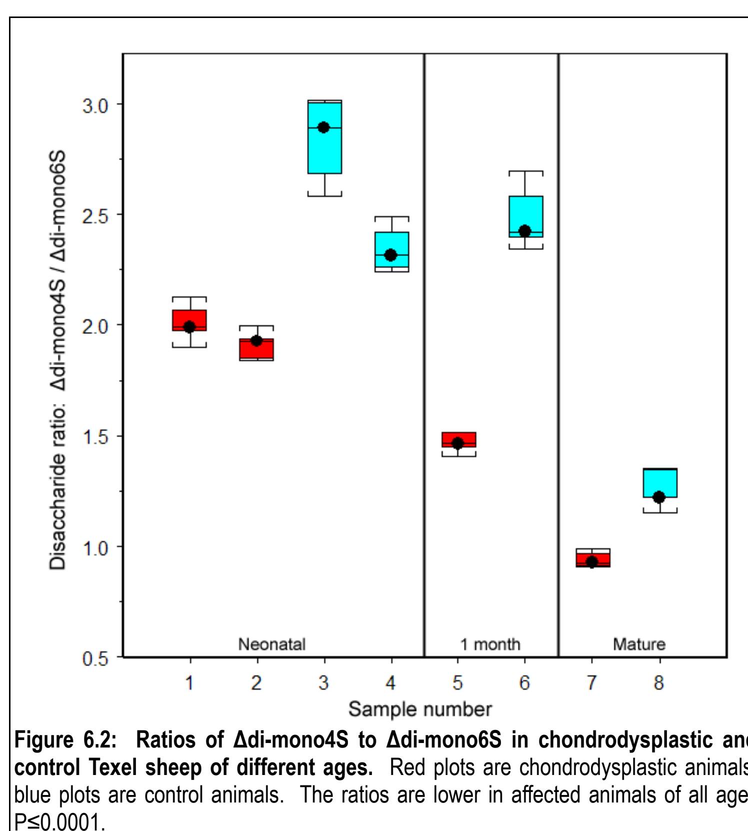


Figure 6.2: Ratios of Δdi-mono4S to Δdi-mono6S in chondrodysplastic and control Texel sheep of different ages. Red plots are chondrodysplastic animals, blue plots are control animals. The ratios are lower in affected animals of all ages $P \leq 0.0001$.

6.4 Discussion

The results of this pilot study indicate that capillary electrophoresis can be used to detect a difference in the profile of cartilage chondroitin sulphate disaccharides in chondrodysplastic Texel sheep when compared with controls, and that the decreased cartilage sulphate content in this disease is most likely due to reduced levels of chondroitin 4-sulphate. Cartilage from Texel sheep with chondrodysplasia had a lower ratio of Δdi-mono4S to Δdi-mono6S than age-matched controls at all

ages measured, supporting the hypothesis that a difference in chondroitin sulphate disaccharides is detectable in affected newborn lambs prior to the development of lesions. A decreased ratio of Δdi-mono4S to Δdi-mono6S has been recorded in chondrodysplasias resulting from sulphate transport disorders due to reduced activity of the DTDST (Rossi *et al.*, 1998), and in mutations in the gene encoding chondroitin 4-sulphotransferase-1, *CHST11* (Klüppel *et al.*, 2005). This finding indicates that *CHST11,* which maps to ovine chromosome 3 at approximately 221.4cM (Australian Sheep Gene Mapping Web Site, 2004), is a potential candidate gene. A naturally occurring decrease in the ratio of Δdi-mono4S to Δdi-mono6S has been described in ageing articular cartilage in humans (Roughley *et al.*, 1981; Bayliss *et al.*, 1999; Lauder *et al.*, 2001). A similar change was evident in the articular cartilage of mature control and chondrodysplastic sheep in this study.

The similarity in Δdi-mono6S concentration between chondrodysplastic and control lambs indicates that a defect in chondroitin 6-0-sulphotransferase-1, encoded by *CHST3*, is unlikely. Loss-of-function mutations in *CHST3* result in spondyloepiphyseal dysplasia Omani type, in which cartilage matrix concentrations of chondroitin 6-sulphate are severely reduced (Thiele *et al.*, 2004). Mutations in *SLC26A2* that decrease DTDST function result in an increase in unsulphated chondroitin disaccharide, along with decreased Δdi-mono4S, and sometimes Δdi-mono6S, in cartilage matrix (Rossi *et al.*, 1997; Rossi *et al.*, 1998). If the unclassified peak identified in the present study is truly representative of unsulphated chondroitin disaccharide, it would suggest that abnormalities in DTDST function are not involved in chondrodysplasia of Texel sheep. This is supported by previous *in vitro* and genetic studies, neither of which were able to support an abnormality of chondrocyte sulphate uptake (see Chapters 4 and 5).

The decreased ratio of Δdi-mono4S to Δdi-mono6S in newborn chondrodysplastic lambs indicates that although affected lambs cannot be distinguished phenotypically from normal or heterozygous lambs at birth, the underlying cartilage abnormality is present. Capillary electrophoresis techniques may therefore be useful in identifying the phenotype of newborn animals that are yet to demonstrate gross or microscopic lesions of chondrodysplasia. DNA and articular cartilages are available from all lambs that could not be included in the previous genetic study (see Chapter 5) because of their undetermined disease status. Although funding constraints do not allow it at this time, the phenotypes of these lambs could be estimated using capillary electrophoresis, thereby increasing the

number of animals available for a renewed genetic investigation. While more samples would be required to confirm the true diagnostic power of the ratio of cartilage Δdi-mono4S to Δdi-mono6S, this study indicates that the information would at least be useful as quantitative trait data associated with the chondrodysplastic phenotype (Lynch & Walsch, 1998).

The newborn affected lambs, which had no gross or microscopic lesions, and the mature affected ram, which had mild lesions of chondrodysplasia, all had ratios of Δdi-mono4S to Δdi-mono6S closer to the values for control animals than did the 1-month old affected lamb, which demonstrated severe lesions of the disease. This suggested that the magnitude of the difference in the ratio of Δdi-mono4S to Δdi-mono6S may have been related to the severity of lesions. It cannot be discerned from this study whether the difference was solely due to the severity of disease, age-related changes in cartilage sulphation specific to chondrodysplasia of Texel sheep, or a combination of the two. Further studies using more animals of different ages representing the full spectrum of severity in this disease would be able to answer this question, as would a study using serial cartilage biopsies. Capillary electrophoresis would be an ideal tool for the latter type of experiment due to the small sample size required (10mg of cartilage) (Price *et al.*, 1996).

The regulation of the proportion of sulphate added to either the 4- or 6-position on the chondroitin disaccharide is poorly understood. While the decrease of Δdi-mono4S but not of Δdi-mono6S found in cartilage from chondrodysplastic Texel sheep in this study intuitively suggests a defect specifically interfering in the addition of sulphate to the 4-position, this is not necessarily the case. In defects of DTDST and PAPSS function, where the available intracellular sulphate and consequently PAPS is reduced (Kurima *et al.*, 1998; Rossi *et al.*, 2003), concentrations of Δdi-mono4S and Δdi-mono6S are not always decreased by equal amounts. In the brachymorphic mouse, which has a mutation in *PAPSS2* and therefore decreased activity of PAPS, there is an increase in unsulphated chondroitin disaccharide and a decrease in both Δdi-mono4S and Δdi-mono6S (Orkin *et al.*, 1976). In cases of diastrophic dysplasia, atelosteogenesis type 2 or achondrogenesis 1b, however, which are all caused by defects in the DTDST, cartilage has increased levels of unsulphated chondroitin disaccharide and tends to show a more marked reduction of Δdi-mono4S than of Δdi-mono6S (Rossi *et al.*, 1997). This difference in chondroitin disaccharide profile was not able to be explained by a difference in PAPS binding affinity of the different

sulphotransferases, as the apparent K_m of chondroitin 4-sulphotransferase has been found to be the same as of chondroitin 6-sulphotransferase (Sugumaran *et al.*, 1986). Because of these past findings, the pattern of altered sulphation of chondroitin disaccharides identified in the present study must be interpreted with care.

The inferences made in this study, while based on striking differences between affected and control animals, are drawn from a very low number of samples. As a result, this study may have underestimated the normal level of variation in concentrations of chondroitin disaccharide species in articular cartilage of sheep. If this is the case, a repeated experiment using cartilage samples from a greater number of animals may reveal less marked differences between control and chondrodysplastic Texel sheep.

6.5 Summary

The concentration of chondroitin 4-sulphate disaccharide and the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate were consistently lower in articular cartilage from chondrodysplastic Texel sheep than in age-matched controls. These differences were detectable in newborn affected lambs that were not showing gross or microscopic lesions of chondrodysplasia, indicating that the underlying cartilage abnormality is present at birth.

7 General discussion

7.1 Introduction

Discovery of a form of chondrodysplasia on a commercial sheep farm in Southland, New Zealand, created an opportunity to investigate the gross, microscopic, biochemical and genetic characteristics of this disease in Texel sheep. Animals for study were generated using a back-cross breeding trial on a Massey University research farm. The results of this trial indicated a simple autosomal recessive mode of inheritance and an apparent defect of cartilage sulphation in chondrodysplasia of Texel sheep (Byrne, 2005). Later studies, discussed in the following sections, generated a more detailed description of the gross and microscopic development of lesions in this disease, and investigated aspects of sulphate metabolism and genetic linkage.

7.2 Development of lesions

Chondrodysplastic Texel lambs were indistinguishable from controls at birth by gross, morphometric or microscopic examination (see Chapters 2 and 3), but appeared to have a lower ratio of chondroitin 4-sulphate disaccharide (Δdi-mono4S) to chondroitin 6-sulphate disaccharide (Δdi-mono6S) in the central articular cartilage of the humeral head (see Chapter 6, Figure 6.2). This finding indicates that although lesions of chondrodysplasia cannot be identified at birth, the underlying biochemical defect is present at that time. While in some genetic diseases the leakage of critical metabolites across the placenta may delay the onset of lesions until postnatal life (Leonard & Morris, 2006), the presence of a biochemical defect at birth in affected lambs suggests that this phenomenon does not occur in chondrodysplasia of Texel sheep. This was also supported by the observation that chondrodysplastic lambs born to affected ewes were identical to those born to phenotypically normal carrier ewes, both at birth and as they grew (see Chapter 2). If the absence of gross and microscopic lesions at birth cannot be explained by metabolic support *in utero*, the question of why the lesions develop so rapidly in the first weeks of life must be raised.

The morphometric analysis of growing control and chondrodysplastic Texel lambs revealed that some disturbance in growth was detectable in the 1-10 day age interval, and there were usually marked differences between affected and control lambs from 11 days of age (see Chapter 3). A similar pattern was seen in the development of microscopic lesions. While subtle lesions were sometimes visible

prior to 5 days of age, the youngest lamb able to be unequivocally diagnosed as chondrodysplastic was 9 days old. These findings suggest that the onset and development of gross lesions in chondrodysplasia of Texel sheep are closely related to the emergence of microscopic lesions. This was also seen in the varying nature of the microscopic lesions, which tended to be milder in the less severely affected animals than in the severely affected animals, and were very mild in the few animals that survived to maturity. The histologically observed rarefaction and cystic degeneration of interterritorial cartilage matrix appeared to be more closely related to the gross phenotype than did the later-developing perichondrocytic collagen rings. The chronology of the development of microscopic lesions in affected lambs was similar to that seen in diastrophic dysplasia, in which longitudinal studies demonstrated relatively early appearance of interterritorial matrix lesions and later appearance of collagen rings (Shapiro, 1992). These microscopic findings support the hypothesis that the development of the gross phenotype in chondrodysplasia of Texel sheep is primarily due to a gradual failure of the structural integrity of the hyaline cartilage matrix, rather than to a disorder of cartilage regulation. This would explain the absence of lesions in neonatal lambs.

There are minimal physical stresses on articular cartilage during fetal development, but forces increase considerably with weight bearing and postnatal growth (Hamrick, 1999). This physical loading, accompanied by compressive and shear stresses, may be a precipitating factor in the development of microscopic and gross lesions in chondrodysplasia of Texel sheep. Credibility is given to this idea by (a) the tendency for lesions to appear earliest and to be most severe in the large joints that combine high loading with extensive mobility; (b) the subjective observation that initially faster-growing lambs seemed to develop severe lesions of chondrodysplasia more rapidly than slower growing lambs; and (c) the preferential appearance of interterritorial matrix lesions in the transitional zone of articular cartilage, which is structurally weaker than the adjacent superficial or radial zones (Bellucci & Seedhom, 2001). Interterritorial cartilage matrix normally contains lower concentrations of sulphated proteoglycans (Ross *et al.*, 1995), which are required for collagen fibrillogenesis (Eyre, 2002). Because the concentration of sulphated proteoglycans in cartilage is decreased in chondrodysplasia of Texel sheep (Byrne, 2005) (see Figure 1.7 and Chapter 6), it is not surprising that the first microscopic lesions appear in parts of cartilage where their concentration would be expected to be lowest. It is possible that the reduction in sulphated proteoglycan

concentration in the interterritorial matrix of the transitional zone of articular cartilage alters the nature of the collagen fibrils, which may further contribute to the weakening of this zone in affected cartilage.

The apparent relationship between lesions of the interterritorial matrix in chondrodysplasia of Texel sheep and severity of the disease did not extend to the perichondrocytic collagen rings. These were present even in mildly affected animals, while the interterritorial matrix lesions tended to be very mild or absent in such cases. Perichondrocytic collagen rings are a distinguishing feature in chondrodysplasia of Texel sheep (Thompson *et al.*, 2005), along with achondrogenesis 1b (Superti-Furga, 1994), diastrophic dysplasia (Stanescu *et al.*, 1984), congenital Mn deficiency (Valero *et al.*, 1990), and apparently in the *SLC26A2* knock-out mouse (Forlino *et al.*, 2006). Immunohistochemistry performed on cartilage from chondrodysplastic Texel lambs revealed that the collagen rings consisted of chiefly type VI collagen (see Chapter 2, Figure 2.14), which, although an important part of the pericellular envelope (Poole *et al.*, 1988b), is not considered to be a major structural component of hyaline cartilage matrix (Wardale & Duance, 1993). Because of this, it may be supposed that the presence of the collagen rings do not have a great effect on the structural integrity of the affected cartilage, and may explain the ability of animals affected by congenital Mn deficiency to recover once a complete diet is available (Valero *et al.*, 1990). This assumes that the collagen rings in congenital Mn deficiency have the same composition as those in chondrodysplasia of Texel sheep. Although defective type VI collagen has been associated with several types of myopathy and muscular dystrophy in human beings (Lamandé *et al.*, 1998; Giusti *et al.*, 2005), there are no published reports of chondrodysplasia either in conjunction with these disorders or as a separate disease.

Many of the arguments in support of a mechanical stimulus for lesion development in chondrodysplasia of Texel sheep could also be applied to a metabolic aetiology, primarily because the metabolic rate of chondrocytes is influenced by mechanical stimulus. From birth until skeletal maturity, articular cartilage is predicted to be under increasing peak pressure due to thinning of cartilage, increased force generation and decreased relative joint size (Hamrick, 1999). High peak pressures in dynamic loading induce chondrocyte division and matrix synthesis (Urban, 1994), possibly in response to mechanically-induced matrix degradation (Archer, 1994). This suggests that as animals become ambulatory and increase in mass,

there will be an increase in metabolic activity of chondrocytes in weight-bearing cartilage. A relationship between local metabolic rate and lesion development was identified in Chapter 3, where the limb-bones with the fastest potential growth rate were also the most severely shortened. It would be reasonable to suppose that cells with a higher metabolic rate would be more severely affected by a disorder involving enzymatic insufficiency or limited substrate. If such a pathogenesis is involved in chondrodysplasia of Texel sheep, it could in part explain the pattern of lesions in this disease, and may be a reason for the marked variability in severity of lesions both between animals, and between sites within animals. Similar variability has been described in diastrophic dysplasia, where relatives with the same *SLC26A2* mutation had differing expression of the disease (Superti-Furga, 2001), potentially due to a varying ability for chondrocytes to use alternative sources of intracellular sulphate to synthesise sulphated glycosaminoglycans (Rossi *et al.*, 2003). Perhaps in chondrodysplasia of Texel sheep there is a similar compensatory mechanism, such as has been proposed in diseases caused by mutations in *PAPSS2*, where the *PAPSS1* gene product may be able to partially recover the deficiency in phosphoadenosine-phosphosulphate (PAPS) (ul Haque *et al.*, 1998). Should the mutation in chondrodysplasia of Texel sheep lie in a sulphotransferase, it would be reasonable to suspect that another sulphotransferase may be able to catalyse the required reaction to varying degrees. In addition, abnormal transforming growth-factor-β (TGF-β) and bone morphogenic protein (BMP) signalling (see Chapter 1, Figure 1.6) have been identified in *CHST11* mutant mice with defective chondroitin 4-sulphotransferase activity (Klüppel *et al.*, 2005), and may provide an increased opportunity for individual genotypes to influence the presentation of a homologous disease. Another explanation for the variable severity of chondrodysplasia in Texel sheep may lie in the different rates of growth, as suggested above, or in the involvement of sulphate in some signalling and regulation pathways, such as the requirement for heparan sulphate in fibroblast growth factor (FGF) signalling (Forlino *et al.*, 2005; Harmer, 2006).

