



ENZYME REPLACEMENT THERAPY IN A FELINE MODEL OF MUCOPOLYSACCHARIDOSIS TYPE VI

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Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
(Faculty of Medicine)

January 1998

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To my husband Phil and to my parents,
for their love and encouragement.

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ABBREVIATIONS

ABC method	Alcian Blue spectrophotometric method
BMT	bone marrow transplantation
BV/TV	bone mineral volume
CHO	Chinese hamster ovary
CNS	central nervous system
CPC	cetylpyridinium chloride
CS	chondroitin sulphate
C3	third cervical vertebra
DEAE method	DEAE anion exchange column chromatography
DS	dermatan sulphate
D520N	aspartic acid to asparagine change at codon 520 of f4S
ED	ethylene diamine
ED4S	ethylene diamine coupled rh4S
EM	electron microscopy
ERT	enzyme replacement therapy
f4S	feline 4-sulphatase
GAG	glycosaminoglycan
H&E	haematoxylin and eosin
HRE	high resolution electrophoresis
HS	heparan sulphate
IV	intravenously
LSD	lysosomal storage disease
L476P	leucine to proline change at codon 476 of f4S
L5	fifth lumbar vertebra
mo	months old
MPS	mucopolysaccharidoses
MPS VI	mucopolysaccharidosis type VI
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PL	poly-L-lysine
PL4S	poly-L-lysine coupled rh4S
rh4S	recombinant human 4-sulphatase
SC	subcutaneously
SD	standard deviation
Tb. Th.	trabecular thickness
Tb. N.	trabecular number
Tb. Sp.	trabecular separation
4MUS	4-methylumbelliferyl sulphate
4S	4-sulphatase
4S/ β hex	4-sulphatase/ β hexosaminidase ratio

THESIS ABSTRACT

Mucopolysaccharidosis Type VI (MPS VI) is an inherited lysosomal storage disorder, due to a deficiency of N-acetylgalactosamine-4-sulphatase (4S), leading to an accumulation of dermatan sulphate (DS) in many tissues. Severely affected human patients exhibit severe skeletal abnormalities causing dwarfism and facial dysmorphism, as well as widespread soft tissue pathology. Death is usually in late childhood. Bone marrow transplantation is currently the only therapy available for these patients, and has alleviated some problems, however improved outcomes by new therapies are needed. Enzyme replacement therapy (ERT) by intravenous administration of artificially produced recombinant human 4S (rh4S) has been proposed as an alternative therapy for MPS VI.

MPS VI has also been described in Siamese cats, and a colony related to these original cats has been established. Disease in the feline model is similar to that in humans. The primary aim of this study was to evaluate the efficacy of ERT with rh4S in feline MPS VI and to test the hypothesis that this form of therapy would reverse or alter the disease course, particularly the bone dysplasia and connective tissue pathologies. Preliminary ERT studies at low enzyme doses in young and adult MPS VI cats suggested that the earlier therapy began, the greater the improvement in bone histomorphometric parameters, although ERT was unable to alter the overall progression of disease. In enzyme distribution studies in normal cats, the majority of enzyme was detected in the liver, however enzyme was also detected in most other tissues except cartilage and cornea.

Subsequent ERT was started at birth at various dose rates. Some cats were treated with rh4S coupled to ethylene diamine or poly-L-lysine to improve penetration of enzyme into cartilage. Efficacy of ERT was evaluated by: clinical examination, radiographs, bone histomorphometry, quantitation of urinary glycosaminoglycans and tissue histology. Plasma antibody titres against rh4S were also measured. ERT resulted in subjective improvements in mobility, overall size and appearance of treated cats. Increased bone lengths and more uniform bone density was observed radiographically, corresponding with improvements in bone histomorphometric parameters. A greatly reduced incidence of spinal cord compression and a dose dependent reduction in urine glycosaminoglycan excretion was also observed. Variable reduction of storage vacuoles was observed in most connective tissues,

including heart valve, however chondrocytes and corneal keratocytes remained unchanged at all doses. Response to therapy was dose dependent, with improved response seen at higher doses. Significant joint pathology was also present in MPS VI cats with or without ERT at 11 months of age. Only one MPS VI cat undergoing intravenous ERT from birth had titres elevated above those in untreated MPS VI and normal control cats.

The original mutation causing the MPS VI phenotype (L476P) was identified during this project, providing a rapid PCR based identification of heterozygotes, which improved breeding within the colony. Subsequent detection of a second mutation (D520N), led to the identification of six genotypes within the same colony, and additional abnormal phenotypes. L476P/L476P cats used in ERT studies exhibit dwarfism, very low leukocyte 4S/ β -hexosaminidase ratios, DSuria, corneal clouding, degenerative joint disease, and lysosomal inclusions in leukocytes and in most connective tissues including chondrocytes. D520N/D520N and L476P/D520N cats have similar biochemical features but have normal growth, lack corneal clouding, and only have inclusions in leukocytes, and some chondrocytes. L476P/D520N cats also have a high incidence of degenerative joint disease. We conclude that L476P/D520N cats have a very mild MPS VI phenotype distinct from the L476P/L476P phenotype.

Prevention or reduction of development of lysosomal storage in the majority of tissues at 1 and 5 mg/kg ERT from birth, suggests that ERT is likely to lead to significant improvements in quality of life in human patients. Improvements in bone growth and structure, and minimal or no lysosomal storage in heart valve, indicates that ERT can target important sites of pathology and alter the disease course. Our studies suggest that the greatest effect of ERT on skeletal disease may occur the earlier the age of onset of therapy. Despite attempts to improve cellular uptake by enzyme modifications, lysosomal storage was still present in cornea and cartilage, which are both avascular tissues. Further study of L476P/D520N and D520N/D520N genotypes will assist in understanding the pathogenesis of joint disease in MPS VI, and may aid in development of therapies to target this site of pathology. They will also improve understanding of genotype to phenotype correlations and the pathogenesis of skeletal dysplasia in MPS VI. These studies have also demonstrated that the feline model of MPS VI is a useful model for evaluation of new therapies.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis being made available for photocopying and loan if accepted for the award of the degree.

DATE: 27/1/98

ACKNOWLEDGEMENTS

I am very grateful to Professor John Hopwood for the enormous input and commitment to this project and for his pivotal role in introducing me to the fascinating world of research. I deeply appreciate the many valuable discussions, his constant encouragement and guidance, and his seemingly endless patience. I will always remember his optimism, and also his drive to reach therapy trials in human MPS patients. I hope this is one step closer towards achieving this goal.

Tony Bourne is acknowledged for the generous use of resources and facilities within the WCH Department of Histopathology, and for helpful discussions regarding histopathology. I also very much appreciated many patient and informative discussions with Sharon Byers about this work, and thank her particularly for her input and guidance regarding cartilage and bone.

I am indebted to a number of people for the tremendous amount of time and effort and care that was involved in major aspects of this work. I am very grateful for the dedicated work by Lesley Powell, Christine Stanford, Vanessa Hobbs, Chris Evans, Karen McDougall and Christine Merrett at different times over a number of years, for daily care of the cat colony. In addition, I thank Julie Bielicki, Liz Isaac, Krystyna Niedzielski, and Chris Boulter for the enormous and monotonous task of enzyme purification over the years, which was integral to this project. I realise that this must not have been an easy job when the cats were growing too quickly and I would “just” give one cat nearly a week’s worth of purified enzyme. I am also grateful to Liz for her major contribution towards screening urine samples from seemingly hundreds of kittens using HRE.

Many thanks also to Barb King, for her very capable, and at times tenacious assistance in holding animals during intravenous infusions over the majority of these studies. I also thank her for her major involvement in the project assaying tissues from the distribution studies and plasma samples for antibody titres. Special thanks to Tom Litjens and Gouri Yogalingam for their dedicated efforts elucidating the molecular defects within the colony, which revolutionised the management of the breeding colony. I am also grateful to Jacquie Nuttall for the detailed and very time consuming work with the bone histomorphometry.

Special thanks to Richard “we can do it” Davey in Electron Microscopy, who’s ideas and enthusiasm and ability to still maintain his calm when I constantly asked “I was just wondering if.....” before I described the next impossible feat to try and attempt. I still think a good motto to stick to is make the person who wants it, try and do it THEMSELVES, and then they really learn how hard (or easy!) things are! I am also indebted to Richard for his very capable and extensive contribution towards figure preparation in this thesis.

I am very grateful for valuable input from Viv Muller, particularly in the early stages of the project, for her patience and guidance (she had the daunting task of introducing me to lab work, showing me such things as how to use a pipette and how to make up 0.9% NaCl), for engendering me with some idea about cataloguing “things” and keeping lab books. Her careful work assaying the leukocyte 4S activities and archiving feline DNA samples over many years is especially acknowledged.

There are a number of other people who have provided helpful advice and friendly encouragement with this work, particularly people within the Department of Chemical Pathology, but also within the Women’s and Children’s Hospital as a whole. This has made my time working on this project enjoyable and interesting and very memorable. In particular I thank Doug Brooks, Wendy Norton, Alvis Jaunzems, Anne Martin, Brian Matthews, Graham Truman and Dora Cross.

Informed opinions from a number of specialist veterinary colleagues were invaluable at a number of stages in this project. Particular thanks is extended to John Finnie (Vetlab, IMVS, Adelaide) for histopathology, Martin Copland, Ruth Reuter and Bill Vernau (Veterinary Pathology Services, Adelaide and Sydney) for clinical pathology, and Graeme Allan (private practice, Sydney) for radiology. Special thanks is also extended to Denise Noonan and Tim Kuchel (IMVS) for general veterinary support with the cat colony when away on leave.

I must also thank Sophie for her friendship during this project, for broadening my blinkered horizons with book titles like “Fascism”, and “The cold war” and “South-east Asian trade

relations”..... and for those nice chats over a cappuccino at Cibo’s, trying to gain inspiration for the day ahead.

This work would not have been possible without the many lovely natured cats who unwittingly gave all of themselves towards our endeavour to improve the quality of many human lives affected by the same disease. Their delightful, affectionate and individual personalities made “clinical observations” (i.e. playing) a sought after activity on those hard to get going Monday mornings. I have many wistful but lovely memories.

I would never have started this project if I hadn’t been given the many opportunities and support throughout my “growing up” by my parents, particularly for their commitment to my education, for which I am very grateful. And finally but definitely the most important person in my life, I thank Phil for his enthusiastic and constant support, great encouragement and helping me to get to “the end”. I wonder how far I would have got without these things. Now the grim reality of sharing the cooking and washing up again can start to set in.....

This work was supported by the WCH Foundation, Channel 7 Children’s Research Foundation, CSL Limited, and the National Health and Medical Research Council of Australia.

1. Introduction and review

1.1 Introduction

Mucopolysaccharidosis (MPS) patients suffer multiple disabilities in many organ systems usually with premature death, due to the deficiency of one lysosomal hydrolase. Bone marrow transplantation (BMT) to supply this deficient enzyme in donor derived marrow is currently the only available therapy for MPS patients, resulting in only limited resolution or arrest of central nervous system and skeletal pathologies. In addition, this is not without significant morbidity and mortality. Therefore, improved outcome from new therapies is needed.

The concept of enzyme replacement therapy (ERT) as an alternative therapy for inherited metabolic disorders, using an external source of normal enzyme to replace the deficient enzyme has been present for a number of years. However at the outset of this work, clinical efficacy of ERT in human Type I Gaucher disease had only recently been demonstrated. This was due to a better understanding of the specific receptor mediated endocytosis needed to target Gaucher storage pathology, and overcoming problems with large scale enzyme production in later years by using recombinant technology.

In Gaucher disease, lysosomal storage occurs predominantly in macrophages resulting in clinical disease. Conversely, in MPS disorders, the enzyme deficiencies are expressed in multiple cell types resulting in multisystem involvement, including skeletal dysplasia in a number of the MPS types. As observed with minimal skeletal improvements with BMT in MPS disorders, skeletal pathology in these and other disorders have generally been anticipated to respond poorly to new therapies. MPS VI was chosen as an appropriate disorder to evaluate skeletal response to developing therapies as skeletal dysplasia is a predominant feature of this disease. In addition, a naturally occurring feline MPS VI model which closely parallels the same disease in humans was available for therapeutic trials.

Therefore the aim of this study was to evaluate the efficacy of ERT with recombinant human N-acetylgalactosamine-4-sulphatase (rh4S) in preventing development of soft tissue and skeletal disease in the feline model of MPS VI.

The following review introduces the general field of lysosomal storage diseases (LSDs) and specifically the MPSs, and reviews the biochemical basis, diagnosis and clinical description of MPS type VI. A description of animal models of LSDs including feline MPS VI, and their use in developing new therapies is also presented, as well as a review of historical, current and future therapies for use in MPS and LSD patients. The review is generally limited to studies reported up to 1993, with more recent reports specific to the work in this thesis included in the discussions in the appropriate chapters.

1.2 Lysosomes and lysosomal storage diseases

Lysosomes are acidified single-membrane bound intracellular organelles containing hydrolytic enzymes (Hopwood and Brooks, 1997). They are the “terminal degradative compartment” (Kornfeld and Mellman, 1989), digesting material obtained intracellularly and extracellularly to a sufficient degree to enable incorporation of components into other biosynthetic pathways or transport out of the cell. Deficiency of one or more of these lysosomal enzymes leads to accumulation of undegraded material within the lysosomes, with subsequent cellular dysfunction. These diseases are termed lysosomal storage diseases (LSDs), with deficiencies of specific enzymes leading to accumulation of specific substrates, both of which form the basis for diagnosis of these disorders. LSDs are genetically inherited with at least 40 currently known disorders found in humans (Hopwood and Brooks, 1997). Several LSDs can also be induced by ingestion of natural and synthetic compounds leading to inhibition of specific enzymes. The trypanocidal drug suramin (Constantopoulos *et al.*, 1980, 1983; Rees *et al.*, 1982) and the immunomodulatory drug tilorone (Luellmann, 1983; Prokopek, 1991) both produce mucopolysaccharidosis-like changes when administered to rats. The natural compound swainsonine found in plants from the genus *Swainsona* causes α -mannosidosis in grazing livestock (Dorling *et al.*, 1978).

Disease characteristics of LSDs vary widely and relate to substrate concentrations in each tissue and their ability to disrupt normal cell and subsequently organ function. Disease severity is also dependent on mutations present in the defective protein, and whether these mutations result in small amounts of protein and residual enzyme activity (Brooks *et al.*, 1991b; Litjens *et al.*, 1996).

1.3 The mucopolysaccharidoses

The mucopolysaccharidoses (MPSs) are a group of 10 LSDs characterised by the inability to degrade glycosaminoglycans (GAGs), previously termed “mucopolysaccharides” (Table 1.1; Whitley, 1993). Clinical phenotype observed within each MPS type varies from relatively mild to extremely severe. The two major sites of pathology in the MPSs are the central nervous system (CNS) and the skeletal system, causing mental retardation and dwarfism respectively. Each MPS type exhibits one or both of these disease outcomes (Table 1.1). Additional disease features found in several of the MPSs includes cardiac valve dysfunction, corneal clouding, joint contractures and hepatosplenomegaly (Whitley, 1993). Children are commonly diagnosed within the first few years of life and disease may lead to their death often before their 10th year (Neufeld and Muenzer, 1995). The overall incidence of LSDs in Australia is estimated to be ~1:7 800 births, with the incidence in the MPSs being 1:22 500 births (Meikle *et al.*, 1998). All MPS types have an autosomal recessive mode of inheritance, except MPS II which is X-linked. An historical review of the identification and characterisation of these disorders is provided by Whitley (1993).

Table 1.1: Classification of the mucopolysaccharidoses*.

MPS type	Enzyme deficiency	Stored substrates [†]	Eponym	Major clinical features [‡]	
I	α -L-iduronidase	DS, HS	Hurler	M [§]	S [¶]
			Hurler/Scheie	$\pm$ M	S
			Scheie	-	$\pm$ S
II	Iduronate 2-sulphatase	DS, HS	Hunter	M	S
IIIA	Sulphamidase	HS	Sanfilippo	M	-
IIIB	α -N-acetylglucosaminidase	HS	Sanfilippo	M	-
IIIC	Acetyl-CoA: α -glucosaminide N-acetyltransferase	HS	Sanfilippo	M	-
IIID	Glucosamine-6-sulphatase	HS	Sanfilippo	M	-
IVA	Galactose-6-sulphatase	KS	Morquio	-	S
IVB	β -D-galactosidase	KS	Morquio	-	S
VI	N-acetylgalactosamine-4-sulphatase	DS	Maroteaux-Lamy	-	S
VII	β -D-glucuronidase	DS, HS, CS	Sly	M	S

* from Hopwood and Morris, 1990; † DS, dermatan sulphate; HS, heparan sulphate; KS, keratan sulphate, CS, chondroitin sulphate;

‡ within each MPS type, disease severity is variable, usually correlating with age at diagnosis and longevity. Historically in MPS I only, these variable phenotypes were given different eponyms which continue to be used;

§ M, mental retardation; ¶ S, skeletal deformities; -, absent;

1.4 Glycosaminoglycans and proteoglycans

Proteoglycans and collagen are the major structural elements of connective tissues. Proteoglycans are a very diverse group of glycosylated proteins which are composed of one or more highly negatively charged GAG chains of varying lengths covalently linked to a protein core (Yanagishita, 1993). GAGs consist of repeating disaccharide units and are the major carbohydrate component of proteoglycans. There are three main groups of GAGs, classified on the basis of their monosaccharide components. Several reviews detail the specific structure of each GAG type (Roden, 1980; Hopwood and Morris, 1990; Yanagishita, 1993). Dermatan sulphate (DS) and chondroitin sulphate (CS) consist of alternating galactosamine and glucuronic residues, with DS containing variable amounts of iduronic rather than glucuronic residues alone. Heparan sulphate (HS) consists of alternating glucosamine and glucuronic or iduronic residues. Lastly, keratan sulphate (KS) contains glucosamine and galactose residues, but no uronic components. Depending on GAG type, variable sulphation positions of these monosaccharide units, such as C2 on the uronic acid residues, and C3, C4 and C6 on hexosamine residues, together with N-acetylation or N-sulphation of glucosamine residues in HS, add to further possible variations.

Following initial proteolysis of the proteoglycans to release single GAG chains, the degradative pathways of each of these GAG types require the sequential action of specific exoglycosidases and exosulphatases within lysosomes to produce inorganic sulphate and monosaccharides (Hopwood and Morris, 1990). These lysosomal enzymes are required for the hydrolysis of sulphate esters or glycosidic bonds from the non-reducing end of the GAG chain (Figure 1.1)(Roden, 1980; Hopwood and Morris, 1990; Neufeld and Muenzer, 1995). A deficiency of any one of these enzymes acting on the non-reducing end of the GAG chain prevents further action by the other enzymes in the degradative pathway, and leads to accumulation of the undegraded substrate within lysosomes. The MPS disorders (MPS I, II, VI and VII) arising from the corresponding enzyme deficiencies leading to DS accumulation, are indicated in Figure 1.1. There are also several known endoglycosidases which may be able to hydrolyse linkages within the GAG chains, and therefore potentially bypass a block in degradation, however it is not known to what degree they contribute to

GAG degradation. These endoglycosidases include hyaluronidase and heparanase which are both known to hydrolyse internal β -glucuronide residues (Hopwood, 1989). In the case of hyaluronidase, activity may only be present in some tissues (see Neufeld and Muenzer, 1995). Due to the elevated levels of the monosaccharides N-acetylgalactosamine-4-sulphate and N-acetylgalactosamine-4,6-disulphate found in the urine from MPS VI patients, an alternative pathway for DS degradation via the action of β -hexosaminidase has been proposed (Figure 1.1)(Hopwood and Elliott, 1985). It is unknown to what degree this pathway may contribute to DS degradation, however the lack of expected excessive chondroitin-4-sulphate accumulation in MPS VI and VII may be attributable to the action of both hyaluronidase and β -hexosaminidase (Roden, 1980; Neufeld and Muenzer, 1995).

The heterogeneous functions of proteoglycans is reflected in their variable structures and locations, with some proteoglycans only present in specific tissues, while others are more widely distributed (Yanagishita, 1993). For example, impaired KS degradation in MPS IV A and B results in characteristic skeletal deformities, distinct from those observed in other MPS types. Aggrecan, the major cartilage proteoglycan, contains up to 200 CS plus KS side chains attached to a central core protein, which itself interacts with hyaluronic acid and a link protein to form large proteoglycan aggregates within the cartilage matrix (Roden, 1980). The heavily sulphated GAG chains result in a highly negatively charged structure which attracts and holds water molecules. This gives cartilage the ability to resist compression and other mechanical stresses (Yanagishita, 1993).

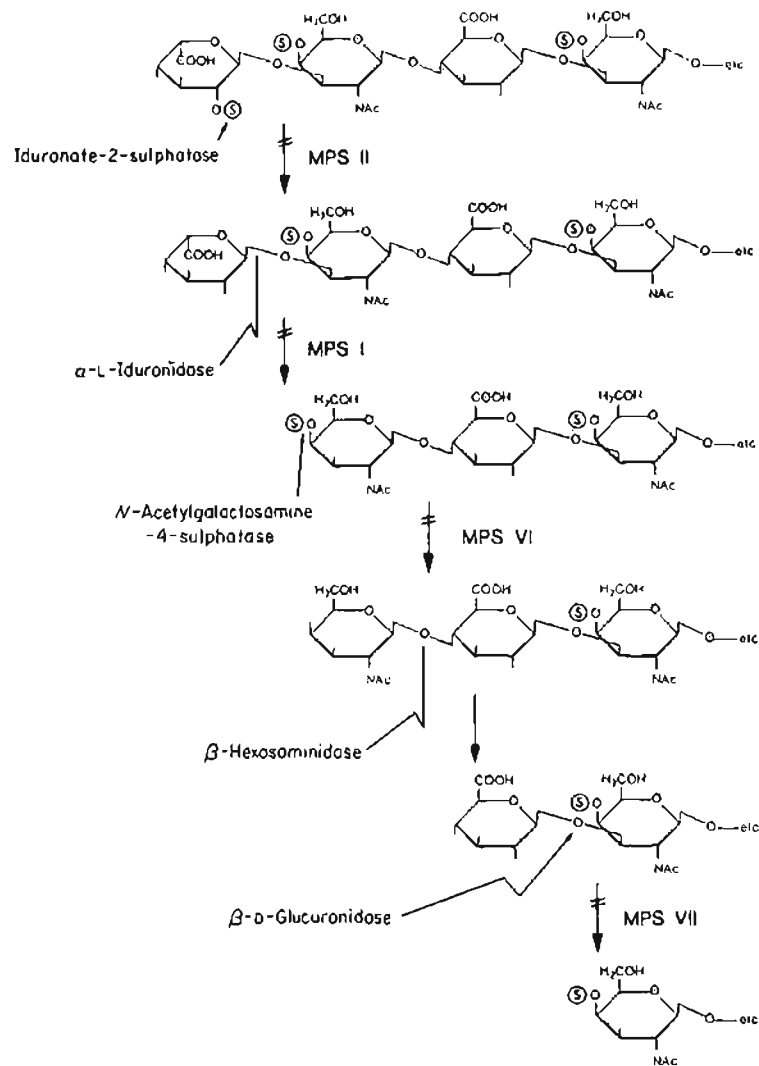
Decorin is a small proteoglycan containing only one CS or DS chain and is found in most extracellular matrices (Yanagishita, 1993). Decorin has been shown to inhibit both type I and type II collagen fibrillogenesis in vitro (Vogel *et al.*, 1984; Vogel and Trotter, 1987). It also has a close association with type I and type II collagen in many connective tissues (Bianco *et al.*, 1990) including cartilage. It has been hypothesised that decorin may play a role in negative feedback control of transforming growth factor- β (TGF- β) (Yamaguchi *et al.*, 1990). As well as decorin, another small DS containing proteoglycan biglycan, has been associated with the growth plate and newly deposited osteoid (Poole *et al.*, 1986; Bianco *et al.*, 1990), however their functions are still unclear.

The main clinical features in each of the MPS types reflects the location and sometimes function of each of the GAG species associated with different proteoglycans. However the role of undegraded GAG in the exact pathogenesis of the disease characteristics in the MPSs has yet to be fully elucidated.

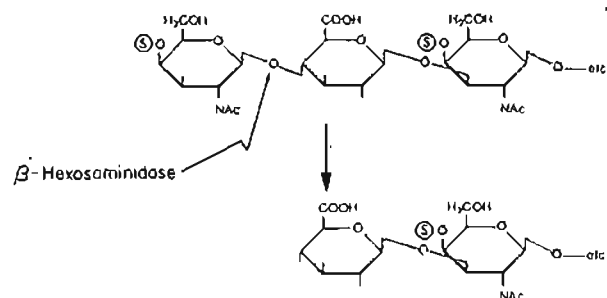
Figure 1.1: Stepwise degradation of dermatan sulphate.

(from Hopwood and Morris, 1990)

Diagrammatic representation of the stepwise degradation of DS oligosaccharides in the lysosome by five exoenzymes. An inability to degrade components of the DS chain due to defective activity of any one of these enzymes results in a specific MPS disorder, indicated alongside the broken arrows. Shown also is an alternate pathway of DS degradation which has been proposed via the action of β -hexosaminidase A or S.



Alternate pathway



1.5 Bone growth

The growth plate is the site at which transformation of cartilage into bone takes place, resulting in longitudinal bone growth, otherwise known as endochondral ossification. Growth plate structure and biology has been reviewed by Howell & Dean (1992). A complex sequence of events involving changes in cell organisation and matrix structure enable mineralisation of the lower regions of the growth plate. This process is not yet completely understood. Resting chondrocytes at the top of the growth plate proliferate, divide and form columns of cells (proliferative zone). This is followed by hypertrophy of these chondrocytes (hypertrophic zone) with vascular invasion and mineralisation of the cartilage septae present between the columns of cells in the lower hypertrophic zone. Osteogenic cells deposit a layer of osteoid on these calcified cartilage septae to form the trabeculae in the primary spongiosa, which are then remodelled to form mature bone.

A number of changes in the composition of the growth plate matrix are known to occur, however how these influence the process of endochondral ossification is not understood. Changes in collagen types occurs within the growth plate, with type II, IX and XI collagens found distributed throughout the cartilage matrix of the growth plate, and type X collagen only found in the regions of the hypertrophic zone undergoing calcification. Type I collagen is found in bone trabeculae emerging from the growth plate, and it is the predominant collagen type found in bone (Howell and Dean, 1992). Aggrecan structure and sulphation pattern is also known to change (Byers *et al.*, 1997b), and certain non-collagenous proteins are expressed by hypertrophic chondrocytes (Howell and Dean, 1992).

Any disruption to the cartilage or new bone matrix structure may impair matrix mineralisation leading to abnormal bone growth. For example, defects in type I and II collagen synthesis can lead to severe skeletal abnormalities (Jacenko *et al.*, 1994). Defects in degradation of other matrix components including those with low abundance can also lead to skeletal abnormalities as seen in some LSDs. The common feature of MPS types I, II VI and VII is that they are all unable to degrade the GAG DS, and all have similar skeletal abnormalities to each other. Impaired KS metabolism in MPS IV results in a different pattern of skeletal disease. Some disorders of oligosaccharide degradation also

have a similar skeletal appearance to the MPS disorders, such as mannosidosis, sialidosis (ML I), galactosialidosis and aspartylglycosaminuria (Whitley, 1993). Skeletal dysplasia may be due to direct effects of the undegraded material or secondary storage products on growth plate processes. Alternatively, massive accumulation of storage material within the lysosomes may impair lysosomal function involved in other processes in the growth plate. Defects in synthesis of matrix components may also result in skeletal abnormalities. For example, undersulphation of GAG components within the cartilage matrix has been observed in mice exhibiting disproportionate short stature (Orkin *et al.*, 1976, 1977, Schwartz *et al.*, 1978) and in a human neonate with lethal short-limbed chondrodysplasia (Superti-Furga, 1994).

1.6 Lysosomal enzyme synthesis and processing

The historical observations by Frattantoni, Hall and Neufeld (1968, 1969) were fundamental to the development of a new understanding of lysosomal enzyme trafficking and targeting. Co-culture of MPS I and MPS II fibroblasts led to cross-correction of storage due to endocytosis of “corrective factors” later understood to be lysosomal enzymes. It also became apparent that cell surface receptors mediated the lysosomal enzyme uptake due to mannose-6-phosphate (M6P) residues attached to the protein, and that these receptors also mediated intracellular transport of lysosomal enzymes (Sly *et al.*, 1981). These observations were fundamental to the concept of replacing the defective enzyme in LSDs with an external source of normal purified natural or artificial enzyme, otherwise termed enzyme replacement therapy (ERT).

The main features of lysosomal enzyme synthesis and intracellular transport are well known, have been widely reviewed, and are summarised below (also see Figure 1.2)(Sly *et al.*, 1981; von Figura and Hasilik, 1986; Kornfeld, 1987; Dahms *et al.*, 1989; Neufeld, 1991; Pfeffer, 1991). Briefly, lysosomal enzyme and secretory protein synthesis takes place in the rough endoplasmic reticulum (RER), with glycosylation of selected asparagine residues. Synthesis is followed by post-translational modifications in the Golgi apparatus, which includes phosphorylation of the mannose residues on lysosomal enzymes. These residues have a high affinity for M6P receptors (M6PR) present in the Golgi and this selectively

targets soluble lysosomal enzymes to the lysosomes, via the prelysosomal compartment. Acidification of this compartment leads to dissociation of the enzyme from the M6PR followed by delivery to lysosomes. The M6PR are then recycled back to the Golgi. A small proportion of the lysosomal enzymes fail to bind to the M6PR in the Golgi, and are secreted to the cell surface, together with other secretory proteins that have alternative oligosaccharide residues, such as sialic acid. Endocytosis of the secreted lysosomal enzymes can occur via the cell surface M6PR, hence the original observations of cross correction of MPS I and II cell storage with used culture medium, or with cells from a different MPS type (Fratantoni *et al.*, 1968, 1969). The presence of M6P independent transport mechanisms of soluble lysosomal enzymes are also recognised. A clear example of this is almost normal intracellular levels of lysosomal enzymes in some cell types in Mucopolipidosis type II and III, a disease in which defective phosphorylation of mannose residues results in a lack of M6P residues on lysosomal enzymes (Glickman and Kornfeld, 1993). Alternative signals have not yet been identified.

Addition of weak bases to culture medium, such as ammonium chloride, is observed to elevate the pH of the acidic compartments and inhibits dissociation of the M6PR from the enzymes. This stops recycling and availability of vacant receptors (von Figura and Hasilik, 1986) and leads to increased secretion of the lysosomal enzymes to the cell surface. This mechanism can be exploited in enzyme expression systems *in vitro*, to produce high levels of recombinant enzyme in culture medium.

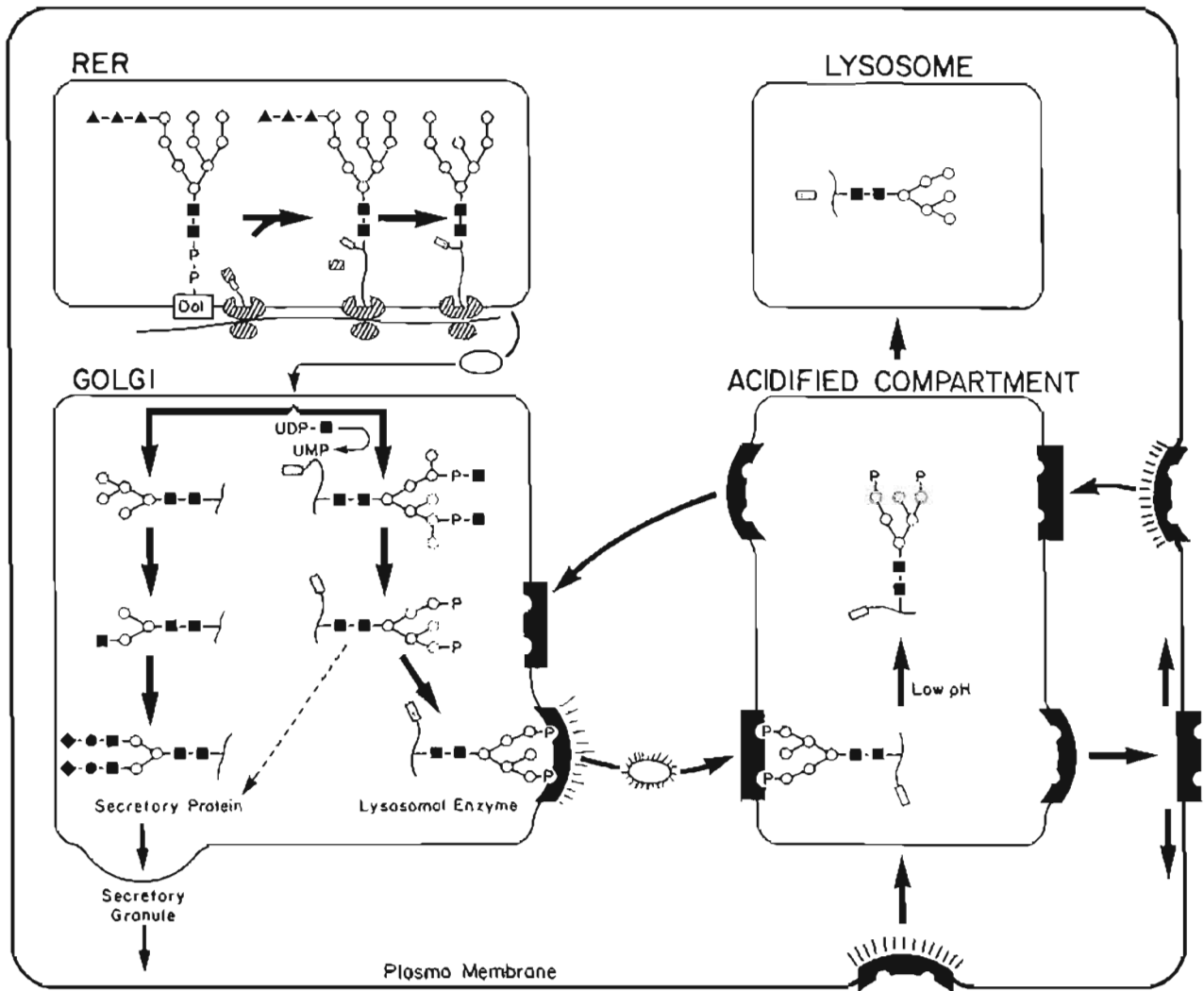
Two distinct M6PR are known: the 300 kDa cation-independent receptor (CI-M6PR), and the 46 kDa cation-dependent receptor (CD-M6PR) (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989; Pfeffer, 1991). In fibroblasts lacking both receptors, it was apparent that both receptors were required for efficient intracellular targeting of lysosomal enzymes and each receptor may interact with different subgroups of lysosomal enzymes (Ludwig *et al.*, 1994). However only the CI-M6PR mediates endocytosis at the plasma membrane. The CI-M6PR is also the receptor for insulin-like growth factor II (Kornfeld, 1992). Moderate elevations in serum lysosomal enzyme levels and abnormal endosomal/lysosomal inclusions due to missorting of lysosomal enzymes were observed in one of two CD-M6PR deficient mouse models reported concurrently (Ludwig *et al.*, 1993). However these mice were

phenotypically normal, indicating the presence of compensatory mechanisms *in vivo* (Koster *et al.*, 1993; Ludwig *et al.*, 1993). Other carbohydrate specific receptors such as mannose-specific and galactose-specific receptors are also present on the cell surface, and some lysosomal enzymes can bind to more than one of these receptors. These alternative receptors can compensate for lysosomal enzyme endocytosis *in vivo* if several receptors are blocked with specific inhibitors (Koster *et al.*, 1994).

Once lysosomal enzymes reach the lysosome, exoglycosidases further trim the carbohydrate residues, which may include removal of the M6P residue. Some proteolysis may also occur, resulting in reduced molecular weight on polyacrylamide gel electrophoresis. Hence the terms “precursor” or “high-uptake,” and “mature” are applied to these two different enzyme forms. These changes are not required for enzyme activation of most lysosomal enzymes (Neufeld, 1991).

Figure 1.2: Lysosomal enzyme synthesis and targeting to lysosomes.

Diagram of the pathway of lysosomal enzyme synthesis and enzyme targeting to the lysosome. See text for explanation of details. RER denotes rough endoplasmic reticulum, mannose-6-phosphate receptors (M6PR) , N-acetylglucosamine (■), mannose (O), glucose (▲), galactose (●), sialic acid (◆), (Kornfeld, 1987).



1.7 4S characterisation, production and purification

The lysosomal enzyme N-acetylgalactosamine-4-sulphatase (4S)(EC 3.1.6.1)(also known as arylsulphatase B) removes the 4-sulphate groups present on N-acetylgalactosamine units in the stepwise degradation of DS and CS (Hopwood and Morris, 1990). A deficiency of 4S leads to accumulation of DS resulting in MPS VI.

4S was originally purified and characterised from human liver (McGovern *et al.*, 1982; Gibson *et al.*, 1987). 4S was found to be present as a 66 kDa precursor which was processed to a 58 kDa mature form composed of 43, 7 and 8 kDa disulphide linked polypeptides (Taylor *et al.*, 1990; Kobayashi *et al.*, 1992). The three dimensional 4S crystal structure has recently been elucidated, which has improved understanding of functional aspects and the active site of 4S and other sulphatases (Bond *et al.*, 1997).

Following the isolation and purification of human 4S, the gene was mapped to the chromosomal region 5q13-q14 (Litjens *et al.*, 1989) and a full length 4S cDNA clone was isolated (Peters *et al.*, 1990; Schuchman *et al.*, 1990). Anson *et al.* (1992) stably transfected Chinese Hamster Ovary (CHO) cells, achieving high levels of expression, which enabled large amounts of human recombinant 4S (rh4S) to be produced in cell culture. A monoclonal antibody column enabled a high degree of purification of rh4S from the collected medium (Anson *et al.*, 1992). M6PR were also shown to mediate uptake of rh4S in fibroblasts, by inhibition studies with M6P, and rh4S was able to degrade stored DS in human MPS VI fibroblasts (Anson *et al.*, 1992). The synthesis and maturation of rh4S from CHO cells was identical to that seen for 4S in normal human fibroblasts, however the rh4S had a significantly shorter half-life in fibroblasts of ~3.5 days than that of endogenous human 4S (9-17 days) (Taylor *et al.*, 1990; Anson *et al.*, 1992). No comment was made by the authors giving a reason for this difference.

1.8 Human MPS VI

MPS VI or Maroteaux-Lamy syndrome was first described by Maroteaux and colleagues in 1963 (Maroteaux *et al.*, 1963), and is characterised by a deficiency 4S. This leads to

impaired degradation of DS (see Section 1.4 above). MPS VI is one of the less common MPSs, with an incidence estimated to be in the order of 1:235 000 births in Australia (Meikle *et al.*, 1998).

1.8.1 Diagnosis

Initial screening detection and then diagnosis of MPS VI and indeed all of the MPS disorders is based on identification of elevated levels of specific GAG species within the urine, and then deficiency of specific enzyme activities in peripheral leukocytes and cultured fibroblasts (Hopwood and Morris, 1990). A variety of methods have been used to perform these tests, some of which have inherent problems or inaccuracies. The most commonly used methods for routine analysis of large numbers of samples will therefore be reviewed briefly.

1.8.1.1 Urinalysis

Three broad categories of laboratory methods for routine analysis of urine samples are used in the diagnosis of MPS disorders: a semi-quantitative screening test, total GAG quantitation, and qualitative identification of the type of GAG excreted. Methods are outlined in extensive reviews by Pennock (1976) and Whitley (1993). An additional review of some other methods is also available (Kodama *et al.*, 1988).

A major factor to take into consideration with semi-quantitative and quantitative methods is that both urine creatinine concentration and urinary GAG excretion vary with age, particularly within the first few years of life (Pennock *et al.*, 1971, 1973; Taniguchi, 1972; Applegarth and Ross, 1975). This makes it imperative that reference ranges for total GAG levels are within specified age ranges. Fluctuation in urinary GAG excretion and creatinine concentration also varies within a 24 hour period, however if these values are calculated from a 24 hour sample collection then the ratio of these two parameters remains relatively constant (Di Ferrante and Lipscomb, 1970). Despite this, "spot" urine samples have been used as a screening method in infants, with results from MPS patients consistently higher than age-matched normal controls (Whitley *et al.*, 1989b).

Some screening tests based on GAG levels in spot urine have in general lost favour due to the high numbers of false positives and negatives in some laboratories (see table, pg 457 in Whitley, 1993). These tests include the Berry spot test, the acid albumin turbidity test, and the cetylpyridinium chloride citrate (CPC) turbidity test (refer to references in Whitley, 1993). Using various cationic dyes, direct spectrophotometric measurement of cationic dye-GAG complex formation is more quantitative. Dyes include Alcian Blue (Gold, 1979), dimethylmethylen blue (Whitley *et al.*, 1989a-b), Stainsall (Homer *et al.*, 1993) and Azure A and B (Thuy and Nyhan, 1992). Instability of the dye-GAG complex can sometimes occur due to high ionic strength (Whiteman, 1973; Gold, 1979), however this can be overcome by diluting the sample. An alternative method of quantitation is CPC precipitation of GAG (Di Ferrante, 1967; Pennock, 1976) followed by uronic acid quantitation (Bitter and Muir, 1962; Blumenkrantz and Asboe-Hansen, 1973). One disadvantage of this method is that no uronic residues are present in keratan sulphate which is the GAG which accumulates in MPS IV (Morquio syndrome)(Table 1.1). CPC precipitation also does not precipitate GAG below approximately 3000 Da (Hopwood and Harrison, 1982), however one method compared ratios of high and low molecular weight GAGs by applying CPC supernatants to anion exchange columns, and this was considered to be of some diagnostic benefit although very time consuming (Di Ferrante *et al.*, 1972).

Qualitative examination of urine GAG species by electrophoretic separation is of the greatest diagnostic value as it indicates which GAG species are present in abnormal amounts and hence, which lysosomal enzymes are possibly deficient (refer to Table 1.1). Several methods have been developed allowing separation and identification of GAG species (Schuchman and Desnick, 1981; Hopwood and Harrison, 1982; Juretic *et al.*, 1991). Even if total urine GAG has been within normal limits, if an MPS disorder is suspected, electrophoresis of urine GAG is important. Total urine GAG within the normal reference range is considered to be a common problem in Sanfilippo (MPS III) and Morquio (MPS IV) patients (Whitley, 1993), and normal total urine GAG has also been observed in less severely affected MPS VI patients. However upon electrophoresis of urine samples, DSuria was present in these patients, confirming the diagnosis of MPS VI and stressing the importance of qualitative urinalysis to achieve diagnosis (Quigley and Kenyon, 1974; Paterson *et al.*, 1982; Tonnesen *et al.*, 1991). The level of DSuria in MPS VI patients

also correlates approximately with clinical phenotype (Hopwood and Morris, 1990). Estimations of the amount of stained GAG on the cellulose acetate strips can be determined using densitometric scanning, or elution of the GAG-dye complex from the strips followed by spectrophotometric quantitation (Hsu *et al.*, 1972; Hronowski and Anastassiades, 1979; Hopwood and Harrison, 1982).

1.8.1.2 Enzymology

An enzymatic diagnosis of MPS VI is based on the ability to demonstrate a deficiency of 4S, usually in peripheral leukocytes or cultured fibroblasts. MPS VI patients have less than approximately 10% residual 4S activity compared with normal controls (Hopwood *et al.*, 1986; Jin *et al.*, 1992; Litjens *et al.*, 1996). Normal levels of other sulphatases rules out a diagnosis of multiple sulphatase deficiency.

Two synthetic substrates, p-nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) and 4-methylumbelliferyl sulphate (4MUS) have been used to assay 4S activity. However other sulphatases (mainly arylsulphatase A and C) also show high reactivity toward these substrates. Hence separation from these contaminating arylsulphatases by chromatography or electrophoresis, or addition of inhibitors of these other enzymes has been used to improve specificity of the 4S assay (reviewed by McGovern *et al.*, 1981; Hopwood *et al.*, 1986; Brooks *et al.*, 1991a). Use of an artificial radiolabelled trisaccharide substrate which closely resembles the natural substrate has been developed and is considered highly specific for 4S activity (Hopwood *et al.*, 1986). More recently, an alternative method of separating 4S from contaminating sulphatases, using immune capture of 4S with a specific monoclonal antibody together with 4MUS substrate has been developed (Brooks *et al.*, 1991a; Brooks, 1993).

Recent studies have demonstrated some correlation between 4S activity, 4S protein, genotype and MPS VI clinical phenotype (Brooks *et al.*, 1991b; Wicker *et al.*, 1991; Litjens *et al.*, 1996), however in another report, 4S activity and clinical phenotype were not correlated (Tonnesen *et al.*, 1991).

Theoretically, MPS VI heterozygotes have half the normal levels of enzyme activity in leukocytes, plasma or cultured fibroblasts, however in most instances a wide overlap is observed between enzyme activity ranges from heterozygotes and normal controls, making carrier detection in individuals difficult (Hopwood and Morris, 1990). Ratios of 4S to arylsulphatase A activities in MPS VI humans (and cats) provided accurate separation of obligate heterozygotes from normal controls, although the lower end of the ratio range observed in obligate heterozygote cats was very close to the highest values obtained from some MPS VI homozygote cats (McGovern *et al.*, 1981). Where mutations are known within a particular family, detection of mutations provides an accurate means of heterozygote detection (Hopwood and Morris, 1990).

1.8.2 Clinical description

The major clinical feature of MPS VI is dwarfism with progressive skeletal abnormalities. In severely affected MPS VI patients, growth is retarded by about 4 years and ceases by about 8 years of age (Whitley, 1993; Neufeld and Muenzer, 1995). Patients exhibit a characteristic facial dysmorphism with progressive corneal opacity which can lead to blindness (Litjens *et al.*, 1992). Progressive joint contractures of shoulders, elbows, fingers, hips and knees are severely incapacitating and lead to claw hands, a semi-crouched stance and eventual inability to walk. Contractures have been observed as early as 6 months of age (Litjens *et al.*, 1992). Cardiopulmonary disease is progressive, and commonly causes death in late childhood, often in association with surgical procedures (Whitley, 1993). Patients lack CNS involvement which is in contrast to most of the other mucopolysaccharidoses where patients show progressive neurological deterioration (see Table 1.1).