In all likelihood, both mechanical and metabolic processes are involved in the development of lesions in chondrodysplasia of Texel sheep.

7.3 Possible aetiology

An abnormality in cartilage sulphate metabolism is likely to be the defect in chondrodysplasia of Texel sheep. This is supported by the microscopic lesions,

which mimic those found in defects of the diastrophic dysplasia sulphate transporter (DTDST) (Thompson *et al.*, 2005) and in congenital Mn deficiency (see Chapter 2). The former reduces the cellular uptake of sulphate (Karniski, 2001), while the latter affects polymerase and galactosyltransferase, enzymes required for synthesis of chondroitin sulphate (Leach *et al.*, 1969; Tsopanakis & Herries, 1976). The pale staining of hyaline cartilage matrix with toluidine blue, compared with equally-thick sections from control animals, suggests that there were fewer negatively charged ions in the abnormal cartilage. This would be expected if there were a deficiency of sulphate in the cartilage matrix. A biochemical study identified a decreased amount of sulphate relative to cartilage protein in chondrodysplastic Texel sheep (see Figure 1.7) (Byrne, 2005), and capillary electrophoresis of articular cartilage chondroitin disaccharides revealed a decreased concentration of chondroitin 4-sulphate in cartilage from chondrodysplastic animals of all ages tested (see Chapter 6).

The pathway involved in the sulphation of cartilage matrix proteoglycans includes several steps (see Chapter 1, Figure 1.3). Initially, sulphate must enter the cell through the DTDST, an antiporter that exchanges intracellular chloride ions for extracellular sulphate (Vincourt *et al.*, 2003). This protein is encoded by *SLC26A2*, and mutations in this gene cause human achondrogenesis 1b, atelosteogenesis type II, diastrophic dysplasia and recessive multiple epiphyseal dysplasia (rMED) (Hästbacka *et al.*, 1996b; Cai *et al.*, 1998; Rossi & Superti-Furga, 2001; Ballhausen *et al.*, 2003), and chondrodysplasia in a transgenic mouse model (Forlino *et al.*, 2005). The importance of this gene in human diseases that share microscopic lesions, and some of their developmental aspects, with chondrodysplasia of Texel sheep made *SLC26A2* the initial candidate for this disease. In light of this, 85.4% of the exonic DNA of *SLC26A2* was sequenced in chondrodysplastic and control sheep, but no mutation was identified (Byrne, 2005). While this study decreased the likelihood of a causative mutation in this gene, it could not rule out a mutation in the remaining 14.6% of exonic sequence, or a mutation in a non-expressed regulatory region. Because of these points, and the strong similarity between microscopic lesions of chondrodysplastic Texel sheep and those described in diseases caused by *SLC26A2* mutations, further investigation of the DTDST was performed via an assay of radiolabelled sulphate uptake by dermal fibroblasts in chondrodysplastic and control sheep. This study did not show a difference in sulphate uptake between chondrodysplastic and age-matched control lambs, but

was limited by the low number of animals able to be used and the omission of results from one animal due to its outlying nature (see Chapter 4). It must also be noted that while identifying a decreased level of sulphate uptake would have provided strong evidence for a defect of DTDST function, failure to detect a difference has occurred in some cases where a disease-causing mutation in *SLC26A2* was known to exist (Karniski, 2001).

Because of the remaining possibility that the causative mutation in chondrodysplasia of Texel sheep involved this gene, the area of ovine chromosome 5 containing *SLC26A2* was included in a microsatellite marker study. While some of the marker alleles used for this chromosome did not amplify as well as expected (one failed to amplify clearly enough to be accurately scored, one contained a suspected null allele, and one was not polymorphic enough to provide useful information), the results of chi-squared testing did not support any loss of heterozygosity in association with the chondrodysplastic phenotype at marker TGLA303, 9.251cM from the *SLC26A2* gene locus (Australian Sheep Gene Mapping Web Site, 2004). Although none of the individual studies performed on DTDST function or the *SLC26A2* gene in chondrodysplasia of Texel sheep were powerful enough to rule out a defect of sulphate uptake of chondrocytes in affected sheep, collectively they suggest that *SLC26A2* is unlikely to contain a causative mutation in this disease.

Given the similarity of lesions and biochemistry in chondrodysplasia of Texel sheep to those caused by *SLC26A2* mutations, a defect within the cartilage proteoglycan sulphation pathway was still considered to be the most likely cause of the disease. Following the uptake of sulphate into cells, it is converted into an "active" form known as PAPS by phosphoadenosine-phosphosulphate synthase (PAPSS), encoded by *PAPSS2* (Superti-Furga, 2001). A defect in this enzyme results in reduced concentrations of PAPS and undersulphation of cartilage proteoglycans (Orkin *et al.*, 1976). Mutations in *PAPSS2* have been identified in spondyloepimetaphyseal dysplasia (SEMD) Pakistani type in humans (ul Haque *et al.*, 1998), and mutations in the orthologous gene, *ATPSK2*, are responsible for brachymorphism in mice (Pennypacker *et al.*, 1981). Microscopic cartilage lesions have not been described in SEMD, Pakistani type, and in the brachymorphic mouse the lesions do not include degeneration of the interterritorial matrix or formation of the perichondrocytic collagen rings typical of chondrodysplasia in Texel sheep (Orkin *et al.*, 1977; Kurima *et al.*, 1998; ul Haque *et al.*, 1998). The profile of

chondroitin disaccharide sulphation has not been described in PAPS deficiency. While the precise location of *PAPSS2* has not been mapped in the ovine genome, it is mapped to chromosome 10q22-24 in the human genome (Xu *et al.*, 2002), corresponding to the tip of ovine chromosome 22 at a position below 15.4cM (Australian Sheep Gene Mapping Web Site, 2004). For this reason, the genetic study included microsatellite markers from the area of ovine chromosome 22, but was unable to detect any difference in the level of heterozygosity between affected and control sheep. This suggests either that chondrodysplasia of Texel sheep is unlikely to be caused by a mutation in *PAPSS2* or that *PAPSS2* in sheep exists at another locus not investigated during the genetic study. Because of this uncertainty, a mutation in *PAPSS2* cannot be excluded with confidence in chondrodysplasia of Texel sheep, although the lack of similar microscopic lesions in the brachymorphic mouse suggests that disturbed PAPSS function may not be involved in chondrodysplasia of Texel sheep.

Following the activation of sulphate to PAPS, sulphate is translocated from the cytosol into the golgi apparatus by PAPS/PAP antiporters, PAPS transporter 1 encoded by *SLC35B2* (Kamiyama *et al.*, 2003) and PAPS transporter 2 encoded by *SLC35B3* (Kamiyama *et al.*, 2006), neither of which have been mapped in the ovine genome (Australian Sheep Gene Mapping Web Site, 2004). No disease-causing mutations in either PAPS transporter have been described. While it is reasonable to suppose that loss of PAPS transporter function could prevent sulphation of cartilage matrix proteoglycans and result in chondrodysplasia, the relatively equal expression of *SLC35B2* and *SLC35B3* in most tissues examined indicates that deficient action of one transporter could be compensated for by the other (Kamiyama *et al.*, 2006). It is therefore reasonable to presume that a disease involving PAPS transporter function would not have a simple autosomal recessive mode of inheritance, and is consequently unlikely to be the cause of chondrodysplasia in Texel sheep.

Once PAPS has been translocated to the golgi apparatus, sulphate is transferred to glycosaminoglycans by various sulphotransferases (Schwartz *et al.*, 1998). In cartilage, chondroitin 4-sulphotransferase, encoded by *CHST11* (Yamauchi *et al.*, 2000), and chondroitin 6-sulphotransferase, encoded by *CHST3* (Tsutsumi *et al.*, 1998), catalyse the addition of sulphate to cartilage glycosaminoglycans. Chondroitin 6-sulphotransferase transfers sulphate from PAPS to position 6 on chondroitin (Tsutsumi *et al.*, 1998). Defects in chondroitin 6-sulphotransferase

caused by mutations in *CHST3* have been identified in humans, and result in spondyloepiphyseal dysplasia Omani type, characterised biochemically by a decreased concentration of chondroitin 6-sulphate in cartilage but unchanged concentrations of chondroitin 4-sulphate and unsulphated chondroitin (Thiele *et al.*, 2004). The results of the capillary electrophoresis study in chondrodysplasia of Texel sheep revealed decreased levels of Δdi-mono4S, unchanged concentrations of Δdi-mono6S, and what appeared to be unchanged levels of unsulphated chondroitin disaccharide, although the identity of that peak could not be confirmed (see Chapter 6). These results suggest that it is unlikely that chondrodysplasia of Texel sheep is caused by a mutation in *CHST3*.

Defects in the other major sulphotransferase in cartilage, chondroitin 4-sulphotransferase, have been induced in transgenic mice, and result in severe chondrodysplasia with fibrillation of cartilage extracellular matrix and a reduced ratio of Δdi-mono4S to Δdi-mono6S (Klüppel *et al.*, 2005). Although the disease in transgenic mice was very severe relative to the milder phenotype of chondrodysplasia in Texel sheep, it was caused by total absence of functional chondroitin 4-sulphotransferase, which may not always simulate a naturally occurring mutation. Because of this, the nature of the lesions and biochemical changes induced may be more informative than their level of severity when comparing the *CHST11*-mutant mouse with naturally occurring diseases. Due to the similar nature of the chondroitin disaccharide sulphation profile in the CHST11-mutant mouse and chondrodysplastic Texel sheep (see Chapter 6, Figure 6.2), *CHST11* is considered a likely candidate gene for this disease.

7.4 Importance of chondrodysplasia in Texel sheep

7.4.1 Relevance to industry

Chondrodysplasia of Texel sheep represents a concern to sheep farmers in New Zealand largely because of the potential for defective genes to be widespread in the population due to a founder effect. This is reasonably likely to be the case, since the Texel breed is comparatively new in New Zealand (released from quarantine in 1990), and upgraded Texel sheep in this country are descended from relatively few founding animals. It is also possible that the frequency of the disease allele could be increased through a selective advantage for carriers, such as occurs in "spider lamb syndrome" (Smith *et al.*, 2006). Although animals carrying a single copy of

the gene for chondrodysplasia in Texel sheep appear normal and have no microscopic lesions of the disease, it is still possible they have a slightly different conformation that may be appealing to Texel breeders, for example, a solid, "blocky" appearance such as occurs in mildly affected mature animals (see Chapter 2, Figure 2.2C). The occurrence of affected lambs on farms would result in economic loss due to the effect of the disease on skeletal development. Affected lambs are smaller than normal and have angular limb deformities that would render the animal undesirable for commercial slaughter (see Chapter 2, Figures 2.1 and 2.2).

Until recently, the Texel breed in New Zealand has been used mainly as a terminal sire and the problem of a recessive disease would be of concern only to Texel breeders. Since the late 1990s, new composite breeds such as Greeline (Greeline Flock and Stud Rams, 2007), Highlander (Rissington Breedline, 2007) and TEFRom (Twin Farm, 2007), in which Texel genetics are included on both sides of the pedigree, have achieved a modest degree of popularity. Because of this, there is potential for the defective gene to become more widespread in the population and that the defect will be exhibited due to the mutation appearing in both sides of the pedigree. A genetic test able to identify animals carrying the gene would become a valuable tool to eradicate this disorder from flocks using composite breeds containing Texel genetics.

7.4.2 Biomedical research

The similarity between the lesions in chondrodysplasia of Texel sheep and those of achondrogenesis 1b and diastrophic dysplasia in humans beings suggests that chondrodysplastic Texel sheep may be a model for these types of diseases (Thompson *et al.*, 2005). While no studies have been able to support a defect in DTDST function, the common features of the pathogenesis may mean that chondrodysplastic Texel sheep could be suitable as experimental models for therapeutic studies, other than gene therapy. Although a mouse model for diastrophic dysplasia already exists (Forlino *et al.*, 2005), this species has less in common with humans in terms of metabolism and joint-loading than do larger animals, such as dogs or sheep (Barsoum *et al.*, 2000; Casal & Haskins, 2005; Pearce *et al.*, 2007). The delay in the development of lesions until postnatal life makes chondrodysplasia of Texel sheep a candidate for research into preventive therapy

Naturally occurring genetic disorders have the potential to reveal new information about complex biological mechanisms. Chondrodysplasia of Texel sheep is an example of a disease closely mimicking the microscopic phenotype of diseases in human beings, but appearing to be caused by a different genetic defect. This provides a potential to increase understanding of the way lesions form in hyaline cartilage, and the metabolic requirements of the tissue.

7.5 Limitations to this study

The primary limitation to the studies on chondrodysplasia of Texel sheep presented here was the level of inbreeding involved in the original back-cross breeding trial. While this design represented the best option for rapidly increasing the number of animals for study and determining the mode of inheritance involved (Byrne, 2005), the closely related nature of the flock generated potential limitations to the phenotypic characterisation and genetic analysis of the disease.

The inbreeding in the original breeding trial was likely to have reduced the level of genetic variation within the experimental flock (Futuyma, 1998), and may have misrepresented the characteristics of this disease. While reduced genetic variation would have been of great importance if studying a disease with a polygenic aetiology, the simple autosomal recessive mode of inheritance in chondrodysplasia of Texel sheep lessened the likely impact of inbreeding on the disease phenotype. Because affected sheep from the back-cross trial had a similar range of lesions to those in the affected lambs originally identified on private farms, the findings from this study can still be considered relevant to chondrodysplasia of Texel sheep outside of the experimental flock.

In the genetic study, the high level of inbreeding generated the potential for spurious linkage of markers with the disease phenotype (Pritchard *et al.*, 2000). Transmission disequilibrium testing (TDT) is a statistical method which can overcome this problem (Spielman *et al.*, 1993), but unfortunately the required samples from non-affected parents of affected lambs were not available to be used in this test. Because no genetic linkage could be identified in the microsatellite marker experiment, however, spurious linkage was not a concern in this study.

Other than the effects of inbreeding, the genetic study was limited by several other factors. The level of genetic information available for the ovine genome is low in some chromosomes, although ongoing research is constantly addressing this

deficiency (Cockett, 2006; Pariset *et al.*, 2006). Poor amplification of some markers, despite optimisation of laboratory conditions, reduced the effectiveness of the genetic study in chondrodysplasia of Texel sheep. Time constraints did not allow for the purchase of primers for alternative microsatellite markers in some cases, and in others, no alternative markers were available. Another limitation in the genetic study was the dependency of linkage disequilibrium analysis on accurate pedigree information, which may have been compromised in this experiment due to the absence of maternal genetic information. Such genotypic data would have identified errors due to lamb-stealing, which is well recognised in sheep at the time of birth (Alexander *et al.*, 1983). Furthermore, the number of samples available for use in the genetic study was relatively low due to the inability to verify the phenotype of newborn lambs at the time of the experiment.

Sample size was also a limitation in both the capillary electrophoresis and the sulphate uptake studies. In the former, this may have resulted in the underestimation of the normal variation between animals in chondroitin disaccharide ratios. For the sulphate uptake study, the number of samples was limited by animal availability and laboratory regulations for radioactive substances. Unsuccessful attempts to culture chondrocytes for sulphate uptake study led to fibroblasts being used as a substitute cell type. Although fibroblasts have been used successfully in this type of study (Rossi *et al.*, 1996b; Cetta *et al.*, 1997; Brenig *et al.*, 2003), the greater sulphate uptake potential of chondrocytes (Rossi *et al.*, 1996a) may have increased the sensitivity of the study, had they been used.