A spectrum of disease severity is observed in MPS VI, with less severely affected patients having increased longevity and exhibiting milder skeletal and soft tissue abnormalities. Some patients almost reach normal heights (Quigley and Kenyon, 1974; Pilz *et al.*, 1979; Wilson *et al.*, 1980; Paterson *et al.*, 1982), however these patients still have significant and often similar problems to more severe patients which also require surgical intervention. At least 20 individual disease causing mutations in the 4S gene have now been identified, contributing to the variable clinical phenotype observed in MPS VI (Wicker *et al.*, 1991; Jin

et al., 1992; Litjens *et al.*, 1992; Arlt *et al.*, 1994; Isbrandt *et al.*, 1994; Voskoboeva *et al.*, 1994; Simonaro and Schuchman, 1995; Litjens *et al.*, 1996). The following disease features described in more detail are not found in all MPS VI patients, and also generally vary in the degree of severity.

1.8.2.1 Skeletal

The very general term “dysostosis multiplex” has been used to describe the widespread skeletal changes in a number of MPS disorders including MPS VI (Eggli and Dorst, 1986). The following is a summary of characteristic radiographic changes in MPS VI which have been reviewed elsewhere (Spranger *et al.*, 1970; Eggli and Dorst, 1986; Whitley, 1993). The skull is usually enlarged with a J-shaped sella turcica. The odontoid process is hypoplastic, and anterior hypoplasia of lumbar vertebrae is often observed. Lumbar kyphosis is sometimes present (Keller *et al.*, 1987; Litjens *et al.*, 1992). The ribs are abnormally shaped and very obvious changes are observed in the pelvis. This includes small flared iliac wings with constriction of the iliac bodies, and small acetabulae. Irregular ossification of the proximal femoral epiphyses sometimes leads to a misdiagnosis of bilateral Perthes disease. Diaphyseal distension of tubular bones with constriction at the metaphysis is also observed, particularly in the humerus and femur. Radiographic changes in the hands and feet are very variable.

1.8.2.2 Neurological

The major neurological problems in MPS VI patients are spinal cord compression and carpal tunnel syndrome due to nerve entrapment (Whitley, 1993; Haddad *et al.*, 1997). Additional neurological complications include hydrocephalus and arachnoid cysts (Watts *et al.*, 1981). The upper cervical spine is the most common site of spinal cord compression resulting in paresis or tetraparesis. Compression is most commonly caused by dural thickening (Wald and Schmidek, 1984), however thickening of the vertebral ligaments in the same region has also been demonstrated at postmortem (Keller *et al.*, 1987). A number of different cases have been reviewed by Wald & Schmidek (1984). Bony compression in the thoracolumbar region has also been reported (Wald and Schmidek, 1984). Decompression and excision of the cervical dura has lead to improved neurological function (Young *et al.*, 1980).

Mental retardation is not normally observed in MPS VI, although it has been reported rarely (Taylor *et al.*, 1978; Vestermarck *et al.*, 1987). However, in both reports, several close relatives without MPS VI also exhibited mental retardation, suggesting additional factors may have caused the mental retardation.

1.8.2.3 Cardiac

Cardiac insufficiency due to valve thickening and stenosis is a major cause of mortality in MPS VI patients. The mitral and aortic valves are usually the most severely affected, necessitating valve replacements in some patients (Wilson *et al.*, 1980; Tan *et al.*, 1992; Wippermann *et al.*, 1995). Tricuspid valve thickening has also been reported in several severely affected patients at postmortem (Spranger *et al.*, 1970; Keller *et al.*, 1987). The initial presenting complaint in several MPS VI patients at less than 1 year of age has been acute cardiomyopathy with endocardial fibroelastosis (Miller and Partridge, 1983; Fong *et al.*, 1987; Hayflick *et al.*, 1992). Endocardial fibrosis has also been observed in an adult MPS VI patient at postmortem (Keller *et al.*, 1987).

1.8.2.4 Respiratory

Tracheal narrowing due to GAG deposition in tracheal and pharyngeal connective tissue is a major cause of anaesthetic complications in MPS patients (Peters *et al.*, 1985; Semenza and Pyeritz, 1988). In addition, the shortened neck in more severely affected patients contributes significantly to respiratory compromise and also to the difficulty of intubation at anaesthesia. Autopsy of a severe MPS VI patient demonstrated severe stenosis of the trachea (Keller *et al.*, 1987), and severe retropharyngeal and retrotracheal swelling had also been described previously in the same patient (Rampini *et al.*, 1986).

1.8.2.5 Other

Other features observed in MPS VI include hepatosplenomegaly, thickened skin, hernias, recurrent otitis media, hearing loss, glaucoma, delayed tooth eruption, multiple dentigerous cysts and gingival hyperplasia (Spranger *et al.*, 1970; Roberts *et al.*, 1984; Whitley, 1993; Neufeld and Muenzer, 1995; Smith *et al.*, 1995).

A useful diagnostic feature of MPS VI is the presence of excessive neutrophil granulation (Alder-Reilly bodies) in peripheral blood smears, with abnormal vacuoles or granules in a smaller number of lymphocytes. Coarse and fine granulation was observed in 90-100% of granulocytes and in 1-50% of lymphocytes (Spranger *et al.*, 1970), and these inclusions stain metachromatically with toluidine blue (Haskins *et al.*, 1979b). Similar prominent inclusions are also observed in neutrophils and other granulocytes in MPS VII patients, however these were found to be negative or weakly metachromatic with toluidine blue in one report (Peterson *et al.*, 1982), while all granulocytes stained positively in another report (Gitzelmann *et al.*, 1978). The presence of metachromatic inclusions in MPS I, II III and IV lymphocytes received a lot of attention in the literature (Belcher, 1972; summarised by Murata *et al.*, 1977) however their low incidence in some patients precludes this as a useful screening method (Ikeda *et al.*, 1982).

1.8.3 Current management of MPS VI patients

Aside from BMT (see Section 1.12), current management of MPS VI patients involves invasive procedures to alleviate progressive skeletal and soft tissue pathologies. Orthopaedic procedures include cervical spine decompression to alleviate dural thickening, cervical vertebral fusion to correct vertebral instability, and bilateral femoral osteotomies to relieve flexion deformities of the hips (Litjens *et al.*, 1992). Bilateral hip replacements have also been performed (Pilz *et al.*, 1979). Other procedures include placement of ventriculoperitoneal shunts to alleviate hydrocephalus (Rampini *et al.*, 1986; Litjens *et al.*, 1992), aortic and mitral valve replacements (Tan *et al.*, 1992), corneal transplants (Schwartz *et al.*, 1985), tonsillectomy and adenoidectomy to alleviate upper airway obstruction (Litjens *et al.*, 1992), and intervention with dental and ear problems. Severely affected patients undergo most of these procedures in their short life-times.

1.8.4 Histological features

Histologically, membrane bound lysosomal inclusions are observed in many cell types, predominantly those of mesenchymal origin. Cell types include fibroblasts (including those from skin, conjunctiva, heart valve and dura), Schwann cells, corneal keratocytes, corneal

epithelium and endothelium, histiocytes, hepatocytes, Kupffer cells, and chondrocytes (Quigley and Kenyon, 1974; Pilz *et al.*, 1979; Suveges, 1979; Young *et al.*, 1980; Schwartz *et al.*, 1985; Keller *et al.*, 1987; Tan *et al.*, 1992; Resnick *et al.*, 1994). Abnormal inclusions have also been observed in lymphocytes, neutrophils, monocytes and platelets (Levy *et al.*, 1980). Inclusions are mainly empty due to the highly water soluble GAG contents being extracted during routine fixation and processing, however inclusions may contain fibrillogranular material or lamellar/myelin-like material. The lipid-like inclusions suggest secondary inhibition of other lysosomal enzymes presumably caused by excessive GAG. Inhibition of lysosomal enzymes by GAGs has been recognised for some time, and GAG inhibition of ganglioside-specific neuraminidase activity in cultured fibroblasts has been demonstrated (see Baumkotter and Cantz, 1983).

No descriptions of growth plate morphology in human MPS VI were found in the published literature, however a brief description of findings in other MPS types is of interest because of the similar pattern of skeletal disease in most of the MPS types. In several reports describing the histological appearance of growth plates in MPS I (one patient diagnosed on clinical grounds only) and in MPS IV, abnormal or reduced numbers of trabeculae were observed emerging from the growth plate (Strauss, 1948; McClure *et al.*, 1986; Silveri *et al.*, 1991). These and some other reports (Bona *et al.*, 1966; probable MPS I also) describe absent or disordered columnar architecture of chondrocytes within the growth plate, however another report observed fairly normal growth plate organisation intermittently disrupted by large areas of loose connective tissue (MPS I and IV)(Rimoin *et al.*, 1974). Variable observations may reflect the heterogeneity observed both within and between MPS types, and also the different growth plates examined. For a review of findings in MPS IV (Morquio) growth plates see McClure *et al.* (1986). Most also commented on the reduced calcification zone at the bottom of the growth plate (in MPS I and IV), however this was not a consistent observation.

1.8.5 Pathogenesis of MPS VI disease

The location of major sites of pathology in MPS disorders such as the CNS and skeletal system merely indicates where undegraded GAG has affected normal cell and organ function. However virtually nothing is understood about the mechanisms of these processes.

In general connective tissues, abnormal intracellular and presumably extracellular concentrations of GAG may disrupt normal biological processes. Evidence of this suggested by the excessive fibrosis observed in some MPS types such as coronary artery intimal thickening (MPS I) and general thickening of the endocardium and heart valves (MPS I, MPS VI) (Renteria *et al.*, 1976; Brosius and Roberts, 1981; Keller *et al.*, 1987), and hepatic fibrosis reported in MPS I, II and III (Parfrey and Hutchins, 1986). In some cases such as in heart valve, it is not possible to determine to what degree the thickening is due to excessive cellular distension due to accumulation of GAG, or due to excessive connective tissue deposition, or both.

It has been postulated that the close association of decorin, a dermatan sulphate proteoglycan, with collagen and its widespread distribution in various connective tissues suggests its role in collagen deposition (Bianco *et al.*, 1990). One possible hypothesis is that decorin is present in excessive amounts in connective tissues in MPS I, II, VI and VII stimulating excessive collagen deposition. However it is unclear if accumulated GAG in these MPS types is present as free partially degraded GAG chains, or still part of a proteoglycan, or both. In addition this does not explain the hepatic fibrosis observed in several Sanfilippo patients (in which only HS accumulates), however it was hypothesised by the authors that the liver pathology in all the cases (MPS I, II and III) may have been caused by a hepatotoxic metabolite (Parfrey and Hutchins, 1986).

Abnormal collagen fibres and collagen organisation (Tabone *et al.*, 1978) have also been observed in a several of the MPS disorders. Objective measurement has shown an increased range of collagen fibril diameters in the corneas of MPS I Hurler and Scheie patients (Quantock *et al.*, 1993; Huang *et al.*, 1996). Conversely, predentin collagen fibrils were abnormally thin in dental tissues from MPS VII mice. Mineralisation defects were also evident in the MPS VII mouse dentin (Gritli Linde *et al.*, 1995). Collagen fibril diameter has

not been systematically quantitated in MPS cartilage or bone, except for an early report in several MPS I and III patients which stated that cartilage fibril diameter was much less than in control samples (Silberberg *et al.*, 1972). This suggests that abnormal extracellular matrix composition in some MPSs may alter the normal process of collagen fibrillogenesis which may affect the process of normal mineralisation. Inhibition of type I and type II collagen fibrillogenesis by decorin or the decorin core protein has been demonstrated *in vitro* (Vogel *et al.*, 1984) as well as inhibition of type II collagen fibrillogenesis by free CS GAG (Kuijer *et al.*, 1985). It is therefore possible that collagen fibrillogenesis may be altered in a variety of connective tissues due to abnormal connective tissue matrix concentrations of free GAG or proteoglycans.

This hypothesis could relate to the skeletal abnormalities observed in the MPS disorders, in particular those with impaired DS degradation (I, II, VI, VII). Decorin or free GAG in the cartilage matrix may inhibit type II collagen fibrillogenesis, resulting in collagen fibrils with a smaller diameter which would in turn affect matrix structure which would alter growth plate function and resulting bone formation. In addition, decorin and biglycan, and the small keratan sulphate proteoglycan fibromodulin bind transforming growth factor- β and it has been hypothesised that they may regulate TGF- β by sequestering it within the extracellular matrix (Hildebrand *et al.*, 1994). Therefore if excessive matrix concentrations of decorin and biglycan are present in these MPS types, this may lead to inhibition of TGF- β function and hence may alter cell growth and differentiation.

1.9 Animal models of LSD and MPS disorders

A large number of inherited animal models of LSDs have now been identified in a number of domestic animals including dogs, cats, rats, mice, goats and cattle (Patterson *et al.*, 1982; Dorling, 1984; Haskins *et al.*, 1991b). Included in these, a number of MPS animal models have been identified, or more recently produced by targeted gene disruption (Table 1.2). Breeding colonies have been established for many of these disorders and various therapeutic strategies have been studied (Section 1.12).

Table 1.2: Animal models of MPS disorders.

Disorder	Species	References
MPS I	Dog	(Shull <i>et al.</i> , 1982, 1984)
	Cat	(Haskins <i>et al.</i> , 1979a, 1981)
	Mouse*	(Clarke <i>et al.</i> , 1997)
MPS IIIA	Dog	(Carmichael <i>et al.</i> , 1996)
MPS IIIB	Emu	(Giger <i>et al.</i> , 1997)
MPS IIID	Goat	(Thompson <i>et al.</i> , 1992)
MPS VI	Dog	(Neer <i>et al.</i> , 1992, 1995)
	Cat	(Jezyk <i>et al.</i> , 1977; Haskins <i>et al.</i> , 1980)
	Rat	(Yoshida <i>et al.</i> , 1993a-b)
	Mouse*	(Evers <i>et al.</i> , 1996)
MPS VII	Dog	(Haskins <i>et al.</i> , 1984, 1991a)
	Cat	(Gitzelmann <i>et al.</i> , 1994)
	Mouse	(Birkenmeier <i>et al.</i> , 1989; Vogler <i>et al.</i> , 1990)

* denotes produced by targeted gene disruption

Animal models of any human disease are useful for understanding the pathogenesis of the disease and for evaluating new therapies because of the obvious ethical constraints of human experimentation. With animal models, objective measurements and extensive samples are possible to collect. Importantly this can usually be done in a number of animals with a uniform genotype, all of which are not possible in humans. Biochemically and histologically, disease in all the MPS animal models is virtually identical to the equivalent disease in humans, however some differences between species is apparent in disease phenotype. Until the pathogenesis of disease in MPS disorders is understood, and any species differences in these processes are defined, it is not possible to determine which animal model best represents the comparable disease in humans. In addition, the equivalent mutation at a molecular level would provide the only truly valid comparison of species differences in disease phenotype. Therefore molecular characterisation is also an important aspect to include in the evaluation on new animal models.

The same MPS type in several different animal species also provides the opportunity to test how consistent responses to new therapies are, and hence will improve understanding of species differences and the anticipated response if the same therapy is applied to humans. Few comparative studies between the different MPS animal models are available, however extensive clinical and histological descriptions are generally available for most models making some comparison possible.

Canine and feline MPS I both exhibit moderate clinical severity, with coarse facial features, skeletal disease, heart valve disease and corneal clouding. In addition, dwarfism and severe joint disease is present in the MPS I dog (Shull *et al.*, 1982), neither of which occur in the MPS I cat (Haskins *et al.*, 1981). The facial dysmorphism and skeletal lesions in the recently described MPS I mouse are apparently more severe than the canine and feline models, although both the canine and murine molecular lesions both result in absence of any iduronidase expression (Menon *et al.*, 1992; Clarke *et al.*, 1997). Severe skeletal disease and mental retardation is a characteristic feature of severely affected human MPS I (Hurler) patients. Histological evidence of neurological lesions are present in all three animal models, however mentation is difficult to assess in animals, with evidence of mild clinical neurological abnormalities only in the MPS I cat, presenting as progressive hindlimb gait abnormalities (Haskins *et al.*, 1983a).

The predominant clinical feature of the four human MPS III subtypes is progressive mental retardation with mild somatic changes. Only brief clinical descriptions are available for three animal models with MPS III. Two related wire-haired dachshunds exhibited adult onset ataxia. Neuronal inclusions were present throughout the central nervous system histologically and biochemical evaluation confirmed a diagnosis of MPS IIIA (Carmichael *et al.*, 1996). A Nubian goat was presented at birth with neurological abnormalities, and a subsequent diagnosis of MPS IIID was made. Delayed motor development, growth retardation and normal behaviour was observed in this animal (Thompson *et al.*, 1992). And recently, MPS IIIB was confirmed in emus exhibiting progressive neurological signs. Biochemical analysis revealed elevated brain gangliosides as well as increased liver HS and a deficiency of serum and hepatic α -N-acetylglucosaminidase (Giger *et al.*, 1997).

The first MPS VI animal model was described in a Siamese cat in 1976/1977 (Cowell *et al.*, 1976; Jezyk *et al.*, 1977)(see Section 1.10 also). Naturally occurring MPS VI was also subsequently described in a Miniature Pinscher in 1992 (Neer *et al.*, 1992), in rats derived from the Ishibashi hairless strain in 1993 (Yoshida *et al.*, 1993a), and recently in 1996 in mice following targeted disruption of the 4S gene (Evers *et al.*, 1996).

All species appeared to be affected by MPS VI to a similar degree, with all demonstrating the same biochemical features, facial dysmorphism, skeletal changes, widespread inclusions in connective tissues and in leukocytes (Haskins *et al.*, 1980; Yoshida *et al.*, 1993b; Neer *et al.*, 1995; Evers *et al.*, 1996). All displayed dwarfism with widespread abnormalities in skull, ribs, pelvis, long bones, and vertebrae. Severity of skeletal disease between the different species was difficult to compare directly from the published material, although reduced bone length appeared to be severe in the dog, cat and rat compared with littermates. However skeletal changes in radiographs of an MPS VI mouse compared with a control did not appear as severe. Growth plate architecture was poorly organised in all four species, and growth plates appeared wider in the MPS VI rat and mouse compared with controls (Neer *et al.*, 1992; Yoshida *et al.*, 1993b; Abreu *et al.*, 1995; Evers *et al.*, 1996).

Other differences were present between the different MPS VI animal models. Degenerative joint disease was very apparent in the MPS VI dog and cats, however no joint changes were observed in the MPS VI rat and no observations were recorded for the MPS VI mouse. Corneal clouding was clinically and histologically apparent in the cat and dog, but was only visible histologically in the rat and mouse, and heart valves appeared grossly thickened in the cat, dog and mouse but not in the rat. Hepatosplenomegaly is observed in human MPS VI however this was not a disease feature in any of the animal models. No obvious CNS pathology was present in any of the models except for hindlimb paresis in MPS VI cats (Haskins *et al.*, 1983b). Distribution of lysosomal storage in various tissues in all four models was virtually identical. Minor differences in affected cell types are tabulated by Yoshida *et al.* (1993b).

A wide range in clinical severity is observed in human MPS VII patients (Whitley, 1993). The disease present in the dog, cat and mouse models of MPS VII all appeared to be moderately severe, with facial dysmorphism, dwarfism and severe skeletal abnormalities (Haskins *et al.*, 1984; Birkenmeier *et al.*, 1989; Vogler *et al.*, 1990; Haskins *et al.*, 1991a; Gitzelmann *et al.*, 1994). Joint laxity was apparent in both the dog and cat, with progressive inability to walk, however hindlimb neurological deficits were only present in the cat. Seizures also developed in the cat. Joint disease was obvious in the dog and mouse, with severe erosion of articular cartilage in the dog. The lack of lesions observed in the cat may have been due to the much younger age at euthanasia. Excessive leukocyte granulation and lysosomal storage in the CNS and connective tissues was present in all the MPS VII animal models.

In a comparative study of craniofacial abnormalities in MPS I and VI cats, and MPS VII dogs, skeletal disease was most severe in the MPS VII dog, then the MPS VI cat, and was the least severe in the MPS I cat (Sheridan *et al.*, 1994). This may suggest significant differences in disease severity due to the particular GAG accumulated in each MPS type, however these differences may also reflect the severity of the mutation, and therefore the amount of enzyme protein produced and resulting residual enzyme activity.

1.10 Feline MPS VI

Naturally occurring MPS VI was originally described in a Siamese cat presented to the University of Pennsylvania for further evaluation due to skeletal and neurological abnormalities (Cowell *et al.*, 1976). Subsequent diagnosis of MPS VI was made by demonstration of excessive DSuria on qualitative urinalysis, presence of metachromatic inclusions in neutrophils on Toluidine Blue stained peripheral blood smears, and significantly lower 4S activities in cultured skin fibroblasts compared with controls (Jezyk *et al.*, 1977). Soon after, MPS VI cats from two additional, apparently unrelated Siamese families were identified and described (Haskins *et al.*, 1979b). Affected cats in at least a total of 7 families of Siamese cats were subsequently identified by the same authors in New York, New Jersey, Pennsylvania, Oklahoma, and Oregon (Haskins *et al.*, 1983b). Pedigree information was not available for most of these families, so it was not possible to determine

if they were related, however the fact that they were all of Siamese ancestry indicated that this was a possibility. MPS VI has since been described further afield in a Siamese cat in Quebec (Canada) (Breton *et al.*, 1983), and in two long-haired Siamese cats in Naples (Italy) (Di Natale *et al.*, 1992). MPS VI has also been observed in Maine (USA) in a domestic long-hair kitten not thought to be Siamese related, although the breed of the sire was unknown (Beekman, 1993). From the descriptions available, severity of disease in the cats from different origins appears to be similar.

Clinical features in MPS VI cats begin to be evident at 6-8 weeks of age and include very mild corneal clouding, a broad face and shortened nose, small ears, short neck and tail, and reduced body length and weight. Variable degrees of pectus excavatum and reduced flexibility of the cervical spine is also observed. Animals have progressive walking difficulty with hindlimb paresis or paralysis developing in some animals. Onset of hindlimb neurological problems is usually between 4-7 months of age and is due to bony compression in the thoracolumbar region. Unlike in humans, no hepatosplenomegaly is present, and the mental state of affected cats appears normal, as observed in human MPS VI patients (Haskins *et al.*, 1979b, 1983b; Di Natale *et al.*, 1992). Seizures were also reported in one cat (Breton *et al.*, 1983).

Severe widespread skeletal disease is observed radiographically, including a generalised osteoporosis, with a coarse trabecular pattern and severe epiphyseal dysplasia of the vertebrae, long bones and short tubular bones. A progressive degenerative joint disease with increasing age also occurs, evidenced by irregular subchondral bone outline with proliferation of bone. Other features include bilateral coxofemoral subluxation with shallow acetabulae and flattened femoral heads, fusion of cervical vertebrae, metaphyseal flaring in vertebrae, hypoplasia of the dens, pectus excavatum, and abnormal sinus and turbinate development in the skull, (Cowell *et al.*, 1976; Breton *et al.*, 1983; Konde *et al.*, 1987).

Detailed necropsy and histological findings have been reported by Haskins *et al.* (1980). At necropsy, affected cats had mild dilation of cerebral ventricles, bony compression of the thoracolumbar spinal cord (one cat) and thickened mitral valves. Histologically, lysosomal inclusions were widespread in tissues including in fibroblasts (skin, cornea, heart valves),

smooth muscle cells, chondrocytes, perithelial cells, Kupffer cells, hepatocytes and retinal nonpigmented tapetal pigment epithelium. Excessive thickening of soft tissues as observed in human MPS VI patients was not observed in MPS VI cats. Abnormal inclusions in leukocytes have also been described by light and electron microscopy (Cowell *et al.*, 1976; Haskins *et al.*, 1979b; Alroy *et al.*, 1989).

Purification of feline liver 4S indicated that it was present as a homodimeric structure compared with human 4S which is present as a monomer (McGovern *et al.*, 1982; Gibson *et al.*, 1987). Further studies with recombinant feline 4S have found no evidence of a homodimer (Yogalingam *et al.*, 1996). The cDNA and gene location of feline 4S have also been reported (Jackson *et al.*, 1992) and the predicted feline 4S protein has greater than 90% homology with human 4S. Several studies have also examined various aspects of residual feline 4S activity in MPS VI cats (Vine *et al.*, 1981, 1982; McGovern *et al.*, 1982, 1985).

Essentially, clinical, histological and biochemical features of disease in the MPS VI cat closely resemble a moderately severe human MPS VI phenotype. The main features are summarised and compared in Table 1.3. The feline model of MPS VI is therefore considered to be a valuable model for evaluation of prospective therapies being developed, ultimately to alleviate disease in human patients. The lack of CNS pathology in the MPS VI cat removes the difficulty of crossing the blood brain barrier which prevents access of large molecules to the CNS.

Research protocols using MPS VI cats have included evaluation of hepatic storage of GAGs to determine the validity of several methods for future therapy evaluations (Haskins *et al.*, 1992), detailed studies of ocular pathology (Aguirre *et al.*, 1983; Mollard *et al.*, 1996), cultured retinal pigment epithelium disease expression (Stramm *et al.*, 1985) and also the correlation between the severity of lysosomal storage and residual 4S activity in cells taken from different regions of the retinal pigment epithelium (Stramm *et al.*, 1986). Reciprocal corneal transplantation between MPS VI and normal cats (Aguirre *et al.*, 1992), and BMT (Gaspar *et al.*, 1984)(see Section 1.12) have also been undertaken. Using these different methodologies, greater understanding of the pathophysiology of feline MPS VI

disease and response to therapies may assist in understanding similar aspects in human MPS VI patients.

Table 1.3: Disease features in human MPS VI compared with feline MPS VI.

Feature	Human MPS VI	Feline MPS VI
autosomal recessive inheritance	✓	✓
phenotype	mild to very severe	severe
N-acetylgalactosamine 4S activity	deficient	deficient
dermatansulphaturia	✓	✓
leukocyte inclusions	✓	✓
facial dysmorphism	✓	✓
dwarfism	✓	✓
epiphyseal dysplasia	“dysostosis multiplex”	✓
corneal clouding	✓	✓
glaucoma	✓	not reported
heart valve thickening	mitral & tricuspid - severe aortic - severe	mitral (mild)
tracheal obstruction	✓	×
hepatosplenomegaly	✓	×
hernias	umbilical and inguinal	not reported
spinal cord compression		
-cervical - dural thickening	✓	×
-thoracolumbar - bony	✓*	✓
carpal tunnel syndrome	✓	not reported
joint mobility	stiffness	?laxity some joints [‡]
degenerative joint disease	✓ [†]	✓ [§]
normal intelligence	✓	✓

* This has been reported in an MPS VI patient (Wald and Schmidek, 1984); ‡ (Langweiler *et al.*, 1978); † Severe arthritic changes has been reported in the hips and shoulders of an MPS VI patient at postmortem (Keller *et al.*, 1987); § (Norrdin *et al.*, 1994);

1.11 Historical therapies in human MPS patients

Following *in vitro* correction of lysosomal storage by co-culture of MPS I and MPS II fibroblasts (see Section 1.6), “enzyme replacement” became a popular concept for the correction of MPS disorders. Some attempts at enzyme replacement were also tested in several heritable lipid storage disorders at a similar point in time (Brady *et al.*, 1982). Major factors limiting the success of this work was lack of availability of sufficient amounts of enzyme at a sufficient level of purity to avoid immunogenic reactions, and inability to target enzymes to specific tissues, in part due to use of enzymes lacking the appropriate oligosaccharide residues for efficient uptake by certain cell surface receptors (Neufeld and Muenzer, 1995). Two forms of enzyme replacement were examined, namely tissue/organ transplantation, and direct enzyme replacement, and these will be outlined briefly.

Liver, spleen and kidney transplantation as a source of enzyme to alleviate symptoms in several lipid storage disorders provided minimal or no clinical improvement, probably due to insufficient amounts of enzyme being released into circulation to reach sites of pathology. Immune mediated complications were also encountered (reviewed by Brady *et al.*, 1982). Fibroblast implantation was also attempted in MPS I, II and III patients with either no change or very limited and transient clinical improvement (reviewed by Neufeld and Muenzer, 1995). Based on the enzyme level present in culture medium, the number of fibroblasts required to provide sufficient glucocerebrosidase for anticipated clinical improvement in a Gaucher patient was calculated to be prohibitively large, and this approach was not attempted in this particular case (Brady *et al.*, 1982).

Enzyme replacement was also attempted using subcutaneously implanted amnion from human placentas. Amnion was chosen as a possible alternative because it was shown to express lysosomal enzymes and had low immunogenicity (reviewed by Muenzer *et al.*, 1992). Systematic and objective biochemical and clinical evaluation in a total of 19 MPS I, II and III patients failed to demonstrate improved clinical function except some improved joint mobility which was viewed sceptically by the authors. Activity of the deficient lysosomal enzyme also did not increase in serum or leukocytes, and transient changes in urinary GAG excretion was observed in only several patients. A foreign body reaction was

observed in biopsies of the implantation site taken 6 months after implantation, with no recognisable amniotic tissue remaining. It was concluded that as with previous tissue or organ transplantation protocols, amnion implantation was not an effective form of therapy for MPS disorders (Muenzer *et al.*, 1992). This was probably due to inability of the amnion to survive long-term *in vivo*, and possibly also the limited ability of amniotic cells to undergo division (Muenzer *et al.*, 1992). The authors also stressed the importance of extensive, systematic clinical and biochemical evaluation to determine the efficacy of any future therapies being tested. This had been an obvious weakness in past studies with new therapies, often leading to premature optimism due to very limited evaluation, which often could not be substantiated in further studies with more patients.

Direct enzyme replacement was originally extremely limited by lack of sufficient amounts of the appropriate purified enzyme. Initial studies in MPS patients utilised plasma and leukocytes infusions as a direct source of enzyme. Clinical responses were transient with these treatments and new methods were sought which involved tissue transplantation described above (Neufeld and Muenzer, 1995). Concurrent studies in patients with lipid storage disorders with intravenous and intrathecal injections of enzyme purified from urine, demonstrated that enzyme clearance from circulation was extremely rapid, and no clinical improvements were observed. Brain biopsies from one patient revealed no increase in hexosaminidase A activity in the brain after infusion, because of the impermeability of the blood brain barrier to large molecules (reviewed by Brady *et al.*, 1982).

It was already apparent from animal studies that exogenously derived enzymes had a very short plasma half-life and the majority was efficiently removed by liver hepatocytes (reviewed by Rattazzi, 1983). This resulted in low levels of enzyme being available to other organs including the CNS, a major site of pathology in many LSDs. Methods developed to target enzyme through the blood brain barrier into the CNS included: concurrent intravenous administration of a hepatocyte receptor inhibitor (for example mannan) to block hepatocyte uptake. This would prolong the time enzyme was in circulation and hence its availability to other organs including the brain; disruption of the blood brain barrier with methods such as cerebral air embolism or hyperosmotic agents to allow enzyme passage into the CNS; and conjugating enzyme with an appropriate recognition marker to improve

uptake by different receptors or different processes, for example tetanus toxin fragments and polylysine (reviewed by Barranger *et al.*, 1979; Rattazzi, 1983; Rattazzi *et al.*, 1987; Rattazzi and Dobrenis, 1991). Most of these methods have limited therapeutic application, and it is still apparent that enzyme replacement will be ineffective for LSDs with CNS pathology unless novel methods of targeting enzyme across the blood brain barrier are developed.

A number of different glycoprotein-specific cell-surface receptors have been identified, including the M6PR which is widespread in many cell types, including fibroblasts, myoblasts and hepatocytes (Bou Gharos *et al.*, 1993), mannose receptors which are found in tissue macrophages, recognising oligosaccharides terminating in mannose and N-acetylglucosamine (Stahl *et al.*, 1984), and galactose and fucose receptors on hepatocytes (Neufeld and Ashwell, 1980).

Following a greater understanding of these cell surface receptors, characterisation of the oligosaccharide structure of the lysosomal enzyme glucocerebrosidase purified from placenta revealed that it contained galactose and N-acetylneuraminic acid terminating oligosaccharides (reviewed by Brady and Barton, 1991). After intravenous administration of this enzyme to rats, the majority was targeted to hepatocytes. However sequential deglycosylation of the native enzyme exposed terminal mannose residues which then selectively targeted the enzyme to the mannose receptors on macrophages constituting the reticuloendothelial system (Furbish *et al.*, 1981). This confirmed that enzymes could be selectively targeted to some tissues. The importance of this was that the reticuloendothelial system is the site of glucocerebroside accumulation (a glycolipid) in Gaucher disease, which is one of the most common LSDs. Hence specific cell types known to accumulate storage material in Gaucher disease could be targeted using direct enzyme replacement and this was theoretically a viable therapeutic option awaiting clinical trials in human patients (see Section 1.14).

1.12 Therapies in animal models of MPS and other LSDs

At the beginning of this study in 1992, the major form of therapy which had been evaluated in animal models of LSDs was BMT. BMT was first tested in the 1970's in several animal models of inherited marrow derived disorders, such as cyclic neutropenia in dogs, and the murine model of congenital osteopetrosis, and some success was observed in several transplanted human patients (reviewed by Desnick *et al.*, 1982). These experiments were soon followed by highly experimental and controversial reports of efficacy of BMT in a human MPS I patient (Hobbs *et al.*, 1981). It was unclear how preventable or reversible CNS and skeletal lesions were, which are the major clinical features present in many LSDs. This led to extensive evaluation of BMT in a number of LSD animal models which predominantly exhibited CNS lesions including: fucosidosis in English Springer Spaniels, Krabbe disease in mice, Niemann-Pick disease in mice and α -mannosidosis in cats. BMT was also evaluated in MPS I dogs and MPS VII mice in which both have CNS and skeletal pathology, and also in MPS VI cats which exhibit predominantly skeletal disease (reviewed by Haskins *et al.*, 1991b).

The rationale of BMT in LSDs is that normal donor marrow can provide a continuous supply of both the deficient enzyme into circulation and also normal cells which can transfer enzyme directly to other cells by cell to cell contact. Widely distribution of donor marrow derived cells around the body in the form of monocytes/macrophages would supply enzyme to many tissues (Whitley, 1993).

An important conclusion from the animal model BMT studies was that the age at transplantation had significant impact on overall outcome, in particular for CNS disease, with greatest improvements seen the earlier animals were transplanted. In untreated fucosidosis dogs, obvious mental deterioration was apparent by 12-18 months of age and gait deficits began at 12-15 months of age. Clinical changes became progressively more severe and euthanasia was necessary before 40 months (Taylor *et al.*, 1987). In an animal transplanted at 4 months of age, only mild signs of fucosidosis were present at 3 years post-transplantation, and in animals given BMT at slightly older ages, onset of neurological disease was delayed but not prevented (Taylor *et al.*, 1989a). BMT performed after the

onset of clinical signs was ineffective in preventing the normal progression of disease (Taylor *et al.*, 1988). Fucosidase activity in the brains of animals with long term marrow engraftment (> 6 months) reached 20% of the levels found in normal control dogs, compared with less than 1% enzyme activity in untreated fucosidosis dogs (Taylor *et al.*, 1989b, 1992). Lower levels of enzyme activity was observed in tissues including the CNS after less than 2 months of marrow engraftment (Taylor *et al.*, 1992). Substantial histological correction was also observed in the CNS in animals given BMT at an early age (Taylor *et al.*, 1989a). These findings indicated that enzyme transfer into the CNS had occurred, probably due to donor derived cells crossing the blood brain barrier, and that the donor-derived macrophages were slow to establish themselves in the CNS compared with other tissues.

Similarly, compared with untreated twitcher mice (a model for Krabbe disease), survival was not altered in twitcher mice exhibiting signs of CNS dysfunction, following BMT at 21-28 days of age (Yeager *et al.*, 1984). However in twitcher mice given BMT at 10 days of age, significantly increased longevity, remyelination of peripheral nerves and a gradual increase in galactosylceramidase activity in the CNS to 15% of normal control levels was observed (Yeager *et al.*, 1984; Hoogerbrugge *et al.*, 1988a). Some neurological symptoms were also improved. A characteristic histological feature of CNS disease in twitcher mice is globoid cell infiltration, and in BMT treated mice these were shown to be donor-derived (Hoogerbrugge *et al.*, 1988b). This may explain the increased CNS enzyme levels found in transplanted mice (Hoogerbrugge *et al.*, 1988a).

Dramatic response to BMT has been observed in α -mannosidosis cats (Walkley *et al.*, 1994). Mild CNS clinical signs in untreated animals are observed at 6 weeks of age and progress to terminal disease by 6 months of age. However three kittens transplanted at 8, 10 and 12 weeks of age exhibited minimal neurological abnormalities at 14 - 26 months of age. Greatest clinical improvement was observed in the animal transplanted at 8 weeks of age which was still alive at the time of the report. Histological examination of the other 2 cats revealed minimal lysosomal storage in neurons and most other cell types in the CNS. Activity of α -mannosidase ranged from 9 to 40% of normal levels in different regions of the CNS.

BMT studies in MPS I dogs demonstrated lower levels of enzyme activity in the brain than in BMT fucosidosis dogs, with only 1-3% of normal control values, however lysosomal vacuolation was absent in glial and perithelial cells and was reduced overall in neurons. Brain GAG levels were also significantly reduced (Shull *et al.*, 1988). In addition to CNS changes, BMT treated dogs had clinically less severe disease and slower disease progression, with milder cardiovascular changes grossly and histologically, clinical and histological improvements in corneal disease, and clearance of storage in kidney and liver. Urine GAG excretion was also significantly reduced. Skeletal disease was reduced in severity with greatly reduced degenerative joint disease resulting in increased mobility, although chondrocyte vacuolation persisted (Shull and Walker, 1988; Breider *et al.*, 1989; Haskins *et al.*, 1991b). This suggested that BMT was also partially effective in altering skeletal disease.

BMT has also been performed in MPS VII mice, with BMT in young adult MPS VII mice resulting in a decrease in lysosomal storage in some CNS cells including neurons and increased enzyme activity in most organs. Transplanted mice also had a greatly increased lifespan compared with untreated controls, although no changes were observed in skeletal disease (Birkenmeier *et al.*, 1991). Improved response was observed in the CNS and skeletal system when BMT was performed in MPS VII neonates, however some radiation side-effects also occurred (Sands *et al.*, 1993). As observed in MPS I dogs, lysosomal storage in corneal keratocytes was reversed, indicating BMT could target enzyme to avascular tissues.

Response to BMT in the MPS VI cat was less obvious compared with the MPS I dog, and neonatal MPS VII mice, with BMT treated cats exhibiting mild resolution of facial dysmorphism, improved mobility, and only slight improvements in skeletal disease. Skeletal improvements were apparent with more objective bone morphometric data reported more recently (Norrdin *et al.*, 1993; Turner *et al.*, 1995), although values from BMT treated cats were still significantly different from normal controls. In addition, variable improvements in corneal clouding and significant reduction in urinary GAG excretion was observed, however enzyme activity was not detected in the brain (Gasper *et al.*, 1984; Wenger *et al.*, 1986;

Haskins *et al.*, 1991b; Dial *et al.*, 1997). Some possible reasons for the different response to BMT in the MPS I dog, MPS VII mouse and MPS VI cat maybe due to species differences, different substrate accumulation or initial severity of disease (genotype).

1.13 Current therapies in human MPS patients - BMT

For the majority of LSDs, the only therapy currently available for patients has been BMT, with most long-term studies described in MPS I patients (Hopwood *et al.*, 1993; Whitley *et al.*, 1993; Field *et al.*, 1994; Tandon *et al.*, 1996; Vellodi *et al.*, 1997). BMT is not without risks and has a significant morbidity and mortality, particularly if an HLA-identical match is not available (Whitley *et al.*, 1993).

Effect of BMT on CNS function has probably been the most important issue, as developmental delay and mental deterioration is the major disease feature of in MPS III and in severely affected MPS I, II and VII patients. A major difficulty in assessing overall outcome due to BMT is the clinical heterogeneity observed particularly in MPS I patients. Developmental delay may be apparent by 12-24 months in MPS I Hurler patients, whereas MPS I Scheie patients have normal intelligence throughout life (Neufeld and Muenzer, 1995)(see Table 1.1 also). Greater understanding of genotype to phenotype correlations and comparing patients with the same genotype has improved evaluation of response to BMT (Hopwood *et al.*, 1993). MPS I patients transplanted at a young age generally maintain intellectual level or show a slow rate of decline compared with the anticipated progressive mental retardation (Hopwood *et al.*, 1993; Whitley *et al.*, 1993). Some improvements in CNS function may be attributed to resolution of hydrocephalus, improved hearing and vision and other physical improvements (Neufeld and Muenzer, 1995). As observed in animal studies, patients transplanted after neurological problems are evident, show no improvements compared with the decline expected in untreated patients (Hoogerbrugge *et al.*, 1995). Overall, in practical terms, this means the greatest CNS response will be seen the earlier that the patient is transplanted, and 2 years of age was recently recommended as the maximal age for transplantation in MPS I (Vellodi *et al.*, 1997).

BMT predominantly in MPS I has resulted in dramatic soft tissue changes which have greatly improved patient quality of life, particularly with regard to joint mobility, and cardiac and respiratory function (Hoogerbrugge *et al.*, 1995). Some resolution of facial dysmorphism and improved long bone growth is observed, however overall progression of skeletal pathology is essentially unchanged even in patients transplanted from an early age. Endochondral ossification is poor, and patients develop osteoarthritic changes particularly in the hips, progressive thoracolumbar kyphosis and hip subluxation (Field *et al.*, 1994; Vellodi *et al.*, 1997). Transplanted patients therefore require ongoing invasive orthopaedic intervention. It can therefore be concluded that a major determinant in outcome of BMT is the extent of CNS and skeletal pathology present prior to BMT (Whitley, 1993).

Although fewer MPS VI patients have undergone BMT, a similar response to therapy is observed as seen in MPS I patients. Because of the lack of CNS involvement, older patients have undergone transplantation and have benefited from resolution of soft tissue problems. Forty months after BMT in a 13 year old MPS VI patient, significantly improved cardiopulmonary function and improved joint mobility was observed. A reduction in hepatomegaly, normalisation of spleen size and reduction in urinary GAG excretion were also observed, however no improvements in skeletal pathology or corneal changes occurred (McGovern *et al.*, 1986). Similar improvements were observed in a patient transplanted at ~8 years of age, however this patient also had some slow increases in height and some improvement in the bilateral corneal clouding (Jurges *et al.*, 1991).

1.14 Future therapies in LSD animal models and MPS patients

In 1990, Barton *et al* demonstrated clinical efficacy of ERT in a type I Gaucher patient, with mannose terminated human placental glucocerebrosidase (Barton *et al.*, 1990)(see Section 1.11). This patient showed reduction of splenomegaly and increase in platelet numbers and haemoglobin concentration. Further clinical trials confirmed these observations (Barton *et al.*, 1991) and gradual improvements in skeletal pathology due to clearance of storage in Gaucher cells in the marrow was also observed (Brady and Barton, 1994). These studies clearly demonstrated that enzyme was targeted to the cell types exhibiting the metabolic defect, resulting in substrate turnover and reversal of pathologies. Purified

placental and recombinant glucocerebrosidase is now available commercially (Ceredase/Cerezyme ® Genzyme). It was unknown if repeated administration of the purified protein would result in development of an antibody response, and neutralising antibody was not detected in any of the original patients studies (Murray *et al.*, 1991). However a recent report describes the presence of neutralising antibody in two patients (Ponce *et al.*, 1997).

Polyethylene glycol modified adenosine deaminase (PEG-ADA) has also been a successful form of ERT used to treat patients with severe combined immunodeficiency caused by adenosine deaminase deficiency. Purified bovine ADA is coupled to PEG to increase plasma half-life which enables catabolism of the toxic metabolite 2'-deoxyadenosine in plasma (Hershfield and Chaffee, 1991; Hershfield, 1995).

ERT is currently unavailable for trials in human MPS patients, and at the beginning of this study in 1992, no MPS animal model had undergone evaluation for the efficacy of ERT. Successful ERT in type I Gaucher patients was greatly encouraging, and this form of therapy also overcomes the graft-vs-host disease problems encountered in BMT. Problems associated with producing sufficient amounts of low abundance enzyme from human tissue have largely been overcome, with the introduction of recombinant technology yielding high levels of enzyme in cell culture. The ultimate success of ERT is dependent on effective delivery of enzyme to sites of pathology in sufficient amounts. The MPS animal models therefore provide the opportunity for extensive evaluation of the efficacy of ERT in MPS disorders.

A future strategy for therapy in human MPS and other disorders is replacing the defective gene by inserting a normal gene (gene therapy) into the host DNA in a variety of somatic cell types. Various approaches are possible depending on the target tissue and choice of gene-delivery vector. The gene may be introduced into cells isolated from the patient and then transplanted back into the patient (*ex-vivo*). Alternatively, the gene may be introduced directly into the affected tissue (*in-vivo*). With the currently available vectors, problems with regulating gene expression, length of DNA that can be inserted into vectors, immune

response against vectors, and vector infection rates of cells to be targeted, need to be overcome before gene therapy can be an effective long term therapy in human patients.

Several different strategies for gene therapy have been examined in MPS animal models. Gene transfer into autologous haematopoietic stem cells has the advantage that it would function in a similar way to BMT except it would overcome the morbidity associated with allogeneic marrow transplantation. In addition, if the most primitive bone marrow progenitor cells (stem cells) which give rise to all other blood cells could be transduced, then these gene corrected cells may last the lifetime of an affected individual (Fairbairn *et al.*, 1996). However these cell types are present in low numbers in marrow and are difficult to infect using retroviral vectors. Long-term gene expression has been demonstrated in transduced human bone marrow from MPS I patients in cell culture (Fairbairn *et al.*, 1996), and correction or reduction of lysosomal storage was observed in the liver and spleen of MPS VII mice transplanted with genetically modified MPS VII mouse bone marrow (Wolfe *et al.*, 1992; Marechal *et al.*, 1993).

An alternative approach is using implantation of genetically modified fibroblasts or other cell types, either as a mass of cells engrafted onto a collagen lattice (neo-organs) implanted in the abdominal cavity, or implanting these cells directly into the tissue of interest. Neo-organs containing genetically modified mouse fibroblasts implanted into MPS VII mice provided continuous enzyme delivery for at least 3 months, with detectable enzyme levels in liver, lung and spleen and complete clearance of lysosomal storage in liver and spleen (Moullier *et al.*, 1993). Gene modified fibroblasts secreting high levels of β -glucuronidase were also directly implanted in the brains of MPS VII mice, resulting in clearance of storage in neurons and glial cells in the vicinity of the implanted cells (Taylor and Wolfe, 1997). A slightly different approach to this was direct implantation of β -glucuronidase expressing neural progenitor cells into the cerebral ventricles of newborn MPS VII mice (Snyder *et al.*, 1995). These cells migrated throughout the brains, became incorporated into the normal brain architecture and also resulted in widespread correction of lysosomal storage in neurons and glial cell types.