7.6 Future studies

The next stage of research into chondrodysplasia of Texel sheep would likely involve sequencing of *CHST11*, the gene encoding chondroitin 4-sulphotransferase-1, in chondrodysplastic and unaffected Texel sheep in an attempt to find a causative mutation and therefore allow development of a diagnostic test. The histological and biochemical studies in this thesis have indicated that the pathology of chondrodysplasia in Texel sheep is consistent with a potential defect in the function of chondroitin 4-sulphotransferase, while current and earlier genetic studies combined with biochemical findings and fibroblast sulphate uptake *in vitro* suggest that genes involved in earlier steps of the cartilage proteoglycan sulphation pathway are less likely to be involved.

Should sequencing of *CHST11* fail to find a mutation causing chondrodysplasia of Texel sheep, another chromosome or genome scanning experiment could be conducted. This would require a new breeding trial designed to provide an increased number of samples from a new pedigree containing a large number of unaffected relations to affected animals, and a greater marker density than that used in the initial linkage disequilibrium study. Ongoing genomics research will result in more markers being identified within the ovine genome, including both microsatellites and single-nucleotide polymorphisms (SNPs) (Cockett, 2006; Pariset *et al.*, 2006). This, combined with the increasing availability and cost-effectiveness of large-scale scanning techniques such as SNP-chips, will increase the potential sensitivity of future scanning studies within the ovine genome. A more cost-effective alternative may be to work closely with Texel breeders known to have had affected animals in their flocks. A major difficulty would lie in farmer compliance, since the identification of a genetic disease on a stud farm generates significant stigma. Additionally, pedigree accuracy may not be assured, although genotyping of parents and offspring would be able to reinforce the pedigree information. A scanning study could then be followed up by candidate gene analysis, preferably supported by knowledge of gene function and its potential involvement with the pathogenesis of the disease. Once the causative mutation has been identified, a genetic test to detect phenotypically normal carrier sheep may be developed, enabling eradication of the mutant gene from farms if the allele frequency is low, or control of the disease by breeding only from tested sires if the allele frequency is very high. A genetic test may also be used to determine whether the gene for chondrodysplasia in Texel sheep can be found in Texel populations in countries such as Denmark and Finland, from which the founding embryos for the New Zealand Texels were sourced.

If a new breeding trial is not feasible, and an appropriate collection of samples cannot be obtained from farms, the difference in chondroitin sulphate disaccharide ratios between chondrodysplastic and unaffected sheep could be used to increase the number of usable samples from the original back-cross trial by providing phenotypic information about the tissue collected from newborn lambs produced in that trial. This would increase the power of genetic analyses even though the pedigree structure was not ideal. To do this, the capillary electrophoresis experiment would ideally be repeated on a larger number of samples from animals of known phenotype in order to adequately assess the normal variation in the ratio

of Δdi-mono4S to Δdi-mono6S between animals. This would determine whether the ratio itself can be used as a diagnostic tool, or if it would be more properly used as a quantitative trait in scanning genetic studies designed to test for quantitative trait linkage (QTL testing). Unfortunately, the generation of newborn lambs known to be affected by chondrodysplasia is difficult since it requires both parents to be affected by the disease, and many chondrodysplastic Texel sheep do not survive to maturity. Because of this, it would be difficult to gain enough samples to thoroughly test the diagnostic power of the Δdi-mono4S to Δdi-mono6S ratio in newborn lambs.

Further biochemical characterisation of chondrodysplasia of Texel sheep in order to help identify candidate genes, either alone or in conjunction with genetic scanning studies, may be of use in ongoing research. These could include an assessment of PAPSS function *in vitro* using radiolabelled sulphate to quantify PAPS (Rossi *et al.*, 1996a). This experiment would test the integrity of the proteoglycan sulphation pathway up to and including PAPSS function. Alternatively, mRNA analysis could be used to measure the level of expression of various enzymes and transporters involved in the sulphation pathway.

A potential future study on the development of chondrodysplasia in Texel sheep could consist of serial capillary electrophoresis studies using cartilage biopsies to determine the way in which the ratio of Δdi-mono4S to Δdi-mono6S changes with age in chondrodysplastic Texel sheep, and whether the deviation from age-matched control cartilage is influenced by the severity of the disease. This would provide more information about the pathogenesis of the lesions in chondrodysplasia of Texel sheep, and may help to elucidate the relationship between the altered ratio in other diseases and their development.

7.7 Conclusion

Chondrodysplasia in Texel sheep is caused by defective sulphation of cartilage matrix proteoglycans, possibly due to a mutation in *CHST11*. The gross lesions of this disease develop over the first few weeks of life. These lesions include rhizomelia with forelimb varus deformity and erosion of articular cartilage in large joints, enlarged costochondral junctions with pectus excavatum, and a twisted trachea with soft, thick tracheal rings prone to collapse. The most distinctive microscopic lesions are perichondrocytic collagen rings and degeneration of

interterritorial cartilage matrix with denuded collagen fibrils. These microscopic lesions are shared by other disorders of cartilage sulphation. The development of gross and microscopic lesions appears to be the result of physical forces on structurally weak cartilage matrix. Cartilage from affected sheep had a lower concentration of chondroitin 4-sulphate than did controls, in conjunction with unchanged levels of chondroitin 6-sulphate.

Chondrodysplasia of Texel sheep is of concern to the sheep farming industry in New Zealand due to the potential for economic loss. Conversely, the disease may be useful as a model for disorders of cartilage sulphation in humans, and could be used in studies exploring preventive therapies for non-lethal forms of these diseases. Identification of the causative mutation in chondrodysplasia of Texel sheep would allow for the development of a genetic test to identify carrier animals on sheep farms, and would provide more information about the pathogenesis of the disease and its usefulness as a model for disease in humans.

Appendix 1 - Defects in extracellular structural proteins

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Achondrogenesis type II-Hypochondrogenesis (Langer-Saldino type achondrogenesis; chondrogenesis imperfecta)	Type II collagen OMIM 200610	Autosomal dominant	Perinatally lethal. Micromelia with short, bowed long bones.	Metaphyseal cupping and flaring, variable ossification of spinal column and pelvis.	Densely packed epiphyseal chondrocytes, disorganised physis, plump, disordered primary trabeculae.	Affects from 1 in 40,000 to 60,000 births. Cartilage contains type I rather than type II collagen.	(Sillence *et al.*, 1979; Eyre *et al.*, 1986; Borochowitz *et al.*, 1988b; van der Harten *et al.*, 1988; Bonaventure *et al.*, 1995)
Kniest dysplasia (Metatropic dwarfism type II; metatropic dysplasia type II)	Type II collagen OMIM 156550	Autosomal dominant	Short trunk and limbs, progressive joint enlargement, flattened face. May have cleft palate and tracheal collapse.	Broad metaphyses, enlarged epiphyses, platyspondyly, progressive kyphoscoliosis and lumbar lordosis.	Degeneration of cartilage matrix, intra-cytoplasmic inclusions within chondrocytes.	Uncommon. May also have severe myopia, recurrent ear infections. Ultrastructurally thin, irregular collagen fibrils. Absence of chondrocalcin from epiphyseal extracellular matrix.	(Horton & Rimoin, 1979; Maumenee & Traboulsi, 1985; Poole *et al.*, 1988a; Hicks *et al.*, 2001)
Torrance type lethal platyspondylic skeletal dysplasia	Type II collagen OMIM 151210	Autosomal dominant	Short limbs and small thorax, brachydactyly, lordosis.	Short ribs, under-developed pubic bones, platyspondyly.	Mixture of hypercellular and degenerate hypocellular areas in epiphyseal cartilage.	Very rare. Lethal due to respiratory insufficiency.	(Neumann *et al.*, 2003; Nishimura *et al.*, 2004; Zankl *et al.*, 2005)
Spondyloepiphyseal dysplasia congenita	Type II collagen OMIM 183900	Autosomal dominant	Kypho-scoliosis, flattened face, short neck and limbs.	Kypho-scoliosis, genu valgum, failure of ossification of pubic bones and knee epiphyses, platyspondyly.	Mildly disorganised physeal chondrocyte columns, PAS-positive diastase-resistant intracytoplasmic inclusions within chondrocytes.	Rare. May also have retinal detachment, myopia, odontoid hypoplasia, hearing loss.	(Yang *et al.*, 1980; Lee *et al.*, 1989; Chan *et al.*, 1993)
Spondylo-epimetaphyseal dysplasia Strudwick type (Strudwick syndrome; dappled metaphysis syndrome; SEMD type I; SEMDC)	Type II collagen OMIM 184250	Autosomal dominant	Short trunk and limbs, normal hands and feet, scoliosis and lumbar lordosis, coxa vara, talipes equinovarus.	Alternating areas of osteosclerosis and osteopenia in long bone metaphyses, pectus carinatum.		Very rare. May also have severe myopia, retinal detachment.	(Anderson *et al.*, 1982; Tiller *et al.*, 1995)
Stickler syndrome type 1 (Vitreous type 1 Stickler syndrome; membranous vitreous type arthro-ophthalmopathy)	Type II collagen OMIM 108300	Autosomal dominant	Flattened face, small mandible, hyperextensible joints with degenerative joint disease.			Affects 1 in 10,000 people. May also have severe myopia, hearing loss, cleft palate.	(Stickler *et al.*, 1965; Wynne-Davies & Gormley, 1985; Williams *et al.*, 1996; Stickler *et al.*, 2001; U.S. National Library of Medicine, 2006)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Spondyloperipheral dysplasia	Type II collagen OMIM 271700	Autosomal dominant	Brachydactyly.	Platyspondyly, short ulna.		Very rare. May have short stature, myopia, hearing loss, mental retardation.	(Kelly *et al.*, 1977; Vanek, 1983; Zabel *et al.*, 1996)
Multiple epiphyseal dysplasia	Type IX collagen, *COMP*, *MATN3* OMIM 600969 132400 607078	Autosomal dominant	Short stature, scoliosis, valgus deformity, early onset osteoarthritis.	Irregular ossification of carpals and tarsals, stippled calcification of epiphyses, subchondral sclerosis, flattened femoral heads.	Chondrocytes have intracytoplasmic inclusions and are surrounded by slightly fibrillar matrix. Epiphyseal cartilage is most severely affected.	Affects 1 in 10,000 people Ultrastructurally dilated chondrocyte rER contains alternating bands of electron dense and lucent material.	(Rasmussen & Reimann, 1973; Wynne-Davies & Gormley, 1985; Ballo *et al.*, 1997; Bönnemann *et al.*, 2000; Maddox *et al.*, 2000; Czarny-Ratajczak *et al.*, 2001; Mäkitie *et al.*, 2004a; U.S. National Library of Medicine, 2006)
Schmid metaphyseal chondrodysplasia (Japanese type spondylometaphyseal dysplasia)	Type X collagen OMIM 156500	Autosomal dominant	Disproportionate dwarfism, bowed legs, coxa vara, lumbar lordosis.	Metaphyses are widened, sclerotic and irregular, occasional mild platyspondyly.	Absence of identifiable hypertrophic chondrocytes, disorganised.	The most common type of metaphyseal chondro-dysplasia.	(Lachman *et al.*, 1988; Hasegawa *et al.*, 1994; Chan *et al.*, 1995b; Ikegawa *et al.*, 1997; Savarirayan *et al.*, 2000; Bateman *et al.*, 2003; Bateman *et al.*, 2004)
Stickler syndrome type II (Vitreous type 2 Stickler syndrome; beaded vitreous type Stickler syndrome)	Type XI collagen OMIM 604841	Autosomal dominant	Short stature, midface hypoplasia, cleft palate, micrognathia, hyperextensible joints, osteo-arthropathy.	Mild epiphyseal dysplasia, wide metaphyses.		Hearing loss, myopia, may develop glaucoma and retinal detachment.	(Ayme & Preus, 1984; Richards *et al.*, 1996; Spranger, 1998)
Marshall syndrome	Type XI collagen OMIM 154780	Autosomal dominant	Short stature, midface hypoplasia, less frequent cleft palate.	Narrowed joint spaces with hip and knee osteophytes.		Very similar to Stickler syndrome type II. Myopia, cataracts. Less frequently detached retina.	(Ayme & Preus, 1984; Annunen *et al.*, 1999a)
Otospondylomega-epiphyseal dysplasia (Chondrodystrophy with sensorineural deafness; Nance-Insley syndrome; Nance-Sweeney chondrodysplasia)	Type XI collagen OMIM 215150	Autosomal dominant and recessive forms	Short limbs and fingers, degenerative joint disease, flat face, small mandible, sometimes cleft palate.	Platyspondyly, progressive fusion of carpal bones.		Very rare. Hearing loss.	(Nance & Sweeney, 1970; Insley & Astley, 1974; Vikkula *et al.*, 1995; Pihlajamaa *et al.*, 1998)
Marfan syndrome	*FBN1* OMIM 154700	Autosomal dominant	Increased height, long digits, anterior chest deformities, joint laxity.	Scoliosis, thoracic lordosis.	Fibroblastic chondrocytes.	Affected 2-3 per 10,000 births. Clinically variable, also has ocular and cardiovascular abnormalities.	(Nogami *et al.*, 1979; Judge & Dietz, 2005; Tekin *et al.*, 2007)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Pseudo-achondroplasia (Pseudo-achondroplastic spondyloepiphyseal dysplasia, formerly pseudoachondroplastic dysplasia III and IV)	COMP OMIM 177170	Autosomal dominant	Disproportionate dwarfism, joint laxity, moderate brachydactyly.	Irregular epiphyses and metaphyses of long bones, delayed epiphyseal ossification.	Disorganised, hypocellular prehypertrophic and hypertrophic zones.	Common skeletal dysplasia. Ultrastructurally, dilated chondrocyte rER cisternae containing lamellar structures.	(Pedrini-Mille *et al.*, 1984; McKeand *et al.*, 1996; Maddox *et al.*, 2000; Hecht *et al.*, 2004)
Spondylo-epimetaphyseal dysplasia	*MATN3* OMIM 608728	Autosomal recessive	Disproportionate dwarfism, bowed legs, enlarged joints.	Pectus excavatum, lumbar lordosis.			(Borochowitz *et al.*, 2004)
Schwartz-Jampel syndrome (SJS myotonic myopathy dwarfism chondrodystrophy and ocular and facial abnormalities; Schwartz-Jampel-Aberfeld syndrome; chondrodystrophic myotonia)	Perlecan OMIM 255800		Short stature, joint contractures, blepharo-phimosis, mask-like facial expression.	Enlarged epiphyses of long bones, platyspondyly.	Regular physes contrasting with thick, irregular primary trabeculae.	Myotonic myopathy, myopia, death may occur due to respiratory complications May be 3 different forms.	(Al-Gazali, 1993; Giedion *et al.*, 1997; Nicole *et al.*, 2000)
Silverman-Handmaker type Dyssegmental dysplasia	Perlecan OMIM 224410	Autosomal recessive	Micromelia, flat face, micrognathia, talipes equinovarus.	Anisospondyly, short bent limb bones.	Thin physes, mucoid degeneration of resting cartilage, short and irregular chondrocyte columns, intrachondrocytic inclusion bodies.	Severe lethal and milder Rolland-Desbuquois forms. Ultrastructurally, granular material in dilated chondrocytic rER.	(Arikawa-Hirasawa et al., 2001a; 2001b)

Appendix 2 - Defects in metabolic pathways

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Achondrogenesis type 1B (Fraccaro type achondrogenesis)	*SLC26A2* OMIM 600972	Autosomal recessive	Micromelia, thoracic hypoplasia.	Minimal ossification of vertebral bodies, poorly formed pelvis, short ribs, flared metaphyses.	Chondrocytes surrounded by rings, disorganised physes, vacuolated matrix with coarse collagen fibrils.	Rare. Perinatally lethal. Reduced sulphation of hyaline cartilage, decreased sulphate uptake by chondrocytes.	(Superti-Furga, 1994; Superti-Furga *et al.*, 1996a; van der Harten *et al.*, 1988)
Atelosteogenesis type II (Neonatal osseous dysplasia type I; De la Chapelle dysplasia; McAlister dysplasia)	*SLC26A2* OMIM 256050	Autosomal recessive	Hichhiker thumbs and toes, kyphoscoliosis, micromelia.	Long-bone hypoplasia, irregular ossification of short tubular bones.	Chondrocytes surrounded by rings, attenuated thread-like interterritorial cartilage matrix.	Extremely rare. Perinatally lethal due to respiratory insufficiency due to airway collapse and pulmonary hypoplasia. Reduced fibroblast sulphate uptake.	(Hästbacka *et al.*, 1996b; Rossi *et al.*, 1996b; Sillence *et al.*, 1997)
Diastrophic dysplasia	*SLC26A2* OMIM 222600	Autosomal recessive	Short limbed dwarfism, severe club feet, joint contractures, hitchhiker's thumb, degenerative joint disease, swollen earlobes.	Deformed tarsal and metatarsal bones, extremely short first metacarpals, other metacarpals broad and flared distally. Broad metaphyses of long bones.	Cartilage matrix degeneration with collagen fibrils traversing cystic spaces.	Common in Finland, carrier frequency of 1-2%. Reduced fibroblast sulphate uptake.	(Langer, 1965; Hästbacka *et al.*, 1992; Shapiro, 1992; Hästbacka *et al.*, 1994; Rimoin *et al.*, 1998; Peltonen *et al.*, 1999)
Recessive multiple epiphyseal dysplasia	*SLC26A2* OMIM 226900	Autosomal recessive	Hand and foot abnormalities, short stature.	Abnormal capital femoral epiphyses, double-layered patellae.		May be relatively common. Reduced fibroblast sulphate uptake.	(Superti-Furga *et al.*, 1999; Rossi & Superti-Furga, 2001; Hall, 2002; Ballhausen *et al.*, 2003)
Pakistani type spondylo-epimetaphyseal dysplasia	*PAPSS2* OMIM 603005	Autosomal recessive	Short stature, short and bowed lower limbs, mild brachydactyly enlarged knees.	Early onset osteoarthropathy, delayed epiphyseal ossification, platyspondyly, mild metaphyseal lesions.		Orthologous disorders to the brachymorphic mouse.	(Ahmad *et al.*, 1998; ul Haque *et al.*, 1998)
Schneckenbecken dysplasia	*SLC35D1* OMIM 269250	Autosomal recessive	Short-limbed dwarfism.	Hypoplastic vertebral bodies and iliac bones, short long-bones with dumb-bell shape.	Hypervascular cartilage with increased cell density.	Lethal perinatally. Impaired chondroitin biosynthesis.	(Camera *et al.*, 1991; Hiraoka *et al.*, 2007)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Spondyloepiphyseal dysplasia Omani type	*CHST3* OMIM 608637	Autosomal recessive	Rhizomelia, genu valgum, mild brachydactyly, mild microdontia.	Progressive scoliosis, arthritic changes in large joints, small epiphyses.		Loss of Chondroitin-6-O-sulpho-transferase-1 function.	(Rajab *et al.*, 2004; Thiele *et al.*, 2004)
X-linked recessive chondrodysplasia punctata (Brachytelephalangic chondrodysplasia punctata)	*ARSE* OMIM 302950	X-linked recessive	Low-normal height, facial dysmorphism.	Punctate calcifications of epiphyseal cartilage in multiple sites, hypoplastic distal phalanges.		Deficiency in activity of Arylsulphatase-E.	(Sheffield *et al.*, 1976; Franco *et al.*, 1995)
CHILD syndrome (Congenital hemidysplasia with ichthyosiform erythroderma and limb defects)	*NSDHL* OMIM 308050	X-linked dominant	Unilateral ichthyosiform skin lesions, limb defects, punctate calcification of cartilage, visceral anomalies.		Accumulations of cytoplasmic lipid in fibroblasts.	Deficiency of 3b-hydroxysteroid-Δ8, Δ7-isomerase activity.	(Emami *et al.*, 1992; Grange *et al.*, 2000; Happle *et al.*, 2000; König *et al.*, 2000)
Chondrodysplasia punctata type 2 (CDPXD; Conradi-Hünermann syndrome; Happle syndrome; Conradi-Hünermann-Happle syndrome)	*EBP* OMIM 302960	X-linked dominant	Asymmetric rhizo-mesomelia, flattened face, ichthyosis, cataracts, kyphoscoliosis, sometimes alopecia.	Stippled epiphyses.	Epiphyseal cartilage contains areas of mucoid/cystic degeneration with fibrous invasion and mineralisation, abnormal physeal cell columns.	Deficiency of 3b-hydroxysteroid-Δ8, Δ7-isomerase activity.	(Rimoin *et al.*, 1976; Braverman *et al.*, 1999; Kelley *et al.*, 1999; Has *et al.*, 2000; Ikegawa *et al.*, 2000)
Rhizomelic Chondrodysplasia punctata type I (RCDP1; chondrodystrophia calcificans punctata)	*PEX7* OMIM 215100	Autosomal recessive	Dis-proportionate dwarfism, rhizomelia, joint contractures craniofacial dysmorpho-genesis, cataracts, ichthyosis.	Stippled epiphyses, abnormal vertebral bodies.	Failure of chondrocyte columnisation with reduced vascular invasion and reduced matrix mineralisation, mucoid degeneration of resting cartilage.	Rare. Deficiency of plasmalogens with accumulation of phytanic acid, decreased dihydroxy-acetone-phosphate acyltransferase activity. Hearing loss.	(Rimoin *et al.*, 1976; Poulos *et al.*, 1988; Heikoop *et al.*, 1992; Moser *et al.*, 1995; Braverman *et al.*, 1997; White *et al.*, 2003)
Rhizomelic Chondrodysplasia punctata type II (RCDP2)	*DHAPAT* OMIM 222765	Autosomal recessive	Rhizomelia, facial dysmorphism, cataracts.	Stippled epiphyses, abnormal vertebral bodies.		Deficiency acyl-CoA:dihydroxy-acetone-phosphate acyl-transferase, mental retardation, hearing loss.	(Wanders *et al.*, 1992; Moser *et al.*, 1995; Ofman *et al.*, 1998)
Rhizomelic Chondrodysplasia punctata type III (RCDP3)	*AGPS* OMIM 600121	Autosomal recessive	Rhizomelia, microcephaly, facial dysmorphism, cataracts, developmental delay, hypotonia.	Stippled epiphyses, abnormal vertebral bodies.		Deficiency of alkyldihydroxy-acetone-phosphate synthase, hearing loss.	(Moser *et al.*, 1995; de Vet *et al.*, 1999)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Zellweger syndrome (Cerebrohepatorenal syndrome)	*PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX12*, *PEX14*, *PEX26* OMIM 214100	Autosomal recessive	Some rhizomelia, facial dysmorphism, cataracts, retinopathy, hypotonia, enlarged liver, renal cysts.	Stippled epiphyses.		Hearing loss, seizures, mental retardation.	(Shimozawa *et al.*, 1992; Dodt *et al.*, 1995; Moser *et al.*, 1995; Reuber *et al.*, 1997; Chang *et al.*, 1999; Zhang *et al.*, 1999; Muntau *et al.*, 2000; Warren *et al.*, 2000; Matsumoto *et al.*, 2003; Shimozawa *et al.*, 2004)

Appendix 3 - Defects in the folding and degradation of macromolecules

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Muco-polysaccharidosis IH (Hurler syndrome)	*IDUA* OMIM 607014	Autosomal recessive	Severe dwarfism, coarse facial features, corneal opacity, hepato-splenomegaly.	Dorsolumbar kyphosis, coarse bone trabeculation, widened metaphyses with small epiphyses.	Large vacuolar structures within lymphocytic cytoplasm, showing red metachromasia with toluidine blue stain.	Affects 1 in 107,000 live births (W Australia) α-L-iduronidase deficiency, cardiac abnormalities, frequent upper respiratory infections.	(Wynne-Davies & Gormley, 1985; Bunge *et al.*, 1995; Wippermann *et al.*, 1995; Beesley *et al.*, 2001; *Leroy*, 2002; Nelson *et al.*, 2003; U.S. National Library of Medicine, 2006)
Muco-polysaccharidosis IS (Scheie syndrome)	*IDUA* OMIM 607016	Autosomal recessive	Near normal stature, slightly coarse facial features, corneal opacity, hepato-splenomegaly.	Lumbosacral spondylo-listhesis.		Rare. α-L-iduronidase deficiency, aortic& mitral valvular disease, carpal tunnel syndrome.	(Wraith & Alani, 1990; Yamagishi *et al.*, 1996; Beesley *et al.*, 2001; Leroy, 2002)
Muco-polysaccharidosis IH/S (Hurler-Scheie syndrome)	*IDUA* OMIM 607015	Autosomal recessive	Short stature, flat face, corneal opacity.	Flat femurs with short metaphyses, sclerotic skull base, development of dentigerous cysts.		α-L-iduronidase deficiency.	(Schmidt *et al.*, 1987; Lee-Chen *et al.*, 1999; Beesley *et al.*, 2001; Leroy, 2002)
Muco-polysaccharidosis II (Hunter syndrome)	*IDS* OMIM 309900	X-linked recessive	Dwarfism, coarse facial features.	Occasional mild kyphosis.	Metachromatic granules within fibroblasts.	Affect 1 in 320,000 live births (W Australia) Iduronate-2-sulphatase deficiency, a mild form exists.	(Danes & Bearn, 1965; Clarke *et al.*, 1992; Leroy, 2002; Nelson *et al.*, 2003)
Muco-polysaccharidosis IVA (Morquio syndrome type A)	*GALNS* OMIM 253000	Autosomal recessive	Severe short-trunked dwarfism, corneal opacity, odontoid hypoplasia.	Dorsal kyphosis, platyspondyly, abnormal epiphyses.		Affects about 1 in 200,000 births. N-acetylgalactos-amine-6-sulphate sulphatase deficiency.	(Lowry *et al.*, 1990; Bunge *et al.*, 1997; Leroy, 2002)
Muco-polysaccharidosis IVB (Morquio syndrome type B)	*GLB1* OMIM 253010	Autosomal recessive	Short stature, corneal opacity, odontoid hypoplasia.			β-galactosidase deficiency. Keratan-sulphaturia.	(Arbisser *et al.*, 1977; Oshima *et al.*, 1991; Leroy, 2002)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Muco-polysaccharidosis VI (Maroteaux-Lamy syndrome)	*ARSB* OMIM 253200	Autosomal recessive	Severe dwarfism, moderately coarse facial features, corneal opacity, hepato-splenomegaly.	Dorsolumbar kyphosis.	Inclusions within circulating leukocytes.	Affect 1 in 320,000 live births (W Australia) N-acetylgalactos-amine-4-sulphatase (aryl-sulphatase B) deficiency. A mild phenotype can occur.	(Wynne-Davies & Gormley, 1985; Litjens & Hopwood, 2001; Leroy, 2002; Nelson *et al.*, 2003; U.S. National Library of Medicine, 2006)
Muco-polysaccharidosis VII (Sly syndrome)	*GUSB* OMIM 253220	Autosomal recessive	Dwarfism, severely coarse facial features, corneal opacity, hepato-splenomegaly.	Dorsolumbar kyphosis.	Inclusions within leukocytes and macrophages.	β-glucuronidase deficiency.	(Tomatsu *et al.*, 1991; Vervoort *et al.*, 1997; Leroy, 2002)
Pycnodysostosis	*CTSK* OMIM 265800	Autosomal recessive	Short stature, bone fragility.	Osteosclerosis.	Decreased bone remodelling with inadequately degraded organic matrix.	Deficient cathepsin-K activity.	(Gelb *et al.*, 1996; Fratzl-Zelman *et al.*, 2004; Donnarumma *et al.*, 2007)
Spondylo-epimetaphyseal dysplasia type II (Spondylo-epimetaphyseal dysplasia, Missouri type)	*MMP13* OMIM 602111	Autosomal dominant	Rhizomelia.	Pear-shaped vertebrae in childhood, bowed femurs and/or tibias.			(Patel *et al.*, 1993; Kennedy *et al.*, 2005)
X-linked spondylo-epiphyseal dysplasia tarda	*SEDL* OMIM 313400	X-linked dominant	Short stature, short trunk from 6-8 years of age.	Progressive osteoarthritis, multiple epiphyseal abnormalities, platyspondyly.		Affects 1 in 500,000 people.	(Heuertz *et al.*, 1993; Gedeon *et al.*, 1999; Whyte *et al.*, 1999; Hall, 2002)

Appendix 4 - Defects in hormones and signal transduction mechanisms

Disease name(s)	Defect/ OMIM #	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Achondroplasia	*FGFR3* OMIM 100800	Autosomal dominant	Short stature with a relatively normal trunk, rhizomelia, megalocephaly, frontal bossing with midface hypoplasia, brachydactyly.	Lordosis, bowed legs, shortened vertebral pedicles with decreased lumbar interpedicular space, abnormal pelvic conformation, small skull base.	Reduced endochondral growth, normal periosteal growth, short but well organised chondrocyte columns.	Affects 1 in 15,000 – 40,000 births, the most common form of short-limbed dwarfism.	(Langer, 1967; Rimoin *et al.*, 1970; Rimoin *et al.*, 1976; Rousseau *et al.*, 1994; Shiang *et al.*, 1994; Bellus *et al.*, 1995a)
Hypochondroplasia	*FGFR3* OMIM 146000	Autosomal dominant	Short stature, megalocephaly with mild frontal bossing, micromelia, lumbar lordosis.	Shortened vertebral pedicles with decreased lumbar interpedicular space.	Well organised endochondral ossification.	Similar to achondroplasia, but milder.	(Rimoin *et al.*, 1976; Bellus *et al.*, 1995b; Ramaswami *et al.*, 1998)
Thanatophoric dysplasia Type I (TD1; platyspondylic skeletal dysplasia, San Diego type)	*FGFR3* OMIM 187600	Autosomal dominant	Extremely short limbs, redundant skin folds, narrow chest, large head with frontal bossing and wide-spaced eyes.	Small ribs, platyspondyly, curved femurs.	Physeal chondrocyte columns are disorganised and short, with metaphyseal bone overgrowing the physes, and short, thick primary trabeculae.	Lethal. Affects 1 in 20,000-50,000 births. Pulmonary hypoplasia. Type I is more common than type II.	(Orioli *et al.*, 1986; Tavormina *et al.*, 1995; Delezoide *et al.*, 1997; Kitoh *et al.*, 1998; Wilcox *et al.*, 1998; Brodie *et al.*, 1999)
Thanatophoric dysplasia Type 2 (TD2)	*FGFR3* OMIM 187601	Autosomal dominant		Small ribs, "clover leaf" skull.			
Severe achondroplasia, developmental delay, and acanthosis nigricans (SADDAN)	*FGFR3* OMIM 134934	Autosomal dominant	Severe rhizomelia, acanthosis nigricans, redundant skin folds, midface hypoplasia, frontal bossing.	Femoral bowing, platyspondyly with reduced interpedicular space, short ribs, pelvic abnormalities.		Rare. Developmental delay.	(Bellus *et al.*, 1999; Tavormina *et al.*, 1999)
Jansen metaphyseal chondrodysplasia (Metaphyseal chondrodysplasia Murk Jansen type, formerly metaphyseal dysostosis)	*PTHR1* OMIM 156400	Autosomal dominant	Short stature, short and bowed limb bones.	Genu valgum, broad metaphyses of long bones.	Delayed maturation of chondrocytes.	Rare. Asymptomatic hypercalcaemia and hypo-phosphataemia. Constitutive PTH/PTHrP receptor activation.	(Charrow & Poznanski, 1984; Schipani *et al.*, 1995; Schipani *et al.*, 1997a; David, 1998; Kozlowski *et al.*, 1999; Hall, 2002; Bastepe *et al.*, 2004)
Blomstrand metaphyseal dysplasia	*PTHR1* OMIM 215045	Autosomal recessive	Severe micromelia, narrow chest, short neck, micrognathia.	Osteosclerosis, advanced skeletal maturation, bowed limbs, wide metaphyses, small vertebral bodies.	Short irregular chondrocyte columns, thickened, dense primary trabeculae.	Rare. Absence of PTH/PTHrP receptors. Pulmonary hypoplasia.	(Jobert *et al.*, 1998; Zhang *et al.*, 1998; Galera *et al.*, 1999; Karperien *et al.*, 1999)