High levels of enzyme expression was achieved in cultured myoblasts from MPS I dogs transduced with the canine α -iduronidase gene. However following implantation back into muscle, elevations in enzyme activity in circulating leukocytes were short-lived, and antibodies against α -iduronidase were demonstrated (Shull *et al.*, 1996). This was considered to be due to the lack of any α -iduronidase protein expressed in MPS I dogs (Menon *et al.*, 1992), hence an immune response was mounted against the foreign α -iduronidase secreted by the transduced myoblasts.

As new approaches to therapy in LSD disorders are trialed in animal models, greater understanding of the potential for disease reversal with these therapies in human patients with similar disorders will be gained. Future studies in animal models will also provide new insights into the pathophysiology of these disorders.

1.15 Aims of the project

The overall objective of this project was to evaluate the efficacy of ERT with recombinant human 4S in the feline model of MPS VI. A number of specific objectives were proposed to achieve this:

1. improve breeding efficiency of an established MPS VI cat colony to produce increased numbers of MPS VI kittens
2. diagnosis of MPS VI kittens at or soon after birth
3. evaluate natural course disease in MPS VI cats from birth to maturity compared with normal control cats to generate baseline data
4. evaluation of rh4S enzyme distribution in normal cats
5. evaluate efficacy of ERT in MPS VI cats

An understanding of the efficacy of ERT in the feline model of MPS VI will be important in anticipating the ability of ERT to prevent the significant skeletal and soft tissue pathology observed in children with MPS VI.

1.15.1 Thesis structure

Following a description of methodologies in Chapter 2, results in Chapter 3 establish baseline data and observations of the natural course of disease in MPS VI cats from birth to maturity. Improved diagnostic methods to identify newborn MPS VI kittens and comparison of the pathology observed in the “Adelaide” MPS VI cats with the “Philadelphia Family 3” MPS VI cats from which our colony was derived, is also outlined.

Chapter 4 presents results of distribution and fate of recombinant human 4-sulphatase in normal cats, and then results from preliminary low dose ERT trials evaluating the viability and efficacy of long term therapy in three MPS VI cats.

Chapter 5 examines the efficacy of weekly ERT from birth in MPS VI cats for a duration of 5-11 months at three different dose rates, and in a second group of MPS VI cats using a twice weekly dose regime and modified enzyme to improve penetration of enzyme to cartilage.

Chapter 6 presents results determining the origin and inheritance of the disease causing mutations present within the colony, and also describes and compares the clinical, biochemical and histological features of the different genotypes within the colony.

Finally, Chapter 7 draws overall conclusions resulting from the work presented in this thesis, and recommendations for future work.

2. Materials and methods

The following chapter describes the materials and general methods used in Chapters 3 to 6. Where specific methods have been employed that are relevant to only one chapter, they have been detailed in the appropriate chapter. Where the method has been performed by other people, this has been indicated at the beginning of each method in italics.

2.1 Materials

2.1.1 Animal vaccinations

2.1.1.1 Live attenuated vaccines

Feline 3 Vaccine®	Arthur Webster Pty Ltd. (Castle Hill, NSW)
Combined Feline Rhinotracheitis-Calicivirus®	Arthur Webster Pty Ltd.

2.1.1.2 Inactivated vaccines

Fevac 3 in 1®	CSL Limited (Parkville, Vic.)
Protech F3I®	Cyanamid Websters (Castle Hill, NSW)

2.1.2 Medications and preventatives

Adrenaline 1:1000 (1mg/ml),	Astra DBL (North Ryde, NSW)
Bethanechol chloride 10mg tablets, Urocarb Tablets®,	Hamilton Laboratories Pty Ltd. (Adelaide, SA)
Cat-lax® (70g contains paraffin 33.3g, malt syrup 33.3g, lecithin 1.4g, cod liver oil 1.1g, caramel 0.4g, vitamin E 0.05g, biotin 0.02g),	Heriot AgVet Pty Ltd. (Rowville, Vic.)
Chlopheniramine maleate 10mg/ml, Niramine®,	Jurox Pty Ltd. (Silverwater, NSW)
Chlopheniramine maleate 4mg tablets, Piriton®,	The Boots Company (North Rocks NSW)
Cyproheptadine HCl 4mg tablets, Periactin®,	Charles E. Frosst (South Granville NSW)

Ensal®, phthalylsulphathiazole 50mg/ml, kaolin 100mg/ml, hyoscine methobromide
 0.2mg/ml, monosodium glutamate 5mg/ml, potassium gluconate 10mg/ml, calcium
 gluconate 20mg/ml, Jurox Pty Ltd.
 Ivermectin 10g/l, Ivomec Antiparasitic Injection for Cattle®, MSD Agvet,
 Aust. Pty Ltd.(South Granville NSW)
 Lincomycin HCl 222g/kg & spectinomycin sulphate 445g/kg, Linco-Spectin Soluble
 Antibiotic Powder®, Upjohn, Pty, Ltd. (Rydalmere, NSW)
 Lufenuron 30mg/kg, Program® oral flea control, Ciba-Geigy Australia Limited
 (Pendle Hill, NSW)
 Pentobarbitone sodium 325mg/ml, Lethabarb®, Virbac (Aust.) Pty Ltd. (Peakhurst, NSW)
 Praziquantel 23mg, Droncit Tapewormer®, Bayer Aust. Ltd. (Pymble, NSW)
 Pyrantel embonate 90mg/g & niclosamide 267mg/g, Felex Plus Allwormer Paste®,
 Pfizer Agricare Pty Ltd. (West Ryde, NSW)

2.1.3 Sedatives and anaesthetics

Alphaxalone 9mg/ml and alphadolone acetate 3mg/ml, Saffan®, Jurox Pty Ltd.
 4-Aminopyridine 2mg/ml & yohimbine hydrochloride 1.25mg/ml, Reverzine SA Injection®,
 Parnell Laboratories Aust. Pty Ltd. (Alexandria, NSW)
 Atropine sulphate 0.65mg/ml, Atrosine Mitis®, Parnell Laboratories Aust. Pty Ltd
 Atropine sulphate 1mg/ml & acepromazine maleate 2mg/ml, Anamav Injection®,
 Mavlab Pty Ltd. (Slacks Creek, Qld)
 Halothane, Fluothane®, ICI Australia Operations Pty Ltd. (Melbourne, Vic)
 Ketamine 100mg/ml, Ketamine Injection ®, Parnell Laboratories Aust. Pty Ltd.
 Xylazine 20mg/ml, Rompun®, Bayer Aust. Ltd.

2.1.4 Enzymes and antibodies

The enzymes and antibodies used in this study were obtained from the following companies:

Ava II (5u/μl)	Boehringer Mannheim (Mannheim, West Germany)
Bsl I (5u/μl)	New England Biolabs (Beverly, MA, USA)

<i>Hae</i> III (10u/μl)	Boehringer Mannheim
<i>Taq</i> DNA polymerase (5u/μl)	Boehringer Mannheim
T4 DNA polynucleotide kinase (10u/μl)	New England Biolabs
FITC-Conjugated Goat Anti-Cat IgG (H & L) 1mg/ml	Southern Biotechnology Associates Inc. (Birmingham, AL, USA)
Peroxidase-conjugated Goat Anti-Cat IgG (H & L) 0.8mg/ml	Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA)

2.1.5 Chemicals

All chemicals were of Analar or HPLC grade and were supplied by the following companies:

Acrylamide	Bio-Rad Laboratories (Hercules, CA, USA)
Ammonium formate	Ajax Chemicals (Auburn, Australia)
Bromophenol blue	BDH Chemicals Ltd. (Poole, England)
Cetylpyridinium chloride	Sigma Chemical Co. (St. Louis, USA)
Chondroitin 4-sulphate type A	Sigma Chemical Co.
Citric acid	Ajax Chemicals
Dextran	BDH Chemicals Ltd.
D-glucuronic acid lactone (glucuronolactone)	Sigma Chemical Co.
EDTA (ethylenediaminetetraacetic acid)	Ajax Chemicals
Ethanol	Ajax Chemicals
Ethidium bromide	Boehringer Mannheim
Formaldehyde	Ajax Chemicals
HiRES gel	Progen Industries Ltd. (Darra, Qld)
Lithium Chloride	BDH Chemicals Ltd.
4-methylumbelliferyl sulphate	Sigma Chemical Co.
Phenol red	Merck (Darmstadt, Germany)
3-phenylphenol (hydroxydiphenyl)	Aldrich Chemical Co., (Milwaukee, USA)
Phosphate buffered saline (PBS) tablets	Sigma Chemical Co.
SDS	Sigma Chemical Co.

Sodium acetate	Ajax Chemicals
Sodium chloride	Ajax Chemicals
Sodium citrate	Ajax Chemicals
Sodium formate	BDH Chemicals Ltd.
Sodium hydroxide	Ajax Chemicals
Sodium pyrophosphate	BDH Chemicals Ltd.
Sulphuric acid	BDH Chemicals Ltd.
Sodium tetraborate	Ajax Chemicals
Tris base	Boehringer Mannheim
Triton X-100	Ajax Chemicals

All other chemicals used in this study were analytical reagent grade.

2.1.6 Buffers and solutions

Buffers and solutions routinely used in this study were as follows:

TAE	40mM Tris-acetate, 1 mM EDTA, pH 8.0
20× SSC	3M NaCl, 0.3M trisodium citrate, pH 7.0
10× loading buffer	50% (v/v) glycerol, 1% (w/v) SDS, 100mM EDTA, pH 8.0, 0.1% (w/v) bromophenol blue
10×PCR buffer	Biotech International Ltd. (Perth, Aust.)
25mM MgCl ₂	Biotech International Ltd.

2.1.7 Miscellaneous materials

ABTS substrate kit	Bio-Rad Laboratories (Richmond, VA, USA)
Affi-Gel 10	Bio-Rad Laboratories
Alcian Blue 8GX	Koch-Light Laboratories, (Colnbrook, Berks, England)
Bovine serum albumin (BSA), fraction V	Sigma Chemical Co.
DEAE Sephacel	Pharmacia LKB Biotechnology (Uppsala, Sweden)
Deoxyribonucleoside triphosphates (dNTPs)	Boehringer Mannheim

Detail intensifying screens (cat. no. 1218775)	Kodak (Rochester, NY, USA)
Digital refractometer (UR-1 urine S.G. meter)	Atago (Japan)
GeneScreen Plus membranes	DuPont-NEN Research Products (Boston, MA, USA)
Herring sperm DNA	Sigma Chemical Co.
Immulon 1 ® Removawell strips	Dynatech Laboratories (Chantilly, Virginia, USA)
Insulin syringe Lo-dose 0.5ml and 1.0ml	Becton Dickson (Franklin Lakes, NJ, USA)
Ovalbumin	Sigma Chemical Co.
γ - ³² P-ATP (2pmole/ μ l)	DuPont-NEN Research Products
Poly-prep chromatography columns	Bio-Rad Laboratories
Polyvinylchloride plates (96 well)	Costar (Cambridge, MA, USA)
pUCHpaII molecular weight markers 500ng/ μ l	Bresatec (Adelaide, Aust.)
Sephadex G10	Pharmacia LKB Biotechnology
Toluidine Blue (Gurr code 34077)	Merck
Urinalysis reagent strips (Ames Neostix ® N)	Bayer Australia Ltd.
X-omatic cassettes 24x30cm	Kodak
Xray film cronex daylight 10T, 24x30cm	DuPont (Aust.) Ltd. (Sydney Aust.)
Ultrafiltration membranes, YM-10	Amicon Inc. (Beverly, MA, USA)

2.2 General methods

2.2.1 Animal maintenance

The colony was originally established in 1984 from five “family 3” heterozygotes (Haskins *et al.*, 1979b) obtained from Dr. M. Haskins at the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, USA. Following quarantine and importation, the cats were housed at the Institute of Medical and Veterinary Science field station at Gilles Plains, Adelaide, in June 1984. External laboratory cats were introduced regularly to minimise inbreeding. The colony was attended to by trained animal care staff who fed, cleaned, weighed and administered routine medications to the animals under veterinary supervision, seven days a week.

Cats were housed in concrete floored pens with a brick enclosed sleeping area (approx. 2.5m long × 1.7m wide × 2m high) that adjoined an outdoor wire run (approx. 6.5m long × 1.7m wide × 2m high) containing climbing ladders and platforms. Pens were directly adjacent to each other, allowing “nose contact” between pens. Litter trays were changed daily and hosed clean. Both inside and outside areas of the pens were hosed down three times weekly, and the inside pen area disinfected between successive litters of kittens. Bedding was washed and changed weekly. Adults cats were fed IAMS Cat Food® daily, and pregnant and nursing queens were fed ad libitum IAMS Kitten Food® plus tinned Whiskas® cat food. Kittens were fed ad libitum IAMS Kitten Food® plus twice daily tinned Whiskas Kitten Food® to ten weeks of age.

2.2.2 Disease prevention

All cats over six months of age were vaccinated twice yearly with live attenuated Feline 3 Vaccine®. If a queen was possibly pregnant, Fevac 3 in 1® or Protech F3I® inactivated vaccine was used. Kittens were initially vaccinated with Feline 3 Vaccine® at 6-8 weeks and 10-12 weeks and with live attenuated Combined Feline Rhinotracheitis-Calicivirus® vaccine at 16 weeks. After several mortalities due to feline parvoviral enteritis (May 1994), the vaccine schedule was altered to Feline 3 Vaccine® at 6, 9, 12 and 16 weeks of age. Additional problems due to feline herpesvirus infection with high morbidity and some mortality in kittens less than 6 weeks of age (October 1995) led to further modification of the vaccine schedule to include two half dose vaccinations of Feline 3 Vaccine® or Protech F3I® at two and four weeks of age. After problems with flea infestation, monthly Program® oral flea control was introduced over spring and summer for all cats except unweaned kittens. Faecal samples from kitten pens were submitted three to four times yearly to the Institute of Medical and Veterinary Science (IMVS) for parasitology instead of intermittent blanket medication against intestinal parasites. Droncit Tapewormer® or Felex Plus Allwormer Paste® were used when parasites were observed or detected. Cats were treated when necessary for ear mite infestation with Ivermectin at 0.2mg/kg subcutaneously.

Following persistent outbreaks of chronic mucopurulent conjunctivitis in younger animals, Chlamydia was positively diagnosed from conjunctival swabs taken from one cat in February 1995. Several two monthly courses of lincomycin (55mg) and spectinomycin (111mg) (Lincospectin®) per cat per day was given mixed in food. This led to some improvement in symptoms, however conjunctivitis was also noted in subsequent herpesvirus outbreaks.

2.2.3 Colony breeding management

From four to five months of age, cats were housed individually or in groups of up to six animals of the same sex. Females 15 months or older were paired with one male cat, up to a maximum of five females per male, until pregnancy was achieved. Three weeks after pairing, females were examined weekly for pregnancy using abdominal palpation, and parturition date was estimated from this or from last recorded heat or observed mating if available. Approximately two weeks prior to expected parturition date, females were separated from the male, vaccinated with either Fevac 3 in 1® or Protech F3I® and housed individually. Females were then checked at least once daily by animal care staff for any signs of kitting. Kitting date was taken as being the date on which the kittens were first observed (day 0). After weaning at 8 weeks, females were housed with other females for a minimum of one month, depending on body condition, before re-pairing with a male. Females were allowed a maximum of two litters per year. Prior to mutation analysis, females considered to be heterozygotes from enzymology were culled after two litters if no MPS VI kittens were produced, unless culling was indicated due to other reasons such as mismothering, cannibalism or small litter size. Post-parturient urinary and faecal retention was a common problem in breeding females, but there was no apparent association between genotype and incidence of this problem. Affected females were treated with bethanechol (2.5 mg P.O. twice daily) and Cat-lax® for up to several weeks post-partum.

Kittens were identified according to coat colour and weighed as soon as possible after birth. An air dried blood film was also made from clipping a nail bed closely. The blood film was then stained routinely and examined to determine if kittens were MPS VI or heterozygote/normal (described further in Chapter 6). Depending on the queens mothering ability and litter size, kittens were often fostered to other queens with similar sized kittens

or euthanased to reduce litter size, particularly if MPS VI kittens were in a litter. Kittens were weighed three times weekly to ten weeks of age, then once weekly to 20 weeks of age to assess any signs of sickness/poor weight gains. Kittens were weaned at eight weeks of age and grouped with similar aged kittens, up to a maximum of eight kittens per pen. Kittens were microchipped if this was necessary to accurately identify individuals. At 90 days, kittens were ear tattooed under general anaesthesia with an individual identification number.

2.2.4 Diagnosis of MPS VI

From 1984 to mid 1993, MPS VI cats were defined as having non-detectable levels of 4S in peripheral leukocytes and excessive amounts of urine DS. 4S activity was assessed using leukocyte 4S/ β hexosaminidase (4S/ β hex) ratios (Section 2.2.7) and urine GAG was characterised by high resolution electrophoresis (HRE) (Section 2.2.17).

New methods were developed in order to diagnose kittens as early as possible, including early urinalysis, blood film examination (mid 1993 to mid 1995) and DNA mutation analysis ($\geq$ Sept. 1995). The results of these methods are described further in Chapter 3 and Chapter 6.

MPS VI affected kittens were randomly assigned to treatment and control groups by assigning each kitten, as it was diagnosed, to the next treatment or control group on a previously determined list. The major limitation of the study was the limited amount of enzyme available, hence the disproportionately large MPS VI control group compared to the ERT treated group (refer to Chapter 5).

2.2.5 Selection of control animals

In the preliminary ERT trials (Chapter 4), littermates with normal physical appearance and no excess DSuria (examined at 2-3 months) were used as controls. Most of these had very low or non-detectable 4S/ β hex ratios. Retrospective mutation analysis has shown these cats

to be L476P/D520N compound heterozygotes, D520N/D520N homozygotes and D520N/normal heterozygotes.

In the ERT studies from birth (Chapter 5), normal cats were selected by the lack of neutrophil granulation on blood films. These cats also had moderate leukocyte 4S/ β hex ratios when subsequently tested. Retrospective mutation analysis has shown all of these cats to be D520N/normal heterozygotes. Genotype analysis is discussed in detail in Chapter 6.

2.2.6 Mutation analysis

The L476P mutation was identified by Tom Litjens and Gouri Yogalingam (Department of Chemical Pathology, WCH), and the D520N mutation was identified by Gouri. The oligonucleotides to amplify the appropriate region and the PCR and ASO conditions were designed and optimised by Tom.

The first mutation identified was a T $\rightarrow$ C base substitution at nucleotide 1427, resulting in a change at codon 476 from CTC (leucine) to CCC (proline)(L476P). A second base substitution at nucleotide 1558, present only in some cats, resulted in an aspartic acid (GAC) to asparagine (AAC) change at codon 520 (D520N). This region was also PCR amplified using the same oligonucleotides as for the L476P mutation.

2.2.6.1 PCR

Genomic DNA was prepared by Viv Muller (Department of Chemical Pathology, WCH).

Venous blood from cats was blotted directly onto Guthrie cards (McEwen and Reilly, 1994) without anticoagulants, and stored dry at room temperature. Three mm diameter punched discs were soaked in 1 $\times$ PCR buffer and 2.5mM MgCl₂ for 30-60 minutes at 4°C, before denaturation at 99°C for 2 hours under mineral oil. A long denaturation time was necessary to overcome high DNAase activity present in some samples. Genomic DNA was prepared from leukocytes isolated from venous blood (Section 2.2.7) collected into EDTA anticoagulant tubes. DNA was extracted using the phenol extraction method (Ausbel,

1994), which involves lysis of red cells, proteinase K digestion of the cell pellet, followed by phenol/chloroform/isoamyl alcohol extraction of protein. DNA was stored at -20°C until use. For PCR, 1/100 dilutions of genomic DNA were made into H₂O, and 1µl was added to the 1×PCR buffer and 2.5mM MgCl₂ mix directly before denaturation for a similar duration as for Guthrie samples.

Then 400µM dNTPs, 330ng each of f4S7 and f4S9 oligonucleotides (Table 2.1) and 1.5 units of *Taq* DNA polymerase were added. The final reaction volume for PCR was 100µl. PCR was performed for 40 cycles consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C, and extension for 45 seconds at 72°C. Following amplification, 10µl PCR product was analysed by electrophoresis on a 5% Hi-Res agarose gel in TAE buffer using pUCHpaII molecular weight markers. Loading buffer was added to the samples which were electrophoresed until bromophenol blue had migrated sufficiently to ensure separation of the PCR product. A 296 base pair product was visualised under UV light after the gel was stained briefly in ethidium bromide (10µg/ml).

2.2.6.2 Allele Specific Oligonucleotides (ASOs)

To detect the L476P allele, one to three µl of each PCR reaction was blotted in duplicate onto GeneScreen Plus membranes and probed with either f4S-Leu⁴⁷⁶ or f4S-Pro⁴⁷⁶ ³²P-labelled oligonucleotides (Table 2.1). ASO oligonucleotides (Table 2.1) (~100ng of each) were 5' end labelled with γ-³²P-ATP by incubating at 37°C for 45 minutes with 10 units T4 DNA polynucleotide kinase according to manufacturers instructions. Following hybridisation of the membranes with the probe (1-10ng/ml) at 42°C for at least 1 hour in 6 × SSC, 0.1% (w/v) SDS, 1.1mM sodium pyrophosphate and 100 µg/ml herring sperm DNA, membranes were washed for 15 minutes in 5 × SSC at room temperature. They were then washed for 5 - 10 minutes at 55°C in 2 × SSC and 0.1% (w/v) SDS, and then autoradiographed.

Table 2.1: Oligonucleotides used for PCR and ASO analysis.

Primer	Sequence	Sense or antisense	Position
PCR amplification			
f4S-7 (3' end)	5'AGAAAAGGTGGTCTGGCTCC3'	–	1649-1630
f4S-9 (5' end)	5'GGCTGTTGGTTTCCTCCGCCG3'	+	1348-1368
ASO primers			
f4S-P476	5'CTCTGGCCCTTTGATATT3' mutant allele	+	1420-1437
f4S-L476	5'GAGACCGAGAAACTATAA3' normal allele	–	1437-1420

2.2.6.3 Restriction Enzyme Digestion

To detect the L476P allele, as an alternative to ASOs, 5-10 µl PCR product was digested overnight at 37°C with 20 units *Hae*III restriction enzyme according to conditions specified by the manufacturer. Reaction volumes were 20-30 µl. PCR fragments were electrophoresed as above in 5% Hi-Res agarose in TAE buffer. The presence of the mutant proline⁴⁷⁶ sequence (CCC) introduced a *Hae*III site at nucleotide 1427, as well as restriction sites in both normal and mutant strands at nucleotides 1591 and 1598. The following fragments resulted (see also Figure 6.1):

normal (L/L) - 245, 51 bp

heterozygote (P/L) - 245, 163, 75, 51 bp

homozygote (P/P) - 163, 75, 51 bp

To detect the D520N allele, 5-10 µl PCR product was digested overnight at 37°C with 15 units *Ava*II or at 55°C with 15 units *Bs*II restriction enzymes, according to conditions specified by the manufacturer. Reaction volumes were 20-30 µl. PCR fragments were electrophoresed as above in 5-7% Hi-Res agarose in TAE buffer. The mutant asparagine⁵²⁰ sequence (AAC) removed the restriction sites for both *Ava*II and *Bs*II enzymes at nucleotide 1558. An additional *Ava*II site was present at nucleotide 1446 in both normal and mutant sequences. The following fragments were present after *Ava*II digestion or *Bs*II digestion (see also Figure 6.1):

normal (D/D) - *Ava*II 114, 93, 89 bp; *Bs*II 203, 93 bp;
heterozygote (D/N) - *Ava*II 203, 114, 93, 89 bp; *Bs*II 296, 203, 93 bp;
homozygote (N/N) - *Ava*II 203, 93 bp; *Bs*II 296 bp;

2.2.7 Leukocyte 4S assays

Isolation of leukocytes and leukocyte 4S/ β hex assays were done by Viv Muller.