Disease name(s)	Defect/ OMIM #	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Acromesomelic dysplasia, Hunter-Thompson type (AMDH)	*CDMP1* OMIM 201250	Autosomal recessive	Shortened bones of the forearms, hands and feet.	Dislocation of large joints.			(Langer *et al.*, 1989; Stelzer *et al.*, 2003)
Grebe Dysplasia (Acromesomelic dysplasia, Grebe type; Brazilian chondrodysplasia)	*CDMP1* OMIM 200700	Autosomal recessive	Short-limbed dwarfism, acromesomelia, absent toes, polydactyly.	Failure of fingers to articulate, absence of tibia and fibula. Hypoplastic humeri.		Rare.	(Al-Yahyaee *et al.*, 2003; Stelzer *et al.*, 2003)
Brachydactyly C (Brachydactyly, Haws type)	*CDMP1* OMIM 113100	Possibly semi-dominant	Short limbs with a variety of digit anomalies.			May have normal ring-fingers and metacarpals.	(Savarirayan *et al.*, 2003; Stelzer *et al.*, 2003; Schwabe *et al.*, 2004)
Brachydactyly A1	*IHH* OMIM 112500	Autosomal dominant	Short proximal phalanges of thumb & big toe.	Shortened or missing middle phalanges.			(McCready *et al.*, 2002; Gao & He, 2004)
Brachydactyly A2 (Brachymeso-phalangy II; Mohr-Wriedt type brachydactyly)	*BMPR1B* OMIM 112600	Autosomal dominant	Shortened index-fingers and middle toes.	Shortened middle phalanges of index-fingers and middle toes.		May be clinically indistinguishable from brachydactyly C.	(Lehmann *et al.*, 2003)
Brachydactyly B1	*ROR2* OMIM 113000	Autosomal dominant	Hypoplasia/ aplasia of distal phalanges, mild syndactyly.	Short middle phalanges, rudimentary/ absent distal phalanges.			(Oldridge *et al.*, 2000; Schwabe *et al.*, 2000)
Brachydactyly D	*HOX13* OMIM 113200		Moderate brachydactyly.	Digital anomalies include hypoplasia, duplication and syndactyly.			(Johnson *et al.*, 2003)
Brachydactyly E	*HOX13* OMIM 113300						

Appendix 5 - Defects in nuclear proteins and transcription factors

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Campomelic dysplasia	*SOX9* OMIM 114290	Autosomal dominant	Micrognathia, bilateral talipes equinovarus, bowed long bones.	Hypoplastic scapulae and cervical vertebrae, missing ribs, kyphoscoliosis.		Type II collagen production may be inhibited.	(Foster *et al.*, 1994; Lefebvre *et al.*, 1997; Mansour *et al.*, 2002)
Cleidocranial dysplasia (Cleidocranial dysostosis)	*RUNX2* (*CBFA1*) OMIM 119600	Autosomal dominant	Short stature, brachydactyly, broad forehead with frontal bossing, midface hypoplasia, joint laxity.	Persistently open skull sutures, hypoplastic clavicles, wide pubic symphysis, short middle phalanx of 5th fingers, vertebral malformation.		Dental anomalies.	(Mundlos, 1999; Cooper *et al.*, 2001)
Ellis van Creveld syndrome (Chondroectodermal dysplasia)	*EVC2* OMIM 225500	Autosomal recessive	Dental abnormalities, polydactyly, absence of fingernails, acromelic dwarfism.		Irregular, hypoplastic epiphyseal cartilage with areas of focal necrosis.	Heart murmur.	(Bohm *et al.*, 1978; Galdzicka *et al.*, 2002)
Léri-Weill Dyschondrosteosis	*SHOX* OMIM 127300	Pseudo-autosomal dominant	Short stature, shortened legs and distal phalanges, depressed nasal bridge, frontal bossing.	Short, bowed radius and ulna.		Delayed psychomotor development.	(Shears *et al.*, 1998; Spranger *et al.*, 1999)

Appendix 6 - Defects in RNA and DNA processing and metabolism

Name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Anauxetic dysplasia	*RMRP*		Extremely short stature.	Small epiphyses.	Deficiency of resting and proliferating chondrocytes, reduced hypertrophic zone.		(Thiel et *al*., 2005)
Cartilage-hair hypoplasia	*RMRP* OMIM 250250	Autosomal recessive	Short limbs, brachydactyly, hypoplastic hair, hyperflexibile joints.	Anterior angulation of the sternum, widened metaphyses.		Relatively frequent in Finland. Abnormal cellular immunity and deficient erythrogenesis. Increased rate of malignancy.	(Sulisalo *et al*., 1993; Glass & Tifft, 1999; Harada *et al*., 2005)
Shwachman-Diamond syndrome (Pancreatic insufficiency and bone-marrow dysfunction; Lipomatosis of pancreas, congenital)	*SBDS* OMIM 260400	Autosomal recessive	Skeletal abnormalities, delayed puberty.	Metaphyseal abnormalities, delayed bone maturation.		Rare. Pancreatic insufficiency, haematologic abnormalities, increased likelihood of malignancy.	(Dror & Freedman, 2002; Boocock *et al*., 2003; Nakashima *et al*., 2004)

Appendix 7 - Defects in cytoskeletal elements

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Atelosteogenesis Type I (Giant cell chondrodysplasia; Spondylo-humerofemoral hypoplasia)	*FLNB* OMIM 108720		Premature birth, severe rhizomelia, midface hypoplasia.	Platyspondyly, unossified fibula, absent/ hypoplastic proximal and middle phalanges.	Hypocellular areas in physeal reserve zone with occasional multinucleate giant cells, abnormal column formation and 1° trabeculae.	Lethal, neonatally.	(Sillence *et al.*, 1982; Sillence *et al.*, 1997; Krakow *et al.*, 2004)
Atelosteogenesis type III	*FLNB* OMIM 108721	Autosomal dominant	Kypho-scoliosis.	Distal tapering of humerus and femur, no fibular ossification, irregular shortening of metatarsals and metacarpals, hypoplastic ischiopubis.			(Sillence *et al.*, 1997; Hall, 2002; Krakow *et al.*, 2004)
Boomerang dysplasia	*FLNB* OMIM 112310		Severe micromelia.	Failure of femur and tibia to ossify, often calcification of a single skeletal element of the lower limbs.	Hypocellular areas in the physeal reserve zone with occasional large multinucleate giant cells.	Lethal, perinatally.	(Sillence *et al.*, 1997; Bicknell *et al.*, 2005)
Larsen syndrome	*FLNB* OMIM 150250	Autosomal dominant	Prominent forehead, midface hypoplasia, non-tapering fingers.	Duplication of carpals and tarsal, luxation of large joints, sternal deformities.		Ranges from very mild deformities to lethal. May involve deafness.	(Becker *et al.*, 2000; Bicknell *et al.*, 2007)
	OMIM 245600	Autosomal recessive					
Asphyxiating thoracic dystrophy 1 (Jeune syndrome; Thoracic-pelvic-phalangeal dystrophy)	*IFT80* OMIM 208500	Autosomal recessive	Narrow thorax, short limbs, polydactyly.	Pelvic abnormalities.		Renal dystrophy and insufficiency, hepatic fibrosis, pancreatic cysts.	(Ho *et al.*, 2000; Beales *et al.*, 2007)

Appendix 8 - Chondrodysplasias of unknown aetiology in humans

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Chondrodysplasia punctata group							
Chondrodysplasia punctata Tibia-Metacarpal type	OMIM 118651		Short stature, midface hypoplasia.	Abnormal vertebral bodies, short tibiae and 2nd and 3rd metacarpals. Lesions improve with age.			(Rittler *et al.*, 1990; Savarirayan *et al.*, 2004)
Chondrodysplasia punctata			Facial abnormalities, rhizomelia.	Cervical platyspondyly, stippled calcifications in endochondral bones.		Psychomotor and developmental delay, weak respiratory muscles, lethal by 2 years.	(Kumada *et al.*, 2001)
Short rib polydactyly syndromes							
Short rib polydactyly I (Saldino-Noonan syndrome; Polydactyly with neonatal chondrodystrophy type I)	OMIM 263530	Autosomal recessive	Micromelia, polydactyly.	Severe thoracic dystrophy, irregular metaphyses, small ilia.		Lethal perinatally. Cardiac defects, visceral abnormalities, imperforate anus.	(Balci *et al.*, 2003; Verma, 2005)
Short rib polydactyly II (Majewski syndrome; Polydactyly with neonatal chondrodystrophy type II)	OMIM 263520	Autosomal recessive	Cleft lip and palate, polydactyly.	Short ribs and long-bones, rounded metaphyses. Oval-shaped tibiae, delayed ossification of sternum and fibulae.		Lethal perinatally. Various visceral abnormalities including cystic kidneys.	(Cooper *& Hall*, 1982; Walley *et al.*, 1983)
Short rib polydactyly III – Verma-Naumoff type	OMIM 264510	Autosomal recessive	Rhizomelia.	Narrow thorax, metaphyseal spurs.	Disorganised endochondral ossification, short cell columns in hypertrophic zone.	Respiratory infections. May be a variant of asphyxiating thoracic dystrophy 1.	(Naumoff *et al.*, 1977; Ho *et al.*, 2000)
Short rib polydactyly IV (Beemer-Langer syndrome)	OMIM 269860	Autosomal recessive	Short, bowed limbs, facial abnormalities, protuberant abdomen.	Narrow thorax, short ribs and long-bones.			(Beemer *et al.*, 1983; Elçioğlu *et al.*, 1996)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Spondylometaphyseal dysplasias							
Achondrogenesis 1A (Houston-Harris type achondrogenesis)	OMIM 200600		Short limbs.	Multiple rib fractures, crenated ilia, little ossification of vertebral bodies and skull, wedge-shaped femurs with metaphyseal spikes.	Hypercellular hyaline cartilage with cell clusters, PAS+ve diastase resistant intrachondrocytic inclusions, abundant vascular channels, failure of columnisation, abnormal 1° trabeculae.	Lethal. Fine, fibrillar material in inclusions is visible ultrastructurally.	(Whitley & Gorlin, 1983; Borochowitz *et al.*, 1988b; van der Harten *et al.*, 1988; Aigner *et al.*, 2007)
Opsismodysplasia	OMIM 258480	Autosomal recessive	Micromelia, respiratory distress.	Severe platyspondyly, delayed ossification, metaphyseal cupping.		Often lethal during the first years of life.	(Cormier-Daire *et al.*, 2003)
Sedaghatian spondylometaphyseal dysplasia (Congenital lethal metaphyseal chondrodysplasia)	OMIM 250220	Autosomal recessive	Microphthalmia, depressed nasal bridge, asymmetric ears, short neck, prominent sternum, micrognathia, acrorhizomelia.	Platyspondyly, irregular flared metaphyses, irregular tarsal ossification centres.		Lethal, neurological abnormalities.	(Elçioğlu & Hall, 1998; Koutouby *et al.*, 2000; Foulds *et al.*, 2003)
Dyggve-Melchior-Clausen dysplasia	*DYM* OMIM 223800	Autosomal recessive	Rhizomelia, short trunk with a barrel-shaped chest, microcephaly, facial dysmorphism. Delayed physical development	Irregular metaphyses, platyspondyly, kyphoscoliosis, hypoplastic iliac crests	Intracytoplasmic inclusions in chondrocytes and fibroblasts, disorganised physes, fibrous hyaline cartilage matrix	Mental retardation, abnormal urinary excretion of acid muco-polysaccharides	(Cohn *et al.*, 2003; Paupe *et al.*, 2004; Bayrak *et al.*, 2005)
Smith-McCort dysplasia	*DYM* OMIM 607326					Rare variant of Dyggve-Melchior-Clausen dysplasia with no mental retardation	
Others							
Desbuquois syndrome (Micromelic dwarfism with vertebral and metaphyseal abnormalities and advanced carpotarsal ossification)	OMIM 251450	Autosomal recessive	Short limbs, mildly bowed long bones, flat face, micrognathia, small thorax, hitchhiker thumbs.	Slightly wide metaphyses, joint luxation, advanced ossification of carpals and tarsals.		Death may occur due to respiratory problems. Also has a mild form. Clinical and genetic heterogeneity is suspected.	(Nishimura *et al.*, 1999a; Hall, 2001; Hall, 2002; Faivre *et al.*, 2004)
Metaphyseal anadysplasia	OMIM 309645	X-linked recessive/ Autosomal dominant	Slightly short stature, with varus of lower limbs.	Large irregular metaphyses of long-bones, curved diaphyses.	Wide proliferative and hypertrophic zones in physeal cartilage.	Lesions regress with age, and disappear after 2 years. Some genetic heterogeneity.	(Maroteaux *et al.*, 1991; Nishimura *et al.*, 1999b; Slama *et al.*, 1999)

Disease name(s)	Defect/ OMIM#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Metatropic Dwarfism (Metatropic dysplasia I)	OMIM 250600 OMIM 156530	Dominant and recessive forms	Rhizomelia, depressed nasal bridge, narrow chest, joint contractures.	Marked metaphyseal flaring, platyspondyly, progressive kyphoscoliosis.	Failure of chondrocytes to hypertrophy and degenerate, absent 1° spongiosa, abnormal metaphyseal vascular invasion, a thin seal of bone at chondro-osseous junctions.	Rare.	(Beck *et al.*, 1983; Boden *et al.*, 1987; Geneviève *et al.*, 2005)
Fibrochondrogenesis	OMIM 228520	Autosomal recessive	Rhizomelia, macrocephaly, hypertelorism, micrognathia.	Short ribs, platyspondyly, dumb-bell shaped femora, hypoplastic ilia.	Fibroblastic chondrocytes, abnormal physeal organisation.	Rare, neonatally lethal.	(Leeners *et al.*, 2004; Kulkarni *et al.*, 2005)
Spondyloepiphyseal dysplasia tarda, Toledo type (Brachyolmia)	OMIM 271630	Autosomal recessive	Short stature becoming apparent during childhood.	Spondylar & pelvic dysplasia.		Corneal opacity, decreased activity of chondroitin 6-sulpho-transferase.	(Toledo *et al.*, 1978; Mourão *et al.*, 1981)
Pyle disease (Metaphyseal dysplasia)	OMIM 265900	Likely autosomal recessive	Genu valgum.	Widened metaphyses of long-bones, cortical thinning, mild sclerosis of cranial bones.			(Raad & Beighton, 1978; Heselson *et al.*, 1979)
Acrodysostosis	OMIM 101800	Likely autosomal dominant	Short nose, prognathism.	Cone-shaped epiphyses, advanced skeletal maturation, shortened tubular bones of hands and feet.		Mental retardation is common.	(Jones *et al.*, 1975; Steiner & Pagon, 1992)
Dyssegmental dwarfism (Anisospondylic camptomicromelic dwarfism, Roland-Desbuquois type; Dyssegmental dwarfism, Roland-Desbuquois type)	OMIM 224400	Autosomal recessive	Narrow chest, reduced joint mobility, short, bowed limbs, hypertrichosis, macrocephaly.	Variably-sized vertebral bodies with abnormal ossification, wide metaphyses.	Disturbed maturation of physeal chondrocytes.	Lethal neonatally.	(Gruhn *et al.*, 1978; d'Orey *et al.*, 1997)

Appendix 9 - Chondrodysplasias of production animals

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Sheep								
Spider lamb syndrome	Suffolk, Hampshire	FGFR3 OMIA 000187	May be in-completely dominant	Long limbs, carpal valgus, skull malformations, kyphoscoliosis, erosion of articular cartilage.	Poor development of anconeal process, cuboidal sternebrae, pectus excavatum.	Thickened, irregular physes (esp. thoracic vertebral), with poorly organised cell columns.	Onset from birth.	(Vanek *et al.*, 1987; Vanek *et al.*, 1989; Oberbauer *et al.*, 1995; Cockett *et al.*, 1999; Beever *et* al., 2006; Smith *et al.*, 2006)
Ancon		OMIA 000302	Likely autosomal recessive	Short stature, elbow varus, carpal valgus.			Extinct.	(Landauer & Chang, 1949, Landauer, 1950))
Norwegian Ancon		OMIA 000302			Bowed femurs.	Thin physes, irregular columnisation.	Onset from birth. Extinct.	(Chang, 1949)
Achondro-plasia	Cheviot	OMIA 000004		Ectrodactyly, exophthalmus, short ears & tail.	Abnormal proximal phalanx, absence of middle and distal phalanges.			(Wray *et al.*, 1971)
Lethal dwarfism				Domed head, short muzzle, micromelia, swollen abdomen, cleft palate.	Narrow thorax with short ribs, bowed long bones with large epiphyses.	Lack of physeal chondrocyte columns, matrix reduced to narrow strands.	Lethal. Thyroid hyperplasia. Scrotal hernia may occur.	(Duffell *et al.*, 1985)
Dwarfism	South Down		Autosomal recessive	Short limbs, bulging cranium, throat & palate abnormalities.		Thyroid follicles lack colloid.	Lethal at 1-2 months.	(Bogart, 1946)
Texel Chondro-dysplasia	Texel		Autosomal recessive	Short, bent limbs, barrel chest, thickened trachea.	Enlarged costochondral junctions.	Rarefaction of cartilage matrix, "collagen rings" around chondrocytes.	May cause tracheal collapse and subsequent death.	(Byrne, 2005; Thompson *et al.*, 2005)
Cattle								
Brachy-cephalic Snorter	Hereford	OMIA 000310	Autosomal recessive	Short head, inferior prognathia, shortened distal long bones.	Early closure of spheno-occipital synchondrosis, bony projections into cranium, ventral flattening of lumbar vertebrae.	Short irregular chondrocyte columns, occasional dilations of ER containing granular material.	Onset from birth.	(Julian *et al.*, 1957; Jones & Jolly., 1982)
Dolicho-cephalic Dwarf	Aberdeen-Angus, Hereford, Shorthorn			Long head.			May be part of a complex with the brachycephalic snorter.	(Gregory, 1956; Julian *et al.*, 1959)
Comprest Dwarf	Small-type Hereford						May be part of a complex with the brachycephalic snorter.	(Gregory, 1956; Julian *et al.*, 1959)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Bulldog calves	Dexter	*ACAN* OMIA 001271	Autosomal in-completely dominant	Short spine, severe rhizomelia, hydrocephalus, reduced maxilla, cleft palate, protruding tongue, umbilical hernia.		Limb bones consist mostly of highly cellular epiphyseal cartilage without distinct physes.	Usually aborted by 7 months gestation. Heterozygotes are short-limbed.	(Harper *et al.*, 1998; Cavanagh *et al.*, 2007)
	Jersey						Jersey, onset from birth	(Mead *et al.*, 1946)
	Holstein-Friesian			Short maxilla, protruding tongue, enlarged cranium, eye deformities, short spine, rhizomelia, umbilical hernia, stenotic trachea, narrow thorax, hypoplastic lungs, bilateral ventricular hypertrophy.		Domination of hypertrophic chondrocytes in epiphyses, poor organisation of physis, cartilage cores in metaphysis.	Gestation in completed to term.	(Bowden, 1970; Agerholm *et al.*, 2004b)
	Guernsey		Autosomal recessive	Short maxilla and lower jaw, dis-proportionate dwarfism.	Rhizomelia.		Complete gestation to term, die minutes after birth.	(Jones, 1961)
Telemark Lethal	Telemark		Recessive	Short heads & maxillae, short crooked legs.			Complete gestation to term, die due to respiratory obstruction.	(Mohr, 1926; Brandt, 1941)
Angus dwarfism	American Angus	PRKG2 mutation	Autosomal recessive	Short limbs.		Disorganised physes.		(Mishra *et al.*, 2004)
Brachy-gnathia and degener-ative joint disease	Angus	OMIA 000150	Likely to be heritable	Brachygnathia superior, erosion of cartilage in major joints.	Osteophytes, reduced joint spaces and sclerosis of subchondral bone.	Degeneration of cartilage matrix with chondrocyte necrosis and fibrillation. Poorly organised physes and abnormal ossification of calcified matrix.	Lethal.	(Jayo *et al.*, 1987)
Chondro-dysplasia	Japanese Brown Cattle	*LIMBIN* mutation OMIA 000305	Autosomal recessive	Short long bones with abnormalities at the ends.		Narrow irregular physis with cartilage extending into spongiosa, hypocellular hypertrophic zone, cystic degeneration of matrix. Irregular resting chondrocytes with vacuolation and acidophilic intracytoplasmic granules.		(Moritomo *et al.*, 1992; Takeda *et al.*, 2002)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Dispro-portionate Dwarfism	Shorthorn			Markedly shortened pelvic limbs, carpal & stifle valgus.		Reduced maturity of chondrocytes, reduced capillary penetration, irregular 1° spongiosa.	Onset from birth.	(Carrig *et al.*, 1981)
Cartilage Defect	Ayrshire			Lameness, articular surfaces mottled & rough.		Irregular physes with eosinophilic patches, focal necrosis, and cartilage remnants in spongiosa. Metachromasia with toluidine blue.	Onset at 2 months of age. Ultrastructurally, chondrocytes in abnormal regions resembles hypertrophic cells.	(Wild & Rowland, 1978)
Dwarfism	Aberdeen-Angus		Autosomal recessive	Low-set, thick body form, short wide head, reduced growth rate.			Onset at 2-3 months of age.	(Baker *et al.*, 1951)
Stumpy	Shorthorn	OMIA 000311	Autosomal recessive	Enlarged carpal joints, lateral twisting of canon, distal phalangeal valgus, poor growth, curly coat, small switch.				(Baker *et al.*, 1950)
Pigs								
Chondro-dysplasia	Danish Landrace	OMIA 000187		Shortened limbs, increased joint mobility, reduced weight gain, long hair coat.	Normal axial skeleton, progressive degenerative joint disease, wide metaphyses of long bones with mushroom-shaped epiphyses.	Inhibited endochondral ossification, reduced physeal proliferative zone, irregular hypertrophic zone.	Onset 1-3 weeks of age.	(Jensen *et al.*, 1984)
Dwarfism		Type X collagen	Autosomal dominant	Shortened long-bones, tongues of physeal cartilage extending into metaphyses.	Abnormal metaphyses with radiodense striation.	Wide physes, disorganised hypertrophic zone, poor staining of matrix with H&E and TB.	Animal model for Schmid metaphyseal chondro-dysplasia.	(Nielsen *et al.*, 2000)
Hereditary dwarfism	Large White X Landrace			Short limbs and muzzle.	Short spine and ribs.	Enlarged physes with granular matrix containing foci of necrosis, irregular metaphyseal trabeculae	Respiratory difficulties may occur	(Mores *et al.*, 1989)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Deer								
Chondro-dystrophy	Fallow			Reduced length of all bones, varus deformity of limbs. Cleft palate, domed skull, large fontanelle.	Distorted epiphyses and sternebrae.	Premature closure of some physes, short physeal chondrocyte columns.		(Baker *et al.*, 1979)
Goats								
Bulldog		OMIA 000005		Large cranial vault, short, wide facial bones, brachygnathia superior, protruding tongue, short neck and limbs, ventral hernia.				(Kaikini & Malkhede, 1969)

Appendix 10 - Chondrodysplasias of companion animals

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Dogs								
Chondro-dysplasia	Alaskan Malamute	OMIA 000300	Autosomal recessive	Shortened limbs, curved antebrachium.	Flared metaphyses with irregular borders.	More matrix than normal between chondrocyte columns, variable physeal organisation, irregular spongiosa.	Onset : 5-6 weeks (gross), 7-10 days (radiographic). Reduced activity of lysyl oxidase in growth plate. Associated anaemia with increased red cell sodium content.	(Sande *et al.*, 1974; Terpin & Roach., 1981; Sande *et al.*, 1982)
Enchondro-dystrophy	English Pointer		Autosomal recessive	Forelimb valgus, enlarged shoulder and stifle joints, dorso-ventral flattening of thorax, “bunny hopping” gait.	Physeal enlargement with metaphyseal flaring, abnormal vertebral bodies, cranial bowing of radius develops.	Thickened hypertrophic zone, disorganised proliferative zone. Degeneration of the mid zone becomes cystic spaces.	Onset: First weeks of life.	(Whitbread *et al.*, 1983; Lavelle, 1984)
Pseudo-achondro-plasia	Miniature Poodle		Autosomal recessive	Enlarged joints, poor growth, recumbency in severe cases, dysplastic acetabula, “bunny hopping” gait, dorso-ventral flattening of thorax, thickened tracheal rings.	Enlarged stippled epiphyses, metaphyseal flaring, short vertebral bodies with abnormal spinous processes.	Delayed ossification, haloes surrounding chondrocytes, poorly organised chondrocyte columns.	Onset: 10-21 days. Under-sulphated chondroitin sulphate.	(Amlöff, 1961; Riser *et al.*, 1980; Bingel *et al.*, 1986)
Chondro-dysplasia	Norwegian Elkhound			Cranial bowing of radius, carpal valgus.	Metaphyseal flaring, increased bone density in region of 1° spongiosa, short vertebral bodies.	Narrow zone of proliferation, variable column formation, chondrocytes contain large AB/PAS+ve inclusions, disorganised spongiosa.	Onset: 5 weeks (radio-graphically). EM shows inclusions of finely granular material bounded by a discontinuous membrane.	(Bingel & Sande, 1982)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Oculo-skeletal Dysplasia	Scottish Deerhound	OMIA 000301	Autosomal recessive	Exercise intolerance, poor growth, short bowed limbs, carpal hyperextension, kyphosis, poor muscle tone.	Abnormal epiphyseal ossification centres, short vertebral bodies.	Thickened irregular physes with abnormal column formation, little proliferative cartilage, chondrocytes have PAS+ve diastase resistant intracytoplasmic inclusions, variably organised spongiosa.	Onset: 4-5 weeks. EM shows dilated rER contains granular/lamellar material.	(Breur *et al.*, 1989; Breur *et al.*, 1992)
	Labrador Retriever		Skeletal lesions autosomal recessive. Ocular lesions incomplete-ly dominant	Shortened long bones, abnormal joint development with DJD, carpal varus, bowing of radius, hip dysplasia, ocular abnormalities.	Delayed development of anconeal process.			(Carrig *et al.*, 1977; Carrig *et al.*, 1988)
	Samoyed		Autosomal recessive	Small stature, elbow varus, carpal valgus, ocular lesions.	Metaphyseal flaring.		Onset: 8-12 weeks (radio-graphically). Increased urinary chondroitin sulphate. Non-allelic with Labrador oculo-skeletal dysplasia.	(Meyers *et al.*, 1983; Du *et al.*, 2000)
Chondro-dysplasia	Irish Setter	OMIA 001296	Autosomal	Bowed radius, carpal valgus, shortened body and limb length.		Well organised endochondral ossification, thickened irregular trabeculae.	Onset: Several weeks of age.	(Hanssen, 1992; Hanssen *et al.*, 1998)
Chondro-dysplasia	Great Pyrenees	OMIA 000187	Autosomal recessive	Short limbs, trunk, & muzzle, poor growth, angular limb deformities.	Poor ossification of vertebral bodies, thickened long bone metaphyses, short ribs with enlarged costochondral junctions.	Disordered column formation, thick irregular trabeculae.	Onset 10 days. EM shows dilated rER of proliferative cells, containing ruthenium red positive granules. Increased urinary chondroitin sulphate & triglycerides. Some pups are deaf.	(Bingel & Sande, 1994)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
GM-1 Ganglio-sidosis	English Springer Spaniels, Portuguese Water Dogs, Alaskan Huskies	*GLB1* OMIA 000402	Autosomal recessive		Irregular intervertebral disc spaces, abnormal physeal ossification.	Delayed lumbar vertebral ossification with cartilage necrosis, vacuolated chondrocytes.	Onset: 2 months (radio-graphically). Reduced activity of lysosomal β-galactosidase. Neurological signs develop at 4 ½ months, vacuolation of many cell types, including lymphocytes in blood smears.	(Alroy *et al.*, 1992; Müller *et al.*, 1998; Müller *et al.*, 2001; Yamato *et al.*, 2002)
Muco-polysaccharid-osis I	Plott-hounds	*IDUA* OMIA 000664	Autosomal recessive	Stunted growth, lameness, poor vision, severe DJD in multiple joints, bilateral corneal opacity.	Lytic areas in femoral condyles and spine, narrowing of intervertebral disc space with osteophyte formation.	Dilated vesicles in many cell types with accumulation of acid muco-polysaccharide.	α-L-iduronidase deficiency.	(Shull *et al.*, 1982; Menon *et al.*, 1992; Stoltzfus *et al.*, 1992)
Muco-polysaccharid-osis VII (Sly Syndrome)		*GUSB* OMIA 000667	Autosomal recessive	Broad short skull, broad chest, corneal clouding, poor growth, appendicular skeletal disease, cardiac abnormalities, hepatomegaly, misshapen trachea.	Epiphyseal dysplasia, hip subluxation.	Cytoplasmic vacuolation of many cell types, vacuoles stain positively with TB.	Onset: 4 weeks. EM shows inclusions are membrane-bound and contain granular or lamellar material. β-glucuronidase deficiency. Excessive urinary excretion of chondroitin-4- and 6-sulphate and dermatan sulphate.	(Haskins *et al.*, 1991; Ray *et al.*, 1998)
Multiple Epiphyseal Dysplasia	Beagle			Swaying gait, narrow trachea, erosions of articular cartilage of femoral head.	Stipples epiphyses, femoral neck & lumber vertebral changes.	Acellular areas staining red with toluidine blue and areas of dense calcium deposition in matrix. Irregular arrangement of chondrocytes.	Onset: Birth.	(Rasmussen, 1971; Rasmussen & Reimann, 1973; Mango *et al.*, 2004)
Osteo-chondro-dysplasia	Bull Terrier			Abnormal hindlimb gait, hip dysplasia.		Femoral neck contained areas of non-ossified cartilage with fibrillar areas, vacuolated chondrocytes.	Onset: 3-4 months.	(Watson *et al.*, 1991)
Chondro-dystrophy	Cocker Spaniel			Short limbs, prominent bony masses at joint peripheries.	Metaphyseal flaring.			(Beachley & Graham, 1973)
Chondro-dysplasia	German Shepherd			Short limbs, forelimb valgus, deviation & hyperextension of metacarpals & phalanges.	Wide radial & ulnar metaphyses, dense irregular trabecular patterns.		Onset: 4 months.	(Roberg, 1979)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Achondro-plasia	Scottish Terrier	OMIA 000299		Abducted limbs, unable to walk, dorsoventral flattening of thorax, enlarged costosternal articulations.		Fatty, hypertrophied cells of adrenal glomerular zone.	Onset: Birth.	(Mather, 1956)
Achondro-genesis	Akita		Autosomal dominant	Micromelia, short trunk, angular limb deformities.	Short, broad ilia, pointed vertebral bodies, flared metaphyses.			(Sande *et al.*, 1994)
Osteo-chondro-dysplasia	Bulldog			Inability to adduct front limbs.	Severe dorsoventral flattening of thorax.		Severe dyspnoea.	(Louw, 1983)
Cats								
Muco-polysaccharid-osis VI	Siamese		Autosomal recessive	Small head, short maxilla, small ears, odontoid hypoplasia, corneal opacity, hip subluxation, pectus excavatum.	Osteoporosis with sclerosis at vertebral end plates and articular facets.	Retarded endochondral ossification with focal necrosis, hypercellular cartilage with intracytoplasmic inclusions that are also in many other cell types.	Onset: 8 weeks. Decreased activity of arylsulphatase-B, increased urinary dermatan sulphate. Leukocytes contain coarse granules staining meta-chromatically with toluidine blue. Possible animal model for human Maroteaux-Lamy Syndrome.	(Jezyk *et al.*, 1977; Haskins *et al.*, 1980b; Haskins *et al.*, 1981; Orgad *et al.*, 1989)
Muco-polysaccharid-osis VII				Retarded growth, corneal clouding, delayed dental eruption, abdominal enlargement, short maxilla.	Fused cervical vertebrae, flattened thorax, pectus excavatum, short vertebrae, coxofemoral subluxation.		Onset: 8 weeks. β-glucuronidase deficiency, increased urinary chondroitin sulphate. Granules in neutrophils stain meta-chromatically with toluidine blue.	(Fyfe *et al.*, 1999)
Osteo-chondro-dysplasia	Scottish Fold	OMIA 001315	Autosomal dominant	Lameness, short misshapen distal limbs, short wide caudal vertebrae. Ankylosis develops.	Irregular punctate areas of tarsal radiolucency, excessive new bone formation.	Irregularly thickened hyaline cartilage with necrotic foci and fibrillation.		(Malik *et al.*, 1999; Chang *et al.*, 2007)
Metaphyseal Chondro-dysplasia		OMIA 000187		Carpal valgus, swelling of distal antebrachii, short limbs.	Metaphyseal flaring.	Irregular thickening of physes.		(Gunn-Moore *et al.*, 1996)

Disease name	Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Chondro-dysplasia				Small body, domed skull, flattened face, protruding tongue, short, bent limbs.	Shortened vertebral bodies, short fibs with flared costochondral junctions, mushroom-shaped long bone epiphyses.	Long bone physes were irregularly organised with necrotic foci, cystoid degeneration of cartilage matrix.	This disorder was described in a kitten with Pelger-Hüet anomaly as well as a littermate with normal leukocytes.	(Latimer *et al.*, 1988)
Congenital hypo-thyroidism	Abyssinian		Autosomal recessive	Reduced growth rate, dis-proportionate dwarfism, kitten-like features, constipation, goitre.		Delayed closure of physes, hyperplastic thyroid gland with small follicles containing little colloid.		(Jones, 1992)
Munchkin	Munchkin			Short, bowed long bones.	No involvement of axial skeleton.			(Munchkin.net, 1998)