Five mls venous blood was collected from kittens under anaesthesia into lithium heparin tubes, and stored at room temperature until processing. Leukocytes were isolated within 8 hours of collection using dextran sedimentation (Kampine *et al.*, 1966), followed by washes in hypotonic saline to lyse contaminating red blood cells. Briefly, whole blood was centrifuged at 1600 g for 5 minutes at room temperature. Plasma was removed and 0.15M NaCl was added to the original total volume. One ml sterile 5% (w/v) dextran, 0.12M NaCl was added and the blood were resuspended evenly and allowed to stand for 15-20 minutes, until most of the red blood cells had resettled out again. The supernatant was then removed and centrifuged at 700 g for 3 minutes. The leukocyte pellet was then washed in 0.03M NaCl for 1 minute to lyse red blood cells, followed by addition of 0.31M NaCl to bring the solution back to isotonicity. Leukocytes were pelleted by centrifugation as above, and then stored at -80°C until assayed.

Leukocytes were assayed for 4S using a radiolabelled trisaccharide substrate with minor modifications from the published method (Hopwood *et al.*, 1986). Briefly, leukocytes were disrupted by six cycles of rapid freeze/thawing in 0.1% (v/v) Triton X-100. Lysates were dialysed against 50mM Na-formate pH 3.5 at 4°C for 16 hours and then were incubated at 37°C for 7.5 hours with the radiolabelled trisaccharide substrate *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-D-*N*-acetyl[1-³H]galactosaminitol 4-sulphate (GalNAc4S-GlcA-GalitolNAc4S; 1000 pmol/reaction), derived from rat chondrosarcoma chondroitin 4-sulphate, and β -hexosaminidase inhibitor 2-acetamido-2-deoxy-D-gluconolactone (10 nmol/reaction). Products and residual substrate were separated by high-voltage electrophoresis at pH 1.7, and peaks were counted by scintillation. Lysates were also assayed for protein using the Lowry method (Lowry *et al.*,

1951), and for β -hexosaminidase using a previously published method (Leaback and Walker, 1961). Results were expressed as a ratio of 4S activity (pmol of product produced/min/mg protein) to β -hexosaminidase activity (nmol/min/mg protein).

The radiolabelled trisaccharide substrate assay was used because it is the most specific assay for 4S activity, since only 4S activity can hydrolyse the 4-sulphate ester on the non-reducing end of the trisaccharide substrate. The 4S product can be further hydrolysed by the action of β -hexosaminidase, but this is inhibited with the addition of 2-acetamido-2-deoxy-D-gluconolactone.

2.2.8 Haematology

Blood smears were prepared directly from freshly collected blood, air dried, and stained with May-Grunwald Giemsa (a Romanowsky stain) within 8 hours of collection. Films were then mounted and examined at $400\times$ magnification. Characteristic morphological changes in leukocytes were assessed visually for any changes in response to ERT in treated MPS VI cats. Blood films from controls and cats undergoing ERT were taken at 1-2 monthly intervals. Details regarding morphology are described extensively in Chapter 6.

2.2.9 Enzyme production and purification

Culture medium containing rh4S was produced by CSL Limited, Parkville, Victoria. Enzyme purification, preparation for injection and assays for activity were performed by Julie Bielicki, Liz Isaac, Krystyna Niedzielski and Chris Boulter (Department of Chemical Pathology, WCH).

A rh4S construct using the human polypeptide-chain-elongation factor-1 α gene promoter (Bielicki *et al.*, 1993) was inserted into Chinese hamster ovary cells to express rh4S. Culture medium was harvested and purified (Anson *et al.*, 1992), using monoclonal antibody F58.3 (Brooks *et al.*, 1994) in place of ASB 4.1. Enzyme, concentrated over a YM-10 ultrafiltration membrane and sterile filtered into phosphate buffered saline (PBS) ready for injection, was assayed for activity within 24 hours of use, using the fluorogenic

substrate 4-methylumbelliferyl sulphate (4MUS) (Gibson *et al.*, 1987). Purified enzyme was stored at 4°C until use. A sample from each batch of purified rh4S was run on SDS-PAGE under reducing conditions. All batches contained a mixture of approximately 70% precursor (67 kDa) and 30% mature (43, 8 and 7 kDa) polypeptides.

2.2.10 Enzyme administration and treatment of anaphylaxis

Sterile enzyme preparations (stored at 4°C) were transported at room temperature. Cats were unstarved and were weighed prior to enzyme administration. Enzyme dose was calculated according to bodyweight, and volume was calculated according to enzyme activity of each preparation. Different enzyme activity assay conditions were used in the preliminary ERT trial (Chapter 4) compared with the ERT trial from birth (Chapter 5), resulting in significantly different specific activities used in each trial. Due to the possibility of anaphylactic reactions to the infused enzyme, antihistamine premedication was administered at least 30 minutes prior to enzyme infusion. Slightly different premedication regimes were used in the preliminary ERT trial (Chapter 4) and in the ERT trial from birth (Chapter 5). Two antihistamines used simultaneously can increase effectiveness (Gershwin, 1992).

Enzyme was normally infused at a rate of approximately 5-10 ml/kg/hour but no more than 90 ml/kg/hour, using insulin syringes to administer doses accurately if enzyme volume was small. Mild signs of anaphylaxis included lip licking, restlessness, miaowing, increased respiratory rate, trembling and pruritis of the skin around the ears and head. More severe signs included vomiting, gelatinous diarrhoea, cyanosis and respiratory distress. If mild signs of anaphylaxis occurred, enzyme infusion was slowed to less than 5 ml/kg/hour or temporarily stopped. More severe signs of anaphylaxis were treated with 0.02 mg/kg Adrenaline diluted into 0.5ml isotonic saline or Hartmanns solution given subcutaneously, and rapid intravenous fluids (Hartmanns) and oxygen if considered necessary. Adrenaline was repeated in 20 minutes if considered appropriate. Rapid intravenous fluids are recommended for anaphylactic shock to counteract the severe drop in blood pressure due to vasodilation (Degen, 1989).

2.2.11 Clinical examination

Animals were routinely examined at 3, 5, 6, 7, 9, and 11 months of age using a check sheet to note specific details. At other times, abnormal observations were noted in a daily record book.

2.2.11.1 *Physical examination*

Physical appearance was noted, particularly in comparison to littermates. Features noted included facial shape, ear size, neck and body length, spinal curvature, physical activity and demeanour.

2.2.11.2 *Neurological examination*

A brief neurological examination was performed which mainly focussed on hindlimb neurological deficits. Observations of gait and ability to correct normally after pushing the hindquarters off balance were the easiest to perform, and animals cooperated more readily with this level of intervention. Wheelbarrowing (forelegs), hopping (forelegs and hindlegs), proprioceptive positioning (forelegs and hindlegs), extensor postural thrust (hindlegs), and where appropriate, muscle tone, muscle atrophy and deep pain perception were also examined, but animals often refused to cooperate after a short time which made these methods of examination difficult. Degree of hindlimb neurological deficits were assessed as follows:

1. normal - rapid ability to correct overbalancing of hindquarters by stepping out with hindlimbs or quickly move hindquarters. Gait was not necessarily normal, as progressive degenerative joint changes and in several cases a fractured patella affected the gait pattern which sometimes made interpretation difficult.
2. mild deficits - subtle changes in gait such as treading heavily, occasional stumbling, slower or more clumsy correction when hindquarters overbalanced.
3. mild to moderate paresis - ability to walk with variable degrees of hindlimb ataxia and falling over, hindquarters easily overbalanced.

4. paralysis - dragging hindlimbs completely, with increased extensor tone and variable degrees of muscle wasting. Deep pain also appeared to be present. Some animals still had some movement of hindlimbs.

2.2.11.3 Flexibility

Flexibility was determined by the ability of animals to move their head laterally, and to extend their hindlimbs caudally. An approximate visual estimation was made of the degrees to which this was possible, with 180° lateral cervical movement being when the nose touched the flank either side of midline, and 180° coxofemoral joint movement being when the hindlimbs were parallel to the body. Most animals tolerated this well and they were not forced to move beyond what appeared comfortable. Results of this aspect of examination were not analysed until the latter part of this study, and therefore the majority of observations were considered to be without obvious bias.

2.2.11.4 Slit lamp examination

Slit lamp examination was performed by an experienced paediatric ophthalmologist, Michael Hammerton (WCH). Dr. Hammerton also had past experience evaluating corneal disease in MPS patients, including three MPS VI children managed at the WCH.

Examination was performed in conscious animals using a portable slit lamp. Corneal clouding was graded using an arbitrary scale of 0 - 4, where 0 = normal, and 4 = complete opacity. This scale was developed from observations made in humans. Animals were evaluated without knowledge of genotype or treatment group, and included MPS VI and normal control animals of all ages. A large group of animals was examined on each of only seven occasions over the span of this study.

2.2.12 Anaesthesia

All radiographs were taken under general anaesthesia. Blood and urine samples were also taken, and kittens were tattooed at the same time. Animals were unstarved and weighed to calculate the appropriate anaesthetic dose. Atropine sulphate at 0.05mg/kg and xylazine at

2.2-3.3mg/kg were given subcutaneously, 15 minutes before administration of ketamine at 20-33mg/kg also given subcutaneously. Some animals were given Reverzine SA Injection ® at 0.25ml/kg intravenously, if recovery was prolonged. MPS VI cats needed higher doses of both xylazine and ketamine to be anaesthetised, and recovery was much faster than other cats anaesthetised at the same time.

2.2.13 Standardised radiographic examination

Standard radiographic views of each cat were taken under general anaesthesia, using a portable Toshiba Type CDC-MA machine (model KCD - 10M6AT) at a constant focal film distance of 133 cm. Cats were positioned with sandbags, foam wedges and tape. Views were as follows: lateral cervical spine, lateral lumbar spine, lateral right and left hindlimb, and ventrodorsal pelvis. The beam was approximately centred over the 3rd cervical vertebra, 5th lumbar vertebra, mid-shaft of the tibia, and sacroiliac joints for each of these views respectively. These were taken using detail intensifying screens. A fixed MaS/variable Kv exposure chart was developed to ensure good quality radiographs. Kv was reduced slightly with 3 month old or younger cats, and also when radiographing the long bones of older MPS VI cats, both due to osteopaenia. Films were processed in an automatic processor (Department of Radiology, WCH). Magnification error was assumed to be minimal and constant, due to the long focal film distance and also as cats in the same age group have similar dimensions in each standard view.

Variable Kv exposure chart:

TISSUE THICKNESS (cm)	Kv
2	50
3	52
4	54
5	56
6	58
7	60
8	62

2.2.14 Standardised bone measurements

Direct measurements were taken from the lateral views of the standardised radiographs using vernier calipers. Measurements were adapted from methods used in human radiology (Keats, 1990) and those previously used in MPS VI cats (Konde *et al.*, 1987). Fifth lumbar and third cervical vertebral lengths were taken as the distance between (and including) the epiphyses at a point midway through the vertebra. Lumbar vertebral width was measured at the narrowest point of the vertebra in a line perpendicular to the length measurement, whereas the cervical vertebral width was taken at the point of bisection of, and perpendicular to, the length measurement, as the cervical vertebrae are almost a parallelogram shape. Patellar length was measured as the greatest distance between the proximal and distal margins, and tibial length was taken as the distance between the articular surfaces of the proximal and distal epiphyses. The corticomedullary ratio of the right tibia was calculated by the addition of the thickness of both cortices where the endosteal borders were parallel to each other (this is usually at their thickest point, that is, at the narrowest point of the medullary cavity) divided by the width between the two endosteal surfaces at the same point. Values in MPS VI cats $\pm$ ERT and normal controls were expressed as a percentage of the mean values obtained from same sex normal control cat groups, to remove sex differences observed between male and female normal control cats (see Chapter 3).

2.2.15 Renal function

Urine was collected from ≥ 3 month old cats under anaesthesia and was stored at -20°C until assayed. Urine creatinine (mmol/l) and protein (g/l) were assayed using an autoanalyser method (Beckman Synchron CX ® Systems). To evaluate renal function, the urine protein/urine creatinine ratio was calculated (Martin Copland, Veterinary Pathology Services, Adelaide, personal communication):

$$\text{Ratio} = \frac{\text{protein}}{\text{creatinine}} \times 8.8$$

where < 0.5 = normal

0.5 - 1.0 = impaired renal function possible

> 1.0 = abnormal

Urine specific gravity was measured using a digital refractometer. Plasma urea (mmol/l) and creatinine (μ mol/l) were assayed using an autoanalyser method (Beckman Synchron CX @ Systems) from plasma collected from heparinised whole blood.

2.2.16 Urinalysis - Alcian Blue spectrophotometric method (“ABC method”)

The ABC method was modified from Gold (1979) and Whiteman (1973). This method was chosen to quantitate total urine GAG as it was already employed in the Metabolic Unit in the Department of Chemical Pathology, WCH, mainly to determine how much GAG to load for HRE.

Alcian Blue 8GX (2mg/ml) was dissolved in 0.5M Na acetate buffer, pH 5.8 and 500 μ l of fresh dye reagent was added to 200 μ l of urine (usually diluted 1/4 with H₂O). Samples were mixed well and allowed to stand for 15-30 minutes at room temperature before absorbances were read at 480 nm, blanking against H₂O. Chondroitin 4-sulphate type A was used as the standard. The standard curve was sigmoidal, and apparently varied according to dye batch number (Wendy Norton, Department of Chemical Pathology WCH, personal communication). The concentration of urinary GAG in the sample was expressed as mg/mmol creatinine. The volume of urine containing 300mg of GAG was calculated if samples were then being prepared for HRE.

2.2.17 Urinalysis - CPC GAG precipitation and high resolution electrophoresis (HRE)

HREs performed on samples in Chapters 3, 4 and 5 were run and scanned by Liz Isaac.

Urine GAG was purified from urine for HRE by the method of Hopwood and Harrison (1982). Briefly, the ABC method was used to determine the volume of urine containing approximately 300mg GAG. The urine GAG was precipitated by cetylpyridinium chloride (CPC) using equal volumes of 0.1% (w/v) CPC in 0.054M Na citrate buffer (pH 4.8 with citric acid) and urine, which was mixed and incubated for 30 minutes at 37°C. Samples were

centrifuged at 1500 g for 10 minutes, supernatant removed and 150 μ l 2M LiCl was added to the pellet and mixed well, followed by the addition of 800 μ l absolute ethanol. Samples were then centrifuged, supernatant removed and pellets containing GAG allowed to drain and then dry overnight. If GAG content of the urine had not previously been assayed using the ABC method, pellets were resuspended in H₂O and the uronic acid content was assayed (see Section 2.2.19). Samples were lyophilised into approximately 10 μ g uronic acid aliquots. Samples were resuspended in 10-20 μ l phenol red (0.5mg/ml, dissolved in H₂O), and approximately 3 μ l was loaded for HRE.

GAG species were then separated by HRE as described (Hopwood and Harrison, 1982). To improve separation of bands, run times were increased to 6, 35 and 17 minutes for samples in Chapter 6. To accommodate this, the size of the cellulose acetate strip was increased to 76 $\times$ 94 mm, and voltage adjusted to 250 volts to maintain the same voltage density across the plate.

Plates were dried at room temperature and then scanned using a Molecular Dynamics Personal Densitometer Scanner and MultiQuant Version 1.0 (1993, Molecular Dynamics Inc.). Densitometric estimations of the approximate relative proportions of two dermatan sulphate species (DS1 and DS2) was made and expressed as a percentage of the total area under the peaks.

2.2.18 Urinalysis - DEAE anion exchange column chromatography (“DEAE method”)

To try and increase sensitivity of total urine GAG quantitation, a new method for quantitating urine GAG was developed using DEAE anion exchange column chromatography based on a previous method (Di Ferrante, 1967). The anion exchange column binds all sulphated GAG, and allows almost complete separation from urine pigments and proteins which interfere with uronic acid quantitation. Some uronic acid content is evident in the flow through, although is not quantifiable due to pigment, with only 5% rebinding when applied to fresh columns.

Briefly, aliquots of 0.375-2ml urine samples were diluted if necessary with deionised water to reduce specific gravity below 1.020, and were mixed with column buffer and pH adjusted to achieve a final concentration of 0.1M Na acetate, and pH 5.0-5.5. Final volume of samples was a maximum of 8mls. DEAE Sephacel was equilibrated in 0.1M Na acetate buffer pH 5 and a 1ml packed volume of beads was allowed to settle in poly-prep chromatography columns. Diluted urine samples were applied to the 1ml DEAE, the column tubes sealed and samples were mixed continuously for at least 30 minutes at 4°C, before allowing DEAE beads to resettle. The supernatant was collected then the bound sulphated GAG was eluted from the DEAE with 2M NaCl/0.1M Na acetate buffer pH 5 over approximately $5 \times 0.5\text{ml}$ fractions. Fractions were assayed for uronic acid content using the hydroxydiphenyl method (Section 2.2.19), and total μg uronic acid/mg creatinine was calculated for each sample.

2.2.19 Uronic acid quantitation

Uronic acid levels were assayed using the hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using D-glucuronolactone (stock solution 0.05mg/ml) as a standard. Briefly, samples were diluted as necessary with H_2O in glass test tubes, to a final sample volume of 200 μl . Samples were cooled on ice and then 1.2ml of 0.0125M sodium tetraborate/concentrated sulphuric acid was added and tubes were mixed well. Tubes were covered aluminium foil to minimise evaporation. Samples were then heated for 5 minutes in a boiling waterbath then cooled in an ice bath. When cool, 20 μl of 3-phenylphenol (1.5mg/ml) diluted in 0.12M NaOH was added and samples mixed well. 200 μl of sample was aliquotted into Immulon 1® Removawell strips and then read on an automated ELISA reader (Ceres 900 Hdi, Bio Tek Instruments, Winooski, VT, USA) at 550nm absorbance.

2.2.20 GAG desalting and gradient gel electrophoresis

Gradient gel electrophoresis was kindly performed by Enzo Ranieri (Department of Chemical Pathology, WCH).

Urine GAG samples purified by both CPC precipitation (Section 2.2.17) and DEAE anion exchange column chromatography (Section 2.2.18) were prepared for gradient gel electrophoresis. The first GAG rich fraction from samples prepared by the DEAE method (containing minimal NaCl) was desalted over a 25cm long $\times$ 0.9cm diameter Sephadex G10 column equilibrated in 0.1M ammonium formate buffer. Approximately 0.4-0.5ml of sample was layered onto the column, and 30 $\times$ 0.5ml fractions were collected. Fractions 9-16 were assayed for uronic acid content, and fractions 12-20 were assayed on a chloridometer (CMT chloride titrator, Radiometer, Copenhagen, Denmark). Salt free fractions containing GAG were pooled and lyophilised. CPC prepared samples (Section 2.2.17) were lyophilised directly after ethanol/LiCl supernatant was removed. Approximately 3.85 μ g uronic acid/lane was then loaded onto a 30-40% gradient polyacrylamide gel. The gradient gel was prepared and run essentially according to previously published methods (Turnbull *et al.*, 1997). The gel was then stained for 30 - 40 minutes with Alcian Blue (0.5g/100ml) in 2% acetic acid (v/v) and destained in 2% acetic acid (v/v).

2.2.21 Antibody titres

The methodology for determination of titres against rh4S was developed by Doug Brooks, and all titres were assayed by Barbara King (Department of Chemical Pathology, WCH).

Blood samples for determination of plasma antibody titre to rh4S were collected into EDTA or lithium heparin anticoagulant directly before rh4S administration in ERT treated cats. Plasma was separated by centrifugation (1600 g for 5 minutes) at room temperature. Plasma was stored at -20°C until assayed.

Polyvinylchloride plates were coated with 0.5 μ g purified rh4S/well of (+rh4S plates), by aliquoting 100 μ l of 5 μ g rh4S/ml (diluted with filtered 0.1M NaHCO₃, pH8.5) into each well. Plates were sealed with adhesive tape and incubated for 2 hours at 37°C, then overnight at 4°C. The plates were then washed three times with 200 μ l 0.02M Tris/HCl buffer, pH 7.0 containing 0.25M NaCl and 1% (w/v) ovalbumin (washing/blocking solution). The final wash was left in the wells and the plates were incubated for at least 2 hours at room temperature to block any remaining reactive sites on the wells. To determine

any reactivity against ovalbumin in the same plasma samples, uncoated plates (-rh4S plates) were also blocked with the wash solution.

Plasma samples were pre-absorbed overnight at 4°C with an equal volume of ovalbumin/BSA coupled Affi-Gel to remove non-specific antibodies. To couple Affi-Gel, equal volumes of Affi-Gel 10 and a solution containing 10mg ovalbumin/ml and 10mg BSA/ml were mixed for 1-2 hours at room temperature then overnight at 4°C. Pre-absorbed plasma samples were diluted serially in 0.02M Tris/HCl buffer, pH 7.0 containing 0.25M NaCl and 1% (w/v) ovalbumin, and after removing the wash solution line by line from the plates, 50µl of each dilution was applied to both +rh4S and -rh4S plates. Plates were incubated for 2 hours at room temperature then washed again three times with 200µl 0.02M Tris/HCl buffer, pH 7.0 containing 0.25M NaCl and 1% (w/v) ovalbumin. Then 100µl of goat anti-cat IgG (1/2000 dilution (v/v)) diluted in 0.02M Tris/HCl buffer, pH 7.0 containing 0.25M NaCl and 1% (w/v) ovalbumin was applied to each well and plates were incubated for 1 hour at room temperature. After plates were washed three times with 200µl 0.02M Tris/HCl buffer, pH 7.0 containing 0.25M NaCl and 1% (w/v) ovalbumin, 100µl/well of ABTS substrate (prepared according to the manufacturers instructions) was applied to the plates, and plates were incubated for 20 minutes at room temperature. Colour development was quantitated on an ELISA reader (Ceres 900 Hdi, Bio Tek Instruments, Winooski, VT, USA) at an absorbance of 414nm.

The antibody titre was determined as being the last dilution at which the absorbance was above background levels. If the -rh4S titre (i.e. titre against ovalbumin) was higher than the +rh4S titre for the same sample, the sample was re-absorbed against the ovalbumin/BSA coupled Affi-Gel and was then re-assayed. A QC sample was also included in each batch to ensure constant colour development, and titre values for this sample varied by only one or two dilutions for the whole time that this assay was in use.

2.2.22 Postmortem procedure and sample collection

Animals were anaesthetised and then euthanased with an overdose of Lethobarb® given intravenously. A standardised postmortem procedure was used in all therapy and control

animals. This involved gross examination of viscera, including heart valves, brain, shoulder, elbow, hip and stifle joints, and spinal cord from C3 to L4 spinal levels. Samples were collected as quickly as possible after certain tissues were photographed.

Any animal that died was autopsied to ascertain the cause of death, and where appropriate samples were submitted to Verlab at the Institute of Medical and Veterinary Science to aid diagnosis.

2.2.22.1 Light microscopy

Samples for light microscopy were processed routinely by staff in the WCH Department of Histopathology.

For light microscopy, tissues were fixed in 10% (v/v) formalin and were processed, embedded in paraffin, cut, and stained with haematoxylin and eosin (H&E).

2.2.22.2 Electron microscopy

All tissue processing, all ultrathin sections and some thick sections were prepared by Richard Davey, Department of Chemical Pathology WCH, and staff in the Electron Microscopy Unit, Department of Histopathology, WCH.

Electron microscopy samples were minced to $\sim 1 \text{ mm}^3$ and fixed in 2% (v/v) glutaraldehyde/ 2% (v/v) formaldehyde in 0.1M sodium cacodylate buffer pH 7.2 overnight at 4°C then postfixed in 1% (v/v) osmium tetroxide for 30-60 minutes, dehydrated through 70%, 90% and 100% ethanol and embedded in Spurr's resin. One micron thick sections were stained with 1% (w/v) Toluidine Blue in 1% (w/v) borax and then ultrathin sections cut and stained with 2% (w/v) uranyl acetate/ 1% (w/v) lead citrate and examined with a Hitachi H-7000 electron microscope.

For examination of leukocytes, venous blood collected into EDTA was centrifuged (1600 g for 10 minutes) and the plasma removed. Fixative (1 % (v/v) glutaraldehyde/ 4 % (w/v) paraformaldehyde in 0.1M sodium cacodylate buffer pH 7.2) was layered gently onto the

buffy coat, which was allowed to harden for ~20 minutes, before being separated from the underlying red cells and placed in fresh fixative for an additional ~60 minutes at room temperature. Alternatively, buffy coats were collected from microhaematocrit tubes after centrifugation, and placed directly in the same fixative as above. Samples were stained *en bloc* with saturated uranyl acetate in 70 % aqueous methanol, then postfixed in 1 % (v/v) osmium tetroxide, dehydrated through graded alcohols and embedded in Spurr's resin. Sections were cut and stained as above.

2.2.22.3 Tissue sampling and section orientation

As this work progressed, it became apparent that good orientation of tissue samples was important, as response to ERT was not uniform in some tissues (see Section 5.3.9). Larger samples of tissue were fixed in EM fixative for several hours so that they were firm before trimming, which enabled more accurate cutting of the tissue. Large Toluidine Blue stained 1µm thick sections of samples prepared for electron microscopy had much better detail by light microscopy than formalin fixed paraffin embedded H&E sections. Therefore, extent of lysosomal vacuolation was evaluated on Toluidine Blue stained thick sections, with selected samples evaluated further by electron microscopy. By electron microscopy, the majority of lysosomal inclusions appeared as empty vacuoles due to extraction of material during processing, however some vacuoles contained fibrillogranular or lamellar material.

In order to orientate mitral heart valve sections, whole valve attached to the papillary muscles and wall of the ventricle at the base of the valve was put in fixative as a whole, then was trimmed heavily once the tissue had fixed for a while, still leaving a small amount of heart muscle at the base of the valve. The valve was then cut in a longitudinal plane to give long narrow pieces extending from the base of the valve to the tip, and these were embedded whole on their side. Thick sections were orientated so that as much as possible of the valve from the base at the heart muscle to the tip of the valve or chordae tendinae were included in the section.

Unfortunately a standard sampling location from somewhere along the length of the aorta was not used. Sections of aorta were orientated so that they were cut transversely through

the full thickness of the aortic wall (tunica intima, tunica media and tunica adventitia). Sections of cornea were cut so that the full thickness of the cornea from the epithelium to the endothelium could be evaluated.

Skin samples were taken from approximately the same region on the left chest wall of each cat. Sections orientated so that storage was evaluated from the epithelium to the dermal layer and where possible also the hypodermis. Some difficulty was experienced with fixation/embedding for electron microscopy due to the dense fibrous nature of this tissue. This problem was experienced less frequently when the fixative was changed from 1% (v/v) glutaraldehyde/4% (w/v) paraformaldehyde to 2% (v/v) glutaraldehyde/2% (v/v) formaldehyde, and fixation times were also increased to overnight at 4°C.

As for skin, similar problems with fixation and embedding were also encountered with hip joint capsule. Samples were also kept fairly large in order to try and orientate the sections so that they extended from the synovium through the dense connective tissue of the capsule, to the outer loose connective tissue, which hampered optimal processing. However, sections were cut as close to the surface of the sample as possible, usually with minimal trimming.

2.2.23 Tissue evaluation

The following grading system was developed to subjectively score the extent and overall distribution of vacuolation due to lysosomal storage in most tissues from 1µm Toluidine Blue stained thick sections evaluated at a magnification of 100-400 ×:

0 = no storage seen (normal)

+/- = very occasional storage vacuoles, almost normal (very mild)

1+ = small numbers of cells with noticeable storage vacuoles with or without distension (mild)

2+ = storage easily seen, with some distended or ballooned cells, but majority of cells less distended due to storage vacuoles (moderate)

3+ = numerous ballooned or distended vacuolated cells throughout; storage easily seen (severe)

These grades were relative to the variable severity of changes seen in different cell types in different tissues. Grading between untreated and treated MPS VI cats was also slightly different as changes in degree of vacuolation were not uniform in some tissues of treated cats. Selected samples were further evaluated under electron microscopy.

2.2.24 Renal immunofluorescence - frozen sections

Tissues were frozen and cut by Mary Carli, Department of Histopathology, WCH. Fluorescent antibody staining was performed under the supervision of Mary who is an experienced technician familiar with evaluation of human renal immunofluorescence.

Kidney samples were snap frozen and stored at -70°C. Fresh cryostat sections were cut onto albumin subbed slides and allowed to dry at 4°C for 30 minutes. FITC labelled goat anti-cat IgG(H&L) was diluted in PBS to a concentration of 0.25mg/ml. Approximately 100-150µl antibody solution was layered gently onto the slides, and then slides were incubated at room temperature in the dark for 30 minutes. Slides were then washed gently in a slide rack immersed in PBS over a magnetic stirrer for 2×5 minutes. Slides were then mounted in glycerol jelly (1 part PBS, 9 parts glycerol) and visualised under a Leitz Orthoplan fluorescent microscope and assessed for degree of immunofluorescence.

2.2.25 Bone histomorphometry

Bone samples were processed and sectioned by Peter McNeil at the Institute of Medical and Veterinary Science (IMVS), Adelaide. Histomorphometric analysis was performed by Jacquie Nuttall, Department of Chemical Pathology, WCH. Histomorphometric analysis of bone samples in Chapter 4 was also performed by Alison Moore (IMVS).

Parameters of trabecular bone development were determined from the fifth lumbar (L5) vertebra. The L5 vertebra was removed, fixed in 10% (v/v) formalin and embedded in

methacrylate resin prior to sectioning using routine methods. Undecalcified tissue sections (5 μm) were stained with von Kossa to visualise mineralised bone. Sections were visually scanned to ensure integrity. Each vertebra was analysed at a magnification of $\times 16$ using an automated image analysis system (Quantimet 520 system, version 4.0, Cambridge Instruments). Mean values were calculated for each vertebra. Bone volume, trabecular thickness, trabecular number and trabecular spacing were calculated using standard morphometric formulae (Recker, 1983).

2.2.26 Statistical analysis

Where sufficient sample numbers allowed, statistical comparison between groups was made using a two-tailed *t*-test assuming equal variance, accepting a *p* value of < 0.05 as statistically significant. To compare variability between groups, the Levene test for equality of variance was used, accepting a *p* value of < 0.05 as statistically significant. One-way analysis of variance and the Tukey-HSD test for post-testing was used for statistical comparison among three or more groups. A *p* value of < 0.05 was considered statistically significant. Statistics were calculated using SPSS for Windows 6.1.3 (Dec. 1995), standard version.

3. Characterisation of the feline MPS VI colony

3.1 Introduction

Disease in the MPS VI cat closely parallels disease observed in human MPS VI patients, and it was therefore considered a valuable model for evaluation of new therapies for possible future use in human patients. Prior to evaluation of ERT in the MPS VI cat as a potential new therapy, it was essential to establish baseline data and observations of the natural course of disease in MPS VI cats from birth to maturity. The aim of this chapter was therefore to describe this progression and to understand the variability between different MPS VI animals, and the extent of differences compared with normal cats. These observations were needed for later comparison with results of ERT studies, which are described in Chapter 5. "Normal" kittens were all kittens other than MPS VI kittens, and they have therefore been designated "heterozygote/normal" throughout this chapter, as they have an undefined genotype. Further characterisation of heterozygote and normal cats is described in Chapter 6.

However before much of this work was possible, improved diagnostic methods to identify MPS VI kittens were needed because of the small numbers of MPS VI kittens being born in the colony. This was largely due to significant difficulty in distinguishing true heterozygote cats from normal cats using leukocyte enzymology. Therefore inappropriate pairing of breeding cats occurred and hence lower numbers of MPS VI kittens were being born. In addition, in order to start ERT at birth, methods to identify newborn MPS VI kittens were needed. Development of diagnostic methods, breeding data and carrier identification is presented in this chapter. Carrier identification is described more extensively in Chapter 6.

The pathology of feline MPS VI has previously been reported (Haskins *et al.*, 1980). Examination of the histological changes observed in MPS VI cats in this chapter was intended to describe obvious features of pathology in selected tissues. These baseline

histological observations were used to provide a comparison for observations in MPS VI cats treated with ERT. In addition, in order to determine the rate at which pathology developed in MPS VI cats, tissues from a mid-term foetus to 6 month and older cats were evaluated. This data was relevant when considering the age of onset of ERT.

In order to achieve these aims, urinary GAG levels, clinical appearance, radiographic skeletal appearance, bone histomorphometry, postmortem changes and tissue histopathology were assessed in MPS VI and heterozygote/normal cats both qualitatively and quantitatively.

3.2 Specific methods

3.2.1 Clinical and biochemical evaluation

Heparinised blood was collected under anaesthesia, and leukocytes isolated and assayed for 4S activity using radiolabelled trisaccharide substrate. Activity was expressed as a 4S/ β hexosaminidase (4S/ β hex) ratio (Section 2.2.7). Blood samples taken without anticoagulant were blotted directly onto Guthrie cards for PCR based mutation analysis (Section 2.2.6) and air-dried blood films were also taken and stained for evaluation of leukocyte morphology by light microscopy (Section 2.2.8). Urine was collected at various ages, and urine creatinine was measured using an autoanalyser method (Section 2.2.15). Total urine GAG was quantitated using the ABC method (Section 2.2.16), and values were corrected to creatinine concentration. The presence of DS was determined by HRE of urine GAG (Section 2.2.17).

Standard clinical examination including evaluation of cervical spine flexibility and hindlimb neurology was performed at 3, 5, 6, 7, 9 and 11 months of age (Section 2.2.11). Slit lamp examination was performed on only several occasions on MPS VI animals at various ages. Standardised radiographic views were taken at 3, 5, 6, 7, 9, and 11 months in MPS VI and heterozygote/normal littermates under general anaesthesia (Section 2.2.13). Radiographs in younger animals were taken following euthanasia. Standardised measurements of bones were taken directly from radiographs (Section 2.2.14). Values from MPS VI animals were

expressed as a percentage of the mean value from age matched, same sex heterozygote/normal cats. Statistical comparison between groups was made using the two-tailed t-test assuming equal variance accepting a p value of < 0.05 as statistically significant. To compare variability between groups, the Levene test for equality of variance was used, accepting a p value of < 0.05 as significantly different.

3.2.2 Pathology

To evaluate development of pathology with age, tissues from an MPS VI foetus (confirmed by mutation analysis) and a control littermate at ~5.5 weeks gestation (age estimated from maternal abdominal palpation and foetal size) (Berman, 1986), and MPS VI cats at 0, 2, 35, and 53 days, and 6, 11 and 30 months of age were collected for electron microscopy (EM) following euthanasia. Tissues were embedded and processed for EM following careful orientation of tissue samples. Orientated 1 μ m thick resin embedded sections were cut and stained with Toluidine Blue and were evaluated and graded for the presence of lysosomal storage (Sections 2.2.22 and 2.2.23).

Standardised postmortem included exposure of spinal cord and joint examination in cats 6 months and older (Section 2.2.22). The L5 vertebra was also removed for histomorphometric analysis, and was processed and analysed as described in Section 2.2.25.

3.3 Results

3.3.1 Diagnosis of MPS VI

Initially MPS VI was defined as having non-detectable leukocyte 4S/ β hex ratios *and* dermatan sulphaturia (DSuria). This definition was used to verify other methods that were later developed. Prior to the commencement of this study in January 1993, all samples were collected from kittens at 70 - 90 days under anaesthesia when a sufficient volume (5 ml) of blood was able to be safely taken for leukocyte isolation and enzymology. Urine was also collected at the same time for GAG analysis. It was important to refine these methods or to

develop new methods in order to be able to detect MPS VI kittens at, or as soon as possible after birth.

3.3.1.1 Enzymology

Many kittens with a normal phenotype (heterozygote or normal) had very low or non-detectable leukocyte 4S/ β hex ratios, and were indistinguishable from MPS VI cats by enzymology (Figure 3.1). These heterozygote/normal kittens bred within the colony overall had much lower 4S/ β hex ratios than outside normal cats (Figure 3.1). This suggested other genetic factors were affecting the 4S/ β hex ratios in colony bred heterozygote/normal cats. This was investigated further and is discussed in Chapter 6. Consequently, kittens were given nominal genotypes according to the following 4S/ β hex ratios:

0 - 0.2	MPS VI or heterozygote
0.2 - 0.6	possible heterozygote
>0.6	normal

3.3.1.2 Urinalysis

To detect MPS VI kittens, urine from kittens in the lowest leukocyte 4S/ β hex ratios range of 0 - 0.2 was then screened for the presence of DS by HRE. A characteristic pattern demonstrating elevated DSuria was observed in MPS VI kittens at all ages examined (10 days and older; data not shown; see Figure 6.3).

From early 1993, in order to screen kittens less than 30 days of age, urine was collected by reflex urination in response to perineal massage. Total urine GAG decreased with increasing age in both MPS VI and heterozygote/normal cats. Overall, GAG levels were higher in MPS VI cats than in heterozygote/normal cats, however MPS VI kittens could only be confidently distinguished from unaffected littermates at less than 40 days of age (Figure 3.2). The ABC assay was subsequently utilised as a rapid screening method in kittens younger than 40 days of age.

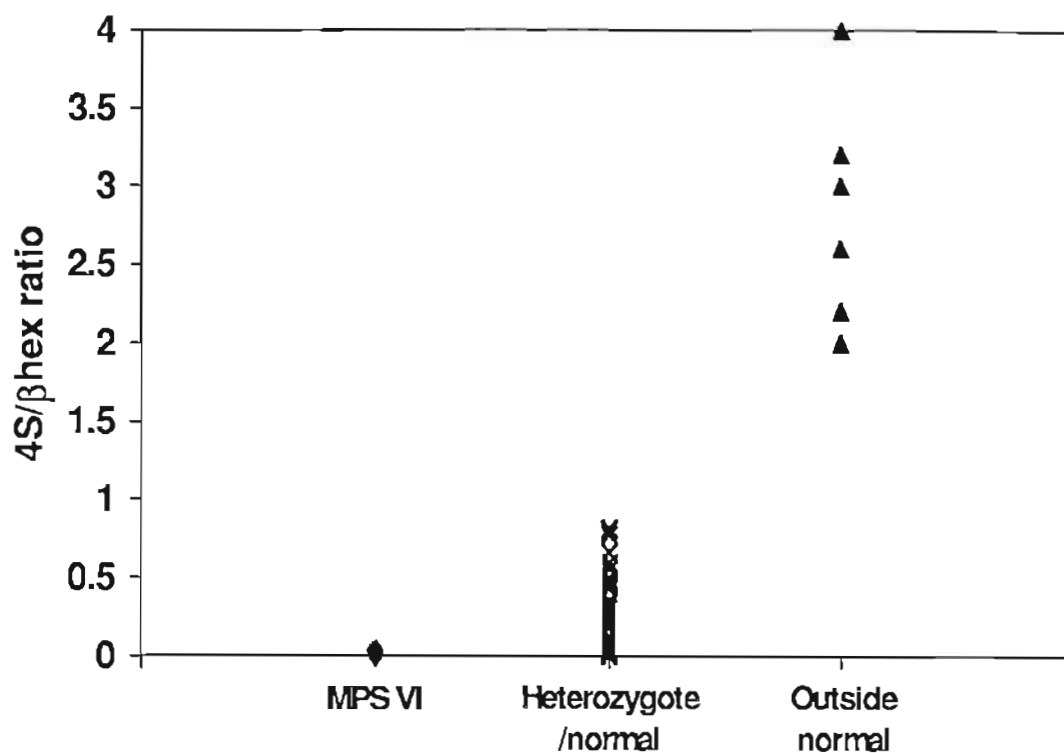


Figure 3.1: Leukocyte 4S/β-hexosaminidase ratios in MPS VI, colony heterozygote/normal and outside normal cats.

Leukocyte 4S/βhex ratios in MPS VI, colony heterozygote/normal and outside normal cats. MPS VI affected cats were enzymologically indistinguishable from some colony heterozygote/normal cats, which also had lower enzyme ratios than outside normal cats.

3.3.1.3 *Leukocyte morphology*

From mid-1993, in order to screen kittens at birth, blood films were examined for the presence of basophilic neutrophil granulation characteristic of MPS VI (Haskins *et al.*, 1979b). Unexpectedly, the majority of kittens examined had coarse basophilic neutrophil granulation. However initially only approximately 7% of kittens (not the anticipated ~25% for an autosomal recessive mode of inheritance) exhibited high total urine GAG content and DSuria confirming the diagnosis of MPS VI. Subsequently it became apparent that the only reliable distinguishing feature between MPS VI and heterozygote/normal kittens was the absence of normal eosinophil morphology in MPS VI kittens. In MPS VI cats at any age (including a mid-term MPS VI foetus), eosinophil cytoplasmic granules lacked any staining, and appeared as clear bubbles or vacuoles (see Figure 6.4). This became a very rapid and extremely accurate (confirmed later by urine HRE and mutation analysis) diagnostic tool, and was subsequently used as the primary method of diagnosis of MPS VI in newborn kittens.

The cause of basophilic neutrophil granulation in kittens other than MPS VI kittens was unknown. When clinically normal cats with basophilic neutrophil granulation were mated with outside normal cats (which had normal neutrophil morphology), all of the offspring lacked any abnormal neutrophil granulation. However, when these first generation offspring were mated back to colony cats which had abnormal neutrophil morphology, the majority of the second generation offspring also exhibited abnormal neutrophil morphology. This suggested a genetic component to the abnormal neutrophil morphology, which was eventually elucidated. Leukocyte morphology and the genetic basis for these observations is illustrated and discussed more extensively in Chapter 6.

3.3.1.4 *Mutation Analysis*

Mutation analysis became available in September 1995, and is discussed further in Chapter 6. All cats which were diagnosed as MPS VI by other means were homozygous for the L476P mutation.

3.3.2 Colony breeding data

Following accurate identification of MPS VI kittens at birth and prior to availability of mutation analysis, breeding data was collected to identify obligate heterozygote breeding cats with limited success. Some cats were already proven to be obligate heterozygotes by producing MPS VI kittens in previous pairings. Cats with unknown genotypes, but thought to be heterozygotes from leukocyte enzymology were also kept for breeding. From the total number of kittens born to obligate heterozygote $\times$ obligate heterozygote, unknown genotype $\times$ obligate heterozygote or unknown genotype $\times$ unknown genotype matings, the incidence of MPS VI kittens born was 15.5% (out of a total of 354 kittens). This is much lower than the ~25% expected for an autosomal recessive mode of inheritance. Following accurate heterozygote identification using mutation analysis (see Chapter 6), it was apparent that only 198 kittens had been born to obligate heterozygote $\times$ obligate heterozygote parents, resulting in an incidence of MPS VI kittens of approximately 1 in 4 (27.8%; Table 3.1). Of these MPS VI kittens, 28 were male, and 27 were female. The disparity between identification of obligate heterozygotes by leukocyte enzymology and mutation analysis is discussed further in Chapter 6.

The total incidence of stillbirths was 9.3%, with the majority in first litters of new queens (Table 3.1). Incidence of stillbirths in MPS VI compared with heterozygote/normal kittens was not ascertained. Deaths in liveborn kittens less than 7 days of age was minimal (5.8% of total liveborn), and of these, only 1/25 deaths was an MPS VI kitten. However the number of total kitten deaths (<7 days old) could have possibly been higher as heterozygote/normal kittens were often euthanased as early as possible, particularly in litters containing MPS VI kittens. The causes of death included mismothering and peritonitis secondary to the umbilical cord being chewed off too short by the mother, but in many cases the cause of death was unknown.

Table 3.1: Breeding outcomes in the MPS VI cat colony from September 1993 to June 1996.

Month-Year	No. Litters	No.kittens Liveborn	No. Stillborn	No. died < 56 days	No. MPS VI	No. kittens born H × H*
9-93 to 6-94	33	121	12	5	16	46
7-94 to 6-95	48	199	14	13	16	74
7-95 to 6-96	28	110	14	13	23	78
TOTAL	109	430	40 (9.3%)	31 [†] (7.2%)	55 (27.8% [‡])	198

* H × H denotes obligate heterozygote × obligate heterozygote mating retrospectively determined by mutation analysis

† 25 (5.8%) of these died at less than 7 days old

‡ percent MPS VI kittens born from total liveborn kittens from H × H mating

3.3.3 Colony general health

Diagnostic veterinary pathology from either whole carcasses or selected tissues was performed by John Finnie at Vetlab, in the Institute of Medical and Veterinary Science, Adelaide, and samples were also submitted to Vetlab for viral isolation, chlamydial detection and parasitology.

Throughout the course of this study there were a number of outbreaks of disease that affected kitten growth to greater or lesser extents in individuals. Transient diarrhoea with some dehydration was observed intermittently in both unweaned and weaned kittens, and was usually responsive to medication with Ensaf®. On occasions, faecal samples were submitted for parasite examination, and coccidia and cryptosporidia were frequently observed in significant numbers.

Initially the greatest problem with kitten health were seasonal outbreaks of severe upper respiratory tract infections due either to feline calicivirus or herpesvirus ("cat flu") and flea infestation. Fleas were quickly controlled with monthly Program® medication, however respiratory disease was endemic despite heavy vaccination. Herpesvirus was isolated from several dead kittens in one outbreak (September 1995), and diagnosis was based on histological grounds thereafter. Virulence of the herpesvirus was high, with high morbidity in kittens less than 3 or 4 months old and high mortality in several litters of young kittens. There appeared to be no difference in morbidity between MPS VI and heterozygote/normal cats with herpesvirus infection. Modification of the vaccine schedule to include vaccinations prior to 6 weeks of age (see Section 2.2.2) reduced the morbidity to a certain degree. Lingual ulceration was observed in several cats which is rare in herpesvirus and is more characteristic of feline calicivirus (Jubb *et al.*, 1985), although this was never isolated or positively identified. In April 1996, herpesvirus was suspected in an outbreak of abortions in at least three cats at approximately 4-5 weeks of gestation, although no causative agent was isolated from aborted material.