Appendix 11 - Chondrodysplasias of laboratory animals

Disease name	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Transgenic mice							
Activating Transcription Factor (ATF) Mutant	*ATF2*		Reduced growth, rhizomelic dwarfism.		Disorganised endochondral ossification with cells in clusters instead of columns, failure of capillary ingrowth. Abnormal CNS histology.	Reduced viability, ataxia, poor hearing, enlarged ventricles in brain, skeletal pathology considered similar to human hypo-chondroplasia.	(Reimold *et al.*, 1996)
β-1 integrin mutant	*ITGB1*		Short, broad long bones, small thorax	Mild lordosis	Abnormally shaped chondrocytes failing to form columns, delayed epiphyseal mineralisation, thick physes.	Abnormal distribution of collagen fibrils with variable diameters and a tendency to form parallel bundles. Neonatally lethal.	(Aszodi *et al.*, 2003)
Cbfa-1 deficient	*RUNX*				Failure of chondrocytes to differentiate to prehypertrophic cells, no bone marrow.		(Inada *et al.*, 1999)
Cbfa-1 overexpression	*RUNX*		Severe dwarfism, domed skull, short limbs and snout, protruding tongue, narrow thorax.	Accelerated endochondral ossification, fusion of most skeletal elements.	Early maturation of chondrocytes.	Neonatally lethal.	(Ueta *et al.*, 2001)
CDK inhibitor p57Kip2 Mutant	*CDKN1C*			Arrested physeal growth.		Failure to express collagen type X.	(Stewart *et al.*, 2004)
CHST11 mutant	*CHST11*	Autosomal recessive	Severe chondro-dysplasia, short limbs, domed skull, thoracic and facial bone hypoplasia.		Disorganised and hypocellular physeal cartilage, extensive fibrillation of extracellular matrix.	Lethal neonatally. Severe decrease in cartilage chondroitin 4-sulphate, some decrease in chondroitin 6-sulphate and unsulphated chondroitin.	(Klüppel *et al.*, 2005)
Crtl-1 mutant	*CRTL1*	Autosomal recessive	Dwarfism.	Delayed bone formation, cervical lordosis. Small epiphyses, flared metaphyses, platyspondyly.	Reduced expression of Ihh in prehypertrophic cells, inhibition of hypertrophic cell apoptosis.	Usually neonatally lethal due to respiratory failure.	(Watanabe & Yamada 1999)
COL2A1 Mutant	*COL2A1*			Multiple joint dysplasia, irregular vertebral endplates, shortened long-bones, spine and skull.		EM revealed thin collagen fibrils.	(Sahlman *et al.*, 2004)

Disease name	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
COLX Transgene	*COLX*		Inset eyes, narrow snout, variable severity of lesions.	Cervical lordosis, thoracolumbar kyphosis.	Little or no matrix in a compressed hypertrophic zone, in some cases absence of cell columns.	EM revealed poorly visible collagen fibrils in hypertrophic zone. Often lethal in perinatal period.	(Jacenko *et al.*, 1993; Jacenko *et al.*, 2001)
PTH/PTHrP Receptor Null	*PTHR1*		Small size, domed skull, short face, protruding tongue, short limbs.		Chondrocyte columns irregular & short. Accelerated mineralisation of endochondral bones.	Often lethal mid-gestation.	(Lanske *et al.*, 1996)
FGF2 Mutant	*FGF2*		Megalocephaly.		Reduced chondrocyte proliferation and increased apoptosis.	Removal of STAT1 function corrected the chondro-dysplasia.	(Sahni *et al.*, 2001)
FGFR3 Mutant	*FGFR3*	Autosomal dominant	Short bowed limbs, curved spine, rounded skull, small thorax with caudal widening.	Decreased ossification, enlarged costochondral junctions & sternebrae.	Hypercellular cartilage, short/absent cell columns, incomplete maturation of hypertrophic cells.	Lethal neonatally, similar to human thanatophoric dysplasia II Constitutive activation of FGFR3.	(Iwata *et al.*, 2000)
FGFR3 Mutant	*FGFR3*	Autosomal recessive	Excessive growth of endochondral bones, long tail, kyphoscoliosis, bowed legs.	Long vertebral bodies and limb bones.	Expansion of physeal hypertrophic zone.	Disruption of FGFR3.	(Deng *et al.*, 1996)
Ihh mutant	*IHH*		Domed skull, shortened snout, mandible, tail and limbs.		Reduced chondrocyte proliferation, inappropriate maturation, degradation of matrix, no cell columns.		(St-Jacques *et al.*, 1999)
Pex7 Knockout	*PEX7*			Impaired ossification of distal limbs, parts of skull and vertebrae.		Model of chondro-dysplasia punctata in humans Impaired oxidation of some fatty acids.	(Brites *et al.*, 2003)
PTHrP Over-expression	*PTHRP*		Shortened limbs & tail, bowed long bones.	Delayed endochondral ossification.	Delayed endochondral ossification, persistent anlage of proliferating & prehypertrophic cells.	Becomes histologically normal by 7 weeks Chondrocyte-specific overexpression of PTHrP.	(Weir *et al.*, 1996)
RANK Null	*RANK*		Small body, short limbs, domed skull, failure of tooth eruption.	Osteopetrotic skeleton.	Thickened physes with disorganised cells, occlusion of medullary cavity.		(Dougall *et al.*, 1999)

Disease name	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
SLC26A2 Mutant	*SLC26A2*	Autosomal recessive	Poor growth, short stature, craniofacial abnormalities, joint contractures, degradation of articular cartilage.	Delayed formation of 2° centres of ossification, long bone osteoporosis, progressive thoracic kyphosis, reduced thoracic volume.	Reduced staining of cartilage with TB, irregular cell size. Good physeal structure early in life becomes disordered.	Model of DTDST-related disorders in humans, backwards walking, usually lethal by 6 months Reduced chondrocyte & fibroblast sulphate uptake, progressive undersulphation of cartilage proteoglycans.	(Forlino *et al.*, 2005; Forlino *et al.*, 2006)
SLC35D1 Deficient	*SLC35D1*	Autosomal recessive	Reduced crown-rump length, extremely short limbs, short snout.	Facial bone and vertebral body hypoplasia, short iliac bones.	Disorganised proliferative zone of physeal cartilage with hypercellularity and scant matrix.	Model of schneckenbecken dysplasia in humans. Lethal in the neonatal period. Failure of chondroitin biosynthesis.	(Hiraoka *et al.*, 2007)
SOX9 Knock-in	*SOX9*		Cleft palate, small thorax, poor growth, domed skull, short face & limbs, bent radius.	Vertebrae may be fused.	Delayed ossification, no columnar organisation.	Often lethal neonatally Excessive expression of SOX9.	(Akiyama *et al.*, 2004)
Tg737^{orpk}	*TG737*	Autosomal recessive	Stunted growth, craniofacial defects, abnormal skeletal patterning.		Small, abnormally organised chondrocytes.	Absent or shortened primary cilia on chondrocytes.	(Zhang *et al.*, 2003; McGlashan *et al.*, 2007)
TNFSF-11 Null	*TNFSF11*				Thickened proliferative zone, no column formation, hypocellular areas, hypertrophic cells fail to mature.		(Odgren *et al.*, 2001)
TRANCE	*TRANCE*		Failure of tooth eruption, reduced growth.	Osteopetrosis.	No osteoclasts, thick irregular physes with no columnisation and a relative increase in hypertrophic cells.	Tumour Necrosis Factor-related, Activation-induced Cytokine (TRANCE) deficiency.	(Kim *et al.*, 2000)
Non-transgenic Mice							
Achondroplasia (cn)	*NPR2*	Autosomal recessive	Similar skeletal changes to human achondroplasia.	Reduced caudal vertebrae, delayed epiphyseal ossification.	Short chondrocyte columns, matrix stains poorly with AB, hypocellular resting zone, scanty 1° spongiosa.	Obvious at birth. EM revealed densely packed collagen fibrils in superficial & pericellular matrix.	(Bonucci *et al.*, 1976; Silberberg *et al.*, 1976; Sannasgala & Johnson, 1990; Tsuji & Kunieda, 2005)

Disease name	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Chondro-dysplasia (cho)	*COL11A1*	Autosomal recessive	Short muzzle, cleft palate, defective trachea.	Short long bones, flared metaphyses.	Little distinction between proliferative and hypertrophic zones, matrix stains poorly with TB, wide fused trabeculae.	Lethal. EM reveals thick collagen fibrils.	(Seegmiller *et al.*, 1971; Li *et al.*, 1995; Fernandes *et al.*, 2007)
Brachymorphism	*PAPSS2*	Autosomal recessive	Short long bones & tail, domed skull.	Small epiphyses.	Short chondrocyte columns, pale staining matrix.	Onset at 4 weeks. Reduced cartilage sulphation, decreased PAPS synthesis.	(Orkin *et al.*, 1976; Orkin *et al.*, 1977; Pennypacker *et al.*, 1981; Kurima *et al.*, 1998)
Brachypodism (bp)	*GDF5* (*CDMP1*)	Autosomal recessive	Reduced length of long bones, irregularly shaped tarsals.	Delayed cartilage and bone formation in feet.		Homozygotes have an abnormal number of phalanges.	(Landauer, 1952; Storm *et al.*, 1994)
Cartilage matrix deficient (cmd)	*ACAN*	Autosomal recessive	Short limbs, tail and snout, cleft palate, thickened tracheal rings.	Thoracic hypoplasia.	Reduced cartilage matrix, occasional degenerate foci, poor physeal organisation.	Absence of aggrecan in cartilage matrix, abnormally thick collagen fibrils.	(Kobayakawa *et* al., 1985; Bell *et* al., 1986; Watanabe *et al.*, 1994; Krueger *et al.*, 1999)
Chubby (cby)		Autosomal recessive	Mild disproportionate dwarfism, short face, acromelia.		Thickened physes, abnormal columnar organisation, necrosis of proliferative cells, poorly developed 1° spongiosa.	Onset at 3 weeks. Chondroitin sulphate chains are 20-30% longer than normal.	(Wikström *et al.*, 1987)
Disproportionate micromelia (Dmm)	*COL2A1*	Incomplete dominant	Disproportionate dwarfism, cleft palate in homozygotes.		Homozygotes have reduced matrix, disorganised physes, cystoid degeneration of epiphyses.	Lethal in homozygotes, heterozygotes develop dwarfism by 1 week of age.	(Brown *et al.*, 1981; Pace *et al.*, 1997)
Cartilage anomaly (can)		Recessive	Small body, reduced growth, short, domed skull, shortened maxilla, ribs and limbs.	Dorso-ventral flattening of thorax, scoliosis.	Thin physes with closely packed chondrocytes and poor organisation of cell columns.	Usually lethal by 10 days of age, reduced proteoglycan content of cartilage.	(Johnson & Wise, 1971)
Dwarfism (dw)		Autosomal recessive	Short maxilla.	Delayed ossification of tibial heads.	Short, hypercellular chondrocyte columns with small cells.	EM reveals thin collagen fibres, diminished matrix ground substance, fibrillation of matrix.	(Silberberg *et al.*, 1966)
Muco-polysaccharid-osis VII			Small body, short limbs, short, thick tail, facial dysmorphism.	Short spine, malformed vertebrae, enlarged weight-bearing joints, narrow thorax, broad ribs with flared metaphyses.	Irregularly hypercellular articular cartilage and physes, enlarged chondrocytes containing vacuoles.	Lesions appear by 21 days. Similar to muco-polysaccharidosis VII in human beings, mice lack β-glucuronidase activity. Vacuoles are apparent in cells from many tissues.	(Birkenmeier *et al.*, 1989; Vogler *et al.*, 1990)

Disease name	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Rats							
Toothless Osteopetrotic Rat	*CSF1*	Autosomal recessive	Incisor teeth fail to erupt.	Osteopetrosis, short club-shaped bones.	Thickened physes, failure of column formation with hypocellular areas, hypertrophic cells fail to mature, small cells, reduced vascular invasion.	Onset at birth (radiographically), 10 days (grossly).	(Seifert, 1996; Odgren *et al.*, 2001; Dobbins *et al.*, 2002; Devraj *et al.*, 2004)
Rabbits							
Pelger Dwarf	OMIA 000783	Incomplete autosomal dominant	Small size, poor growth, short curved long bones, short ribs, poor mobility.	Enlarged epiphyses.		Identifiable at birth. Reduced leukocyte segmentation in heterozygotes, homozygotes have none. Homozygotes show skeletal abnormalities and poor viability.	(Nachtsheim, 1950)
Achondroplasia (da; New Zealand White)	OMIA 000189	Autosomal recessive	Short bent limbs, a cartilaginous papilla at external auditory meatus.	Abnormal scapula, ventrally-directed foramen magnum.		Onset at 1 week of age.	(Crary & Sawin, 1952)
Chondro-dysplasias (ac)		Incomplete dominant	Shortened extremities, domed skull, protruding tongue.			Identifiable at birth. Mitochondria lack one phosphorylation site so no ATP is generated at the cytochrome oxidase region. Neonatally lethal.	(Crary *et al.*, 1958; Bargman *et al.*, 1972; Mackler *et al.*, 1972)
Chondro-dystrophy (cd)		Autosomal recessive	Large rounded head, cleft palate, short bowed long bones, compressed tracheal cartilage.	Flared metaphyses, enlarged epiphyses.		Identifiable at birth. Lethal. Possible model for human metatropic dwarfism.	(Fox & Crary, 1975)
Non-human Primates							
Short-limbed dwarfism	Rhesus (Macaca mulatta)			Marked rhizomelia, curved limbs, large head, flat face.	Flared metaphyses, short, flared ribs.	Disorganised endochondral ossification, no chondrocyte columns or orderly vascular invasion, poorly organised primary spongiosa.	(Zeman & Baskin, 1986)

Appendix 12 - Chondrodysplasias of birds

Disease name	Species/ Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Nanomelia	domestic chicken, Gallus gallus	*ACAN*	Described as autosomal recessive, but this may not be the case if hetero-zygotes also show lesions	Extremely short, twisted limbs, bent tibiotarsus, brachycephaly, parrot-like beak with inferior brachygnathia.	Mushroom-shaped proximal head of the humerus.		Lethal during incubation, markedly reduced synthesis of aggrecan and chondroitin sulphate. Heterozygotes may have slightly shortened long bones.	(Landauer, 1965; Mathews, 1967; Stirpe *et al.*, 1987; Primorac *et al.*, 1994)
Fowl chondro-dystrophy	domestic chicken, Gallus gallus	OMIA 000189	Autosomal recessive	Deformed beak and limbs, shortened long bones.			Lethal due to inability to hatch.	(Lamoreux, 1942)
Fowl micromelia	domestic chicken, Gallus gallus	OMIA 000648	Autosomal recessive	Parrot-like beak with inferior brachygnathia, domed skull, micromelia.				(Asmundson, 1942)
Chondro-dystrophy	Japanese quail, Coturnix japonica		Autosomal recessive	Short, bent long-bones, flattened head, bulging eyes, inferior brachygnathia.			Usually lethal within 4 days of hatching.	(Hermes *et al.*, 1990)
Micromelia	Japanese quail, Coturnix japonica		Autosomal recessive	Short, broad head with bulging eyes, inferior brachygnathia, broad trunk, generalised micromelia.			Lethal during incubation.	(Hill *et al.*, 1963)
Early embryonic chondro-dystrophy	Japanese quail, Coturnix japonica		Autosomal recessive	Short curved upper mandible with virtually no lower mandible, short curved tibia and tarso-metatarsus with no evident hock joint.			Lethal during incubation. Normal axial skeleton.	(Collins *et al.*, 1968)
Short leg	Turkey, Large White, Bourbon Red, Bronze		Autosomal recessive	Shortened tarso-metatarsus, apparent thickening of long bones.			Reduced hatchability and postnatal viability, Survivors in Large White form are reproductively sterile.	(Asmundson, 1939; Asmundson, 1944; Nestor, 1978)