Two confirmed outbreaks of feline parvoviral enteritis occurred in December 1993 and May 1994 and caused the death of one heterozygote/normal and three MPS VI kittens. All the MPS VI kittens were undergoing ERT. No other deaths were positively attributed to parvoviral infection.

Prior to positive diagnosis of herpesvirus, persistent conjunctivitis became an endemic problem in the majority of kittens born, and one heterozygote cat with persistent symptoms was diagnosed with Chlamydial conjunctivitis after repeated conjunctival swabbing of a number of affected cats. Significant improvement was observed after several 2 month long "campaigns" of daily oral medication of all cats within the colony with lincomycin/spectinomycin.

3.3.4 Clinical Findings

The onset of some clinical features and their subsequent development was variable among MPS VI cats. A number of MPS VI kittens were examined systematically at certain ages, and were compared with observations in heterozygote/normal kittens.

3.3.4.1 *Physical appearance*

At birth, MPS VI kittens were indistinguishable from their unaffected littermates. Corneal clouding was first grossly visible at 5 - 6 weeks of age, and clearly obvious by 7 weeks. By 6 - 8 weeks of age, kittens appeared to have a slightly broader face and body when compared with littermates with changes in physical appearance becoming clearly apparent by 3 months of age. Distinguishing features becoming more obvious with increasing age, included very mild corneal opacity, small ears, short neck and tail, reduced body length and bodyweight, abdominal distension (due to the reduced bodylength), reduced cervical spine flexibility, variable degrees of pectus excavatum (which resulted in a broad chest), and kyphosis of the thoracolumbar spine. Long bone growth (forelegs and hindlegs) did not appear to be as severely affected (reduced) as vertebral growth, so that most cats appeared to have similar heights, although several individuals had noticeably shorter statures.

Corneal clouding assessed by slit lamp examination was mild and varied slightly between individuals. The oldest MPS VI cat examined was 2 years old, and compared with younger cats, clouding did not appear to increase in severity with increasing age.

The vast majority of MPS VI cats maintained a bright and affectionate demenour, seeking interaction with animal care staff despite physical limitations as disease progressed.

3.3.4.2 *Bodyweights*

In MPS VI males and females, significantly lower bodyweights were observed at 3 months compared with heterozygote/normal of the same sex (2-tailed t-test (equal variance), $p < 0.007$ males; $p < 0.002$ females). This difference became greater as heterozygote/normal

cats continued at a similar or greater rate of growth, while growth rate in MPS VI cats began to reduce after 3 months (Figure 3.3).

Differences in mean bodyweights between heterozygote/normal males and females was evident at 45 days (2-tailed t-test, (equal variance) $p < 0.05$), and became more obvious with increasing age (at ≥ 3 months, $p < 0.05$ to $p < 0.01$). Bodyweight in MPS VI males compared with MPS VI females was only statistically different at 6 months ($p < 0.05$). Growth rate was approximately linear up to 5 months and then began to plateau off (Figure 3.3). Heterozygote/normal males continued significant weight gains and reached approximate adult weight by 11 months. Mean bodyweight of adult heterozygote/normal males (>11 months) in the colony was 4625 ± 506 grams ($n=7$).

Obvious variability in disease severity was observed in both male and female MPS VI cats, with some cats appearing small and stunted in growth, while others appeared much larger and heavier. Using the Levene test for equality of variance to compare variability between MPS VI and heterozygote/normal cats of the same sex, no statistically significant difference between the two groups was measured. This indicated that the variability observed in MPS VI bodyweights was similar to the variability observed in heterozygote/normal cats.

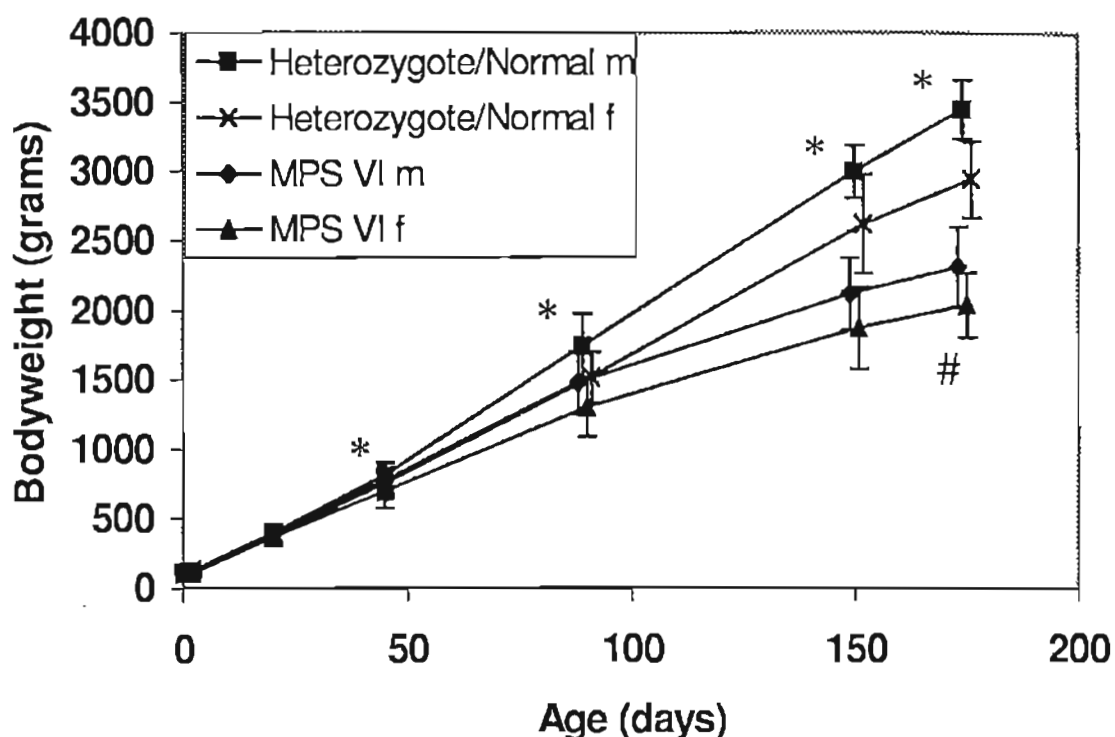


Figure 3.3: Mean bodyweights from birth to 6 months of age.

Mean bodyweights (grams) in heterozygote/normal and MPS VI, male (m) and female (f) cats from birth to 6 months of age. Error bars = 1 SD. Number of animals at each point in each group usually 10 or more except for 0-20 days of age where some groups contain only 2-5 animals. Statistically significantly lower bodyweights in MPS VI cats were observed at 3 months and older, compared with heterozygote/normal of the same sex ($p < 0.01$).

* denotes significant difference in mean bodyweights between heterozygote/normal males and females ($p < 0.05$ to $p < 0.01$).

denotes significant difference in mean bodyweight between MPS VI males and females ($p < 0.05$).

3.3.4.3 Flexibility

When first examined at 3 months, MPS VI cats showed mild reduction in cervical spine flexibility of $\sim 140^\circ$ lateral movement (range $130^\circ - 170^\circ$; normal is 180°). Flexibility was reduced further at 5 and 6 months to $\sim 70^\circ$ (range $45^\circ - 130^\circ$) and $\sim 50^\circ$ (range $30^\circ - 100^\circ$) respectively. By 11 months, the range was further reduced to $\sim 45^\circ$ (range $20^\circ - 80^\circ$) lateral movement. In heterozygote/normal cats, 180° lateral movement was possible at all ages examined.

Extension of the hips in MPS VI and heterozygote/normal cats was quite variable, with some animals in both groups appearing tense and reluctant to cooperate. However, overall there was a general reduction in flexibility among MPS VI cats with increasing age (range $90 - 180^\circ$) compared with heterozygote/normal (150 - 180°).

There were no obvious differences in physical activity or ability to climb in MPS VI kittens compared with unaffected littermates at younger ages, however after 3-4 months of age, MPS VI kittens gradually became less active, playing less vigorously and for shorter periods of time compared with littermates.

3.3.4.4 Neurology

Subtle hindlimb gait deficits due to spinal cord compression at multiple levels were first observed in some MPS VI cats as early as 75 - 80 days of age, but onset was more commonly seen between 4 and 5 months of age (Table 3.2). Deficits were initially most apparent when the hindquarters were pushed off balance and on turning, with affected animals unable to maintain balance by stepping out with a hindlimb. Animals also treaded heavily and tended to sit down quickly after walking to avoid overbalancing in the hindlimbs. Progression of neurological deficits was variable, with some animals progressing to severe paresis or paralysis over several weeks, and some progressing over more than 4 weeks. As the spinal cord compression progressed, the hindquarters became more unstable during walking, with wide based hindlimb stance, the metatarsals were held a lot lower (i.e. no longer walking just on phalanges), and toe scuffing, until gait became a combination of

dragging hindlegs or a wobbly, sometimes “marching” gait. Climbing was still possible in some cats at this stage using forelimbs.

Some cats progressed to full hindlimb paralysis, dragging hindlimbs completely with increased extensor tone and some had noticeably enlarged bladders due to neurogenic urine retention. Some cats still maintained or intermittently showed some (very limited) voluntary movement of one or both hindlimbs, and this was still termed paralysis because of lack of ability to walk using the hindlimbs. Several cats had unexpected partial recovery from hindlimb paralysis (see footnotes to Table 3.2). These cats had been dragging hindlimbs fully, one to the extent of developing skin abrasions on the hocks. Deep pain appeared present, with increased extensor tone and slight muscle atrophy. Thoracic limbs appeared neurologically normal.

There did not appear to be any correlation with degree of metaphyseal flaring of the vertebrae on radiographs (see Radiological features, Section 3.3.4.6), and development of severe paresis or paralysis in the hindlimbs.

Table 3.2: Development of hindlimb neurological deficits in MPS VI cats.

Age (months)	normal	mild deficits	mild-moderate paresis	paralysis	n
3	16	3	2	0	21
5	5	4	7	5	21
6	2	2	10*	7	21
7	1	0	2	2†	5
9	1	0	2‡	1	4
11	1	0	3§	0	4

* 1 cat (239f) had full hindlimb paralysis between 120 and ~150 days, but by 150 days had moderate hindlimb paresis.

† 1 cat (163m) with hindlimb paralysis was euthanased at 7 months with bladder enlargement and urine retention due to hypertonicity of the bladder sphincter.

‡ 1 cat (222f) was paralysed at 7 months but was then able to walk (severe paresis) at 9 months.

§ 1 cat (223m) was paralysed at 9 months but was then able to walk (severe paresis) at 11 months.

n, number of animals examined

3.3.4.5 Other findings

Routine anaesthesia at 3 months of all kittens revealed that MPS VI cats tolerated higher doses of both xylazine and ketamine administered subcutaneously, and that anaesthesia was maintained for a significantly shorter duration. This phenomenon was not evaluated using different anaesthetic agents or routes of administration.

3.3.4.6 Radiological features

From radiographs taken at birth (0-2 days old, heterozygote/normal, n = 6; MPS VI, n = 1), no secondary centres of ossification were present in any kitten. There were no obvious

differences in skeletal appearance (i.e. primary centres only) in the MPS VI kitten compared with the heterozygote/normal, but when closely compared, the dorsoventral width of the lumbar vertebrae was possibly slightly increased, and the metaphyses of several long bones were possibly slightly wider. By 20 days of age (heterozygote/normal, $n = 4$; MPS VI, $n = 2$), secondary centres of ossification were obvious in all cats in the proximal and distal femur, tibia and humerus, and in the distal radius and ulna, and were just becoming apparent in several vertebrae. When MPS VI kittens were compared with littermates, the secondary centres of ossification were slightly smaller, but due to variation between litters, overall it was generally not possible to confidently distinguish MPS VI kittens on this basis. Some mild changes in dimensions were observed in the primary centres of ossification although changes were still subtle and variable between the two MPS VI kittens examined (both kittens were from different litters).

By 53 days of age (heterozygote/normal, $n = 4$; MPS VI, $n = 4$), differences in skeletal appearance between heterozygote/normal and MPS VI kittens were obvious. In MPS VI kittens in general, flaring or widening of the metaphysis of the primary centres of ossification, and slightly smaller secondary centres of ossification than normal, made the epiphyses appear smaller and incongruent in size relative to the primary centre. This was very obvious in the lumbar vertebrae, but could also be seen in the proximal humerus. Cervical and lumbar vertebrae also appeared shorter and broader, and in particular, the endplates in the cervical vertebrae appeared smaller and slightly irregular. Actual sizes of the main secondary centres of the humerus, femur and tibia were marginally smaller in three of the MPS VI cats compared with heterozygote/normal. In the fourth MPS VI cat, secondary centres were much smaller, and metaphyseal flaring of the primary centres and reduced length, was very obvious and much more severe than in the other three MPS VI kittens, one of which was a littermate.

Similar changes were observed at 3 months of age compared with 53 days, except flaring of metaphyses in long bones was not obvious. In addition, epiphyses in cervical vertebrae were more irregular, and the subchondral bone surface of the proximal humeral epiphysis was irregular. A coarse trabecular pattern in the distal femoral epiphysis was now also more obvious. Variable density in the lumbar vertebra was becoming apparent, with radiodense

vertebral endplates and radiolucent vertebral bodies. Variability in MPS VI cats was very obvious in lumbar vertebral appearance, but only subtle variations were observed in the long bones and cervical vertebrae between different MPS VI cats.

At 6 months of age, skeletal changes were widespread and obvious, again with variable degrees in severity (Figure 3.4). In particular, cervical vertebrae appeared to be more severely affected than lumbar vertebrae, with very irregular epiphyses and shortened overall length (Figure 5.6). The shorter and wider lumbar vertebrae had a characteristic increased radiodensity at the epiphyses with a radioopaque (osteopaenic) vertebral body, and the epiphyses were also irregular. Pedicles of the dorsal vertebral arches were also broader (seen in the ventrodorsal view)(Figure 5.5). Variable degrees of degenerative changes were seen in the lumbar vertebral articular facets, which appeared as increased radiodensity, and resulted in variable degrees of thoracolumbar kyphosis. The majority of MPS VI cats had obvious flaring of the lumbar vertebral epiphyses, although this varied quite considerably in severity (Figure 3.4). A number had ventral lumbar spondylosis at several intervertebral disc spaces, and several cats had obvious ventral spondylosis in the cervical and thoracic spine. Sternebrae were short and broad, and most had broader costochondral junctions. MPS VI cats had a very obvious coarse trabecular pattern in the distal femoral and generally in the proximal humeral epiphyses, with irregular (fluffy) subchondral bone contours, increased joint space, and incomplete development or extensive remodelling of the caudal aspects of the proximal humeral and distal femoral condyles. These and other epiphyses were generally smaller than normal. There was variable degrees of coxofemoral subluxation, with variable sized proximal femoral epiphyses and variable remodelling of the epiphyses.

In older MPS VI cats (11 and 30 months), skeletal changes gradually increased in severity, including a generalised osteopaenia. Progressive cortical bone thinning was particularly evident in the iliac wings of the pelvis (ventrodorsal view) and long bones, together with some bone remodelling. A coarse trabecular pattern became more obvious in epiphyses with increasing age, as well as more irregular subchondral bone contours and osteophyte formation in some joints, with subchondral bone sclerosis. Thoracolumbar kyphosis increased in severity with increasing age largely due to degenerative changes in the vertebral dorsal articular facets.

Other abnormalities which were commonly present in the MPS VI cats were pectus excavatum, and lack of fusion of the odontoid. Several cats also had a unilateral fractured patella, one cat fractured the proximal humeral epiphysis in one shoulder, and one cat had severe inversion of the sternum.

Figure 3.4: Variability in radiographic appearance of skeletal disease in MPS VI cats at 6 months of age.

Lateral radiographic view of the lumbar spine in 6 month old MPS VI cats. (A) Cat 106f exhibited probably the most severe skeletal changes of all MPS VI cats examined at 6 months of age; (B) The changes present in cat 163m are representative of the majority of 6 month old MPS VI cats examined; (C) Cat 159f was one of two MPS VI cats with the mildest skeletal changes. The severity of skeletal disease on the views shown reflected the severity of overall clinical and skeletal disease in each cat. All cats exhibit characteristic increased radiodensity of the vertebral epiphyses with radioopaque (osteopaenic) vertebral bodies. Variability in lumbar vertebral shape (both length and width), regularity of vertebral epiphyses, and degree of degenerative changes in the vertebral articular facets is obvious among the three different cats. Metaphyseal flaring and vertebral spondylosis is also present in (A).



3.3.4.7 Skeletal measurements - male vs female

Comparison of bone dimensions between male and female cats from both MPS VI and heterozygote/normal groups was made at 3, 5, 6, and 11 months of age. Dimensions were taken from approximately 10 animals in each group at 3-6 months of age and only 1 male and 3 female MPS VI cats at 11 months of age. Greater numbers of heterozygote/normal females were measured, with ~25 animals at 3-6 months and 8 animals at 11 months of age. There were many statistically significant differences in bone dimensions between heterozygote/normal males and females, but only a few significant differences between MPS VI males and females.

In heterozygote/normal males and females, these differences generally became more significantly different with increasing age. From the first measurements at 3 months of age, C3 and L5 width, tibial length, nose to occiput length and bodyweight were significantly higher in males than in females. At 5 months, C3 length and patella length also were significantly higher in heterozygote/normal males, and this was generally maintained at 6 (Figure 3.5) and 11 months. L5 length was significantly higher in males only at 11 months. No differences in the tibial corticomedullary ratio was observed between heterozygote/normal males and females, and this parameter also had a large standard deviation. In contrast, the only significant differences between MPS VI males and females was in the nose to occiput dimensions at ≥ 3 months of age. Statistical significance was also almost reached for tibial length at 5 months ($p < 0.075$) but at 6 months, the p value was 0.10 indicating less differences between the two groups. Statistical comparison was not performed between the 11 month old MPS VI male and females as parameters were only available from a single MPS VI male at this age.

In both heterozygote/normal and MPS VI, a large standard deviation (% of mean: 12-28 % MPS VI males and females; 12-23 % heterozygote/normal males and females) was observed for the tibial corticomedullary ratio. This partly reflected the clearly visible differences in cortical thickness observed in different animals, but also probably reflected difficulty in measuring the same region in each animal due to things slight variations in rotation of the tibia, and different tibial lengths.

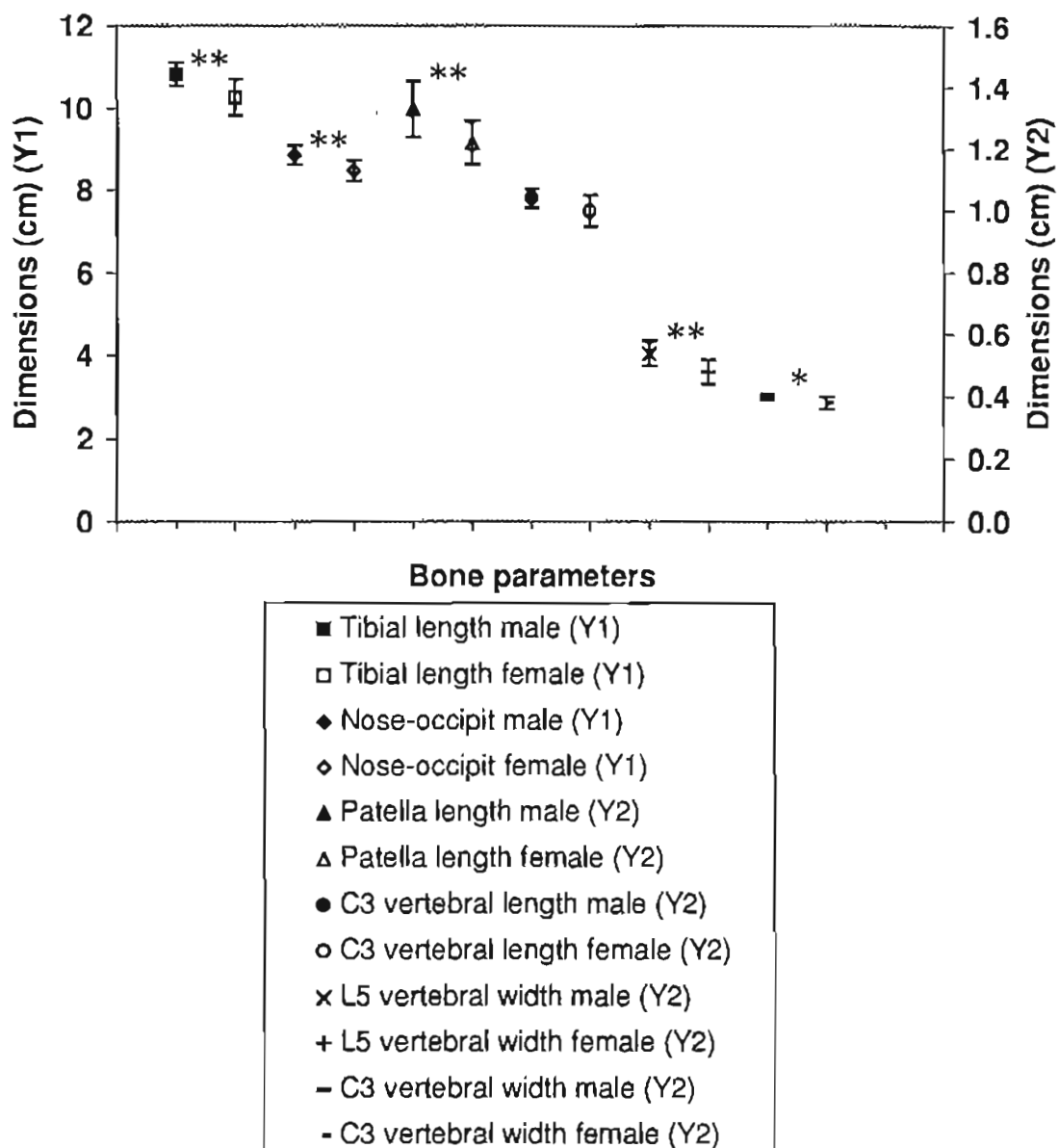


Figure 3.5: Comparison of mean bone dimensions in male and female heterozygote/normal cats at 6 months of age.

Comparison of mean bone dimensions in male (n=7) and female (n=25) heterozygote/normal cats at 6 months of age. Mean L5 vertebral length and tibial corticomedullary ratios were not significantly different at 6 months of age and were not included in this figure. Error bars = 1 SD. * denotes $p < 0.05$, and ** denotes $p < 0.01$, males vs females. Note: Y1 and Y2 axis used.

3.3.4.8 Skeletal measurements - MPS VI vs heterozygote/normal

Further comparison between heterozygote/normal and MPS VI cats of the same sex is presented in Table 3.3, with bone dimensions in MPS VI cats expressed as a percent of the mean value in heterozygote/normal cats. Due to the sex differences in heterozygote/normal cats shown above, data was kept separate between both heterozygote/normal and MPS VI males and females.

Of the bones measured, almost all exhibited significant differences