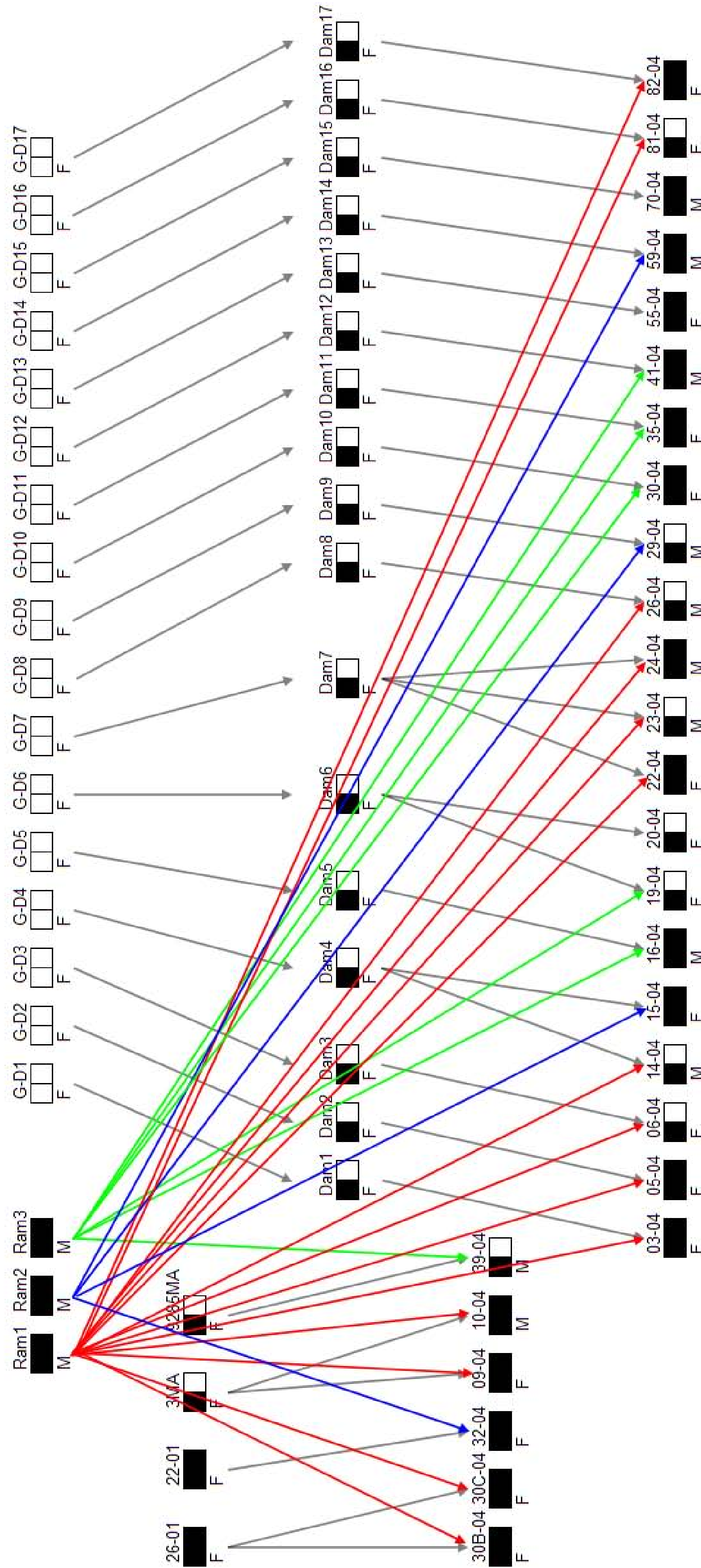
Disease name	Species/ Breed	Defect/ OMIA#	Heritability	Gross lesions	Radiology	Histology	Other notes	Reference
Lethal chondro-dystrophy	Turkey	OMIA 000189	Autosomal recessive	Micromelia, bent femur and tibia, brachycephaly, bulging eyes, brachygnathia inferior.			Lethal during incubation.	(Gaffney, 1975)
Achondro-plasia	Pigeon			Extremely short limbs and beak.			Lethal.	(Hollander, 1945)
Micromelia	Pekin duck		Autosomal recessive	Shortened limbs and upper mandible, subcutaneous oedema, abnormal, curled feathers, epidermal thickening.		Little cellular organisation of epiphyseal cartilage and no evidence of chondrocyte hypertrophy.	Lethal during incubation.	(Ash, 1966; Ash *et al.*, 1970)

Appendix 13 - Microsatellite primer sequences

Chr.	Marker	Locus (cM)	Forward primer sequence	Reverse primer sequence
1	BMS2833	14.2	5'-ACCAGATGACCAGACCTTGTG-3'	5'-TGATCTGTGGATCCTCCCTC-3'
1	BMS835	45.3	5'-TCATGTGCATGGGGTTTG-3'	5'-ATCTGCCTACCTGGGCATC-3'
1	MCMA41	68.1	5'-TTCCTGTTTTCTCTTAGATTTGGC-3'	5'-TGGCAAGTGTGTTCTTTACCAC-3'
1	CSRD108	86.1	5'-CATGGAATCACAAAGAGTTGGACA-3'	5'-CCTGGTAAGACAGTCAGTATACAA-3'
1	MCM58	112.1	5'-CTGGGTCTGTATAAGCACGTCTCC-3'	5'-CAGAACAATAAACGCTAAACCAGAGC-3'
1	BMS482	131.2	5'-ACTTCCCCAGTCTTCCCAGT-3'	5'-TGGTGGACAGTCCCATACAG-3'
1	MAF64	164	5'-AATAGACCATTCAGAGAAACGTTGAC-3'	5'-CTCATGGAATCAGACAAAAGGTAGG-3'
1	INRA11	210.6	5'-CGAGTTTCTTTCCTCGTGGTAGGC-3'	5'-GCTCGGCACATCTTCCTTAGCAAC-3'
1	BM8246	241.1	5'-AATGACAAATTGAGGGAGACG-3'	5'-AGAGCCCAGTATCAATTCTTCC-3'
1	BM864	263.6	5'-TGGTAGAGCAATATGAAGGCC-3'	5'-GGAAATCCAAGAAAGAGGGG-3'
1	BM3205	300	5'-TCTTGCTTCCTTCCAAATCTC-3'	5'-TGCCCTTATTTTAACAGTCTGC-3'
1	EPCDV13	348.4	5'-CCATCTGTGCTGTCAGTCA-3'	5'-GAGCCTGGTGGGTTATAGT-3'
5	TGLA303	40.8	5'-CTTGTGTGCCAGACCCAGGAATCC-3'	5'-CATAAGTCAAAGTAACAGTTTAGATGTCC-3'
5	BM741	52.2	5'-GCCCCTGAAGGAATGGTG-3'	5'-CCAAAAGGTCCTATCTCCAAA-3'
5	BM7247	64.3	5'-AAGGCCTGCAGTATTTATATCC-3'	5'-AATCTTTCCCTAGAACTTACAAAGG-3'
5	JAZ	66.7	5'-CCCTGTCCCTTCGCCTGTAAAT-3'	5'-GCAGTCTGGCTCATGGGAGAAAT-3'
6	CP125	2.6	5'-GCAAATAGCCTCTTGTATGATCCTTGG-3'	5'-ACCAAAACAAGACCTTTATTTTTCATGG-3'
6	MCM53	29.7	5'-CATGGAGTTGTAGAGTCAGACATGA-3'	5'-GAGCAAAGGTCATGTCAGGTGT-3'
6	HH55	54.6	5'-GTTATTCCATATTCTTTCCTCCATCATAAGC-3'	5'-CCACACAGAGCAACTAAAACCCAGC-3'
6	JMP1	76.1	5'-CAAAGAGTCAGACATGACTGAACGAG-3'	5'-CAAAAGGACAAAGCTTGTCTGAGGCAC-3'
6	BM4311	111.6	5'-TCCACTTCTTCCCTCATCTCC-3'	5'-GAAGTATATGTGTGCCTGGCC-3'
6	JMP8	135.5	5'-CGGGATGATCTTCTGTCCAAATATGC-3'	5'-CATTTGCTTTGGCTTCAGAACCAGAG-3'
13	BMC1222	12.3	5'-CCAATTTTGCAGATAAGAAAACA-3'	5'-CCTGAGTGTTCCTCCTGAGT-3'
13	SCYAMS	37.3	5'-CTGAGCAGGAGACCATAGCACT-3'	5'-CAAAAGCAGTAATCCCAAAGCACA-3'
13	MCMA2	58	5'-TCACCCAACAATCATGAAAC-3'	5'-TTAAATCGAGTGTGAATGGG-3'
13	BL42	74.4	5'-CAAGGTCAAGTCCAAATGCC-3'	5'-GCATTTTTGTGTTAATTTCATGC-3'
13	BL1071	106.6	5'-AGAAGGACAGAGACCACAGGC-3'	5'-TTGAGGTGAAGAGGTCCACC-3'
13	BMS995	125.9	5'-AATTCTTCCAACCTCCAGTGC-3'	5'-ACTTTTCAAGCAGGGCTCAC-3'
22	BMS651	0	5'-AATATGTGAAAACAAGTCAAAGCA-3'	5'-CCTGGCAAGCAACAGTTAAT-3'
22	BMS907	13.8	5'-AGTTTCTACTCTGCCACTGTCC-3'	5'-TAAAGTCCTGTCTGCCTCTTTC-3'
22	FASMC2	15.4	5'-GAAGATGGGCCCTATAGCTG-3'	5'-AAATGCCACACATTCAAACTC-3'
22	HEL11	30	5'-CTTTGTGGAAGGCTAAGATG-3'	5'-TCCCACATGATCTATGGTGC-3'
22	BM6041	57.7	5'-GGCTGCTGCATGTCAGTG-3'	5'-GACTTGAGCTCCTCCAGGG-3'
22	BM7237	71.6	5'-TTTCTGCTAATGGCATCATTT-3'	5'-TGGATAAAGAAGATGTGGTGTG-3'

Note that all forward primers had FAM-5'-TGAAAAACGACGGCCAGT-3' added to the 5' end.

Appendix 14 - Pedigree information



Pedigree information attained from microsatellite results. The boxes below animal identifiers indicate whether the sheep was chondrodysplastic (2 black boxes), a known carrier (1 black box, 1 white box) or believed to be homozygous normal (2 white boxes). The three sire rams are at the top-left, they sired dams 1-17, and in the back-cross trial performed by Byrne (2005) sired the lambs used in this study. Ewes 26-01, 22-01, 3MA and 9285MA were not daughters of there rams, but were either affected by chondrodysplasia or had in the past produced chondrodysplastic offspring. Red arrows indicate progeny of Ram1, blue arrows indicate progeny of Ram2, and green arrows indicate deduced progeny of Ram3.

Appendix 15 - Microsatellite results

	Status	BMS2833	BMS835	CSRD108	MCM58	BMS482	MAF64	INRA11	BM8246	TGLA303	JAZ
Ram1	A	1,2	4,5	2,2	4,7	1,2	1,4	1,1	1,2	1,2	2,2
Ram2	A	1,1	3,5	1,2	4,9	1,1	4,6	2,4	2,5	2,2	2,2
03-04	A	2,3	4,4	2,2	4,7	1,2	4,4	1,1	2,2	1,2	2,2
05-04	A	1,3	1,5	2,2	1,4	2,4	1,5	1,1	2,2	1,1	2,2
06-04	C	1,1	5,5	2,2	4,4	1,1	1,4	1,1	2,6	1,1	1,2
09-04	A	1,2	3,5	2,3	4,8	1,2	1,1	1,3	1,4	1,1	2,2
10-04	A	1,3	3,5	2,2	4,8	1,2	1,1	1,2	2,2	2,2	2,2
14-04	C	1,3	2,4	2,2	1,4	1,2	4,4	1,2	2,5	1,2	2,2
15-04	A	1,3	2,3	1,2	1,9	1,2	4,6	1,4	2,3	1,2	2,2
22-04	A	1,5	2,5	2,2	4,7	2,3	4,5	1,2	2,2	1,1	2,3
23-04	C	1,2	4,5	2,3	4,4	1,3	1,4	1,2	1,2	1,1	2,2
24-04	A	1,2	4,5	2,3	4,4	1,3	4,5	1,2	2,5	1,2	2,2
26-04	C	2,2	4,4	2,2	7,7	1,3	1,5	1,2	2,5	1,1	2,2
29-04	C	1,1	5,5	2,3	7,9	1,4	4,5	2,4	2,5	1,2	2,2
30B-04	A	1,4	3,5	2,2	4,7	2,2	4,7	1,1	1,2	n	2,2
30C-04	A	1,4	4,4	2,2	7,9	2,2	4,7	1,1	1,1	1,2	2,2
32-04	A	1,1	5,5	2,3	4,7	1,1	4,6	2,5	4,5	1,2	2,2
59-04	A	1,1	3,3	2,2	4,9	1,5	4,6	1,2	n	2,2	2,2
81-04	C	1,1	3,5	2,2	4,4	1,1	1,1	1,1	n	n	2,2
82-04	A	2,3	4,5	2,3	3,4	1,1	1,4	1,4	1,5	1,1	2,2

	Status	CP125	MCM53	HH55	JMP1	BM4311	JMP8	BMC1222	MCMA2	BL42	BL1071
Ram1	A	1,4	1,2	3,4	3,3	2,2	n	2,2	2,2	1,1	n
Ram2	A	3,4	2,2	4,5	n	2,5	n	n	n	1,1	2,5
03-04	A	4,6	2,2	4,4	3,3	2,2	4,4	n	n	1,1	3,5
05-04	A	1,1	1,2	1,4	1,3	2,2	4,4	n	2,3	1,1	3,3
06-04	C	1,1	2,2	4,4	3,4	2,2	2,4	2,2	2,3	1,3	3,6
09-04	A	4,5	2,3	2,4	3,3	2,4	4,4	2,4	2,2	1,2	3,4
10-04	A	4,5	2,3	4,5	2,3	2,3	2,4	2,4	2,2	1,1	3,4
14-04	C	4,4	2,2	3,4	3,4	2,5	1,4	2,2	2,2	1,1	2,3
15-04	A	4,4	2,2	4,4	3,4	2,5	1,2	2,4	2,2	1,1	2,5
22-04	A	4,4	1,2	3,3	3,5	1,2	4,5	1,2	2,3	1,1	3,3
23-04	C	4,6	2,2	3,4	1,3	2,2	4,5	1,2	2,3	1,1	n
24-04	A	4,4	1,2	3,4	3,5	2,2	4,5	1,2	2,3	1,1	1,1
26-04	C	1,4	1,2	3,4	3,3	2,2	4,4	2,3	2,3	1,4	n
29-04	C	4,4	2,3	4,4	1,3	5,5	1,4	2,4	2,2	1,3	5,6
30B-04	A	4,4	2,4	4,5	3,4	2,2	3,4	1,2	n	1,2	1,1
30C-04	A	4,4	2,4	3,5	1,3	2,2	3,4	n	2,2	1,2	1,3
32-04	A	3,5	2,4	3,4	4,4	2,5	4,4	2,4	2,2	1,1	2,2
59-04	A	3,3	2,2	n	3,3	2,4	2,4	4,4	3,3	1,1	2,2
81-04	C	1,3	1,2	n	3,3	2,2	4,4	n	2,3	n	3,5
82-04	A	1,4	2,3	1,4	1,3	2,2	4,5	2,2	2,2	1,1	3,3

	Status	BMS995	BMS651	BMS907	FASMC2	HEL11	BM6041	BM7237
Ram1	A	2,5	2,3	1,5	1,1	1,1	1,5	1,3
Ram2	A	4,5	2,2	3,5	1,5	1,4	2,6	1,4
03-04	A	5,5	2,3	1,5	1,1	n	1,1	3,3
05-04	A	2,5	2,2	5,5	1,6	1,6	2,5	1,5
06-04	C	5,5	2,2	2,5	1,4	1,2	2,5	1,1
09-04	A	1,5	2,2	2,5	1,3	1,2	4,5	1,5
10-04	A	2,2	2,3	1,2	1,3	1,2	4,5	1,5
14-04	C	5,5	3,3	2,5	1,4	1,2	1,5	3,4
15-04	A	5,5	2,3	2,3	n	2,4	1,6	4,4
22-04	A	4,5	2,2	3,5	1,6	1,2	4,5	1,1
23-04	C	2,4	2,3	1,3	1,6	1,2	1,4	1,3
24-04	A	2,5	2,3	3,5	1,6	1,2	1,4	1,3
26-04	C	2,2	2,3	1,4	1,1	1,5	1,5	1,3
29-04	C	5,5	2,3	1,3	n	3,4	2,2	1,4
30B-04	A	2,5	2,3	1,5	1,6	n	5,5	1,1
30C-04	A	4,5	2,2	5,5	1,6	1,1	1,5	1,1
32-04	A	2,4	2,2	5,5	1,6	1,1	4,6	1,5
59-04	A	2,4	n	n	n	1,4	2,2	1,4
81-04	C	2,3	2,3	n	1,1	1,1	5,6	n
82-04	A	1,5	2,3	5,5	1,1	1,1	1,2	1,3

A = affected animal, C = carrier animal, n = genotype not available.

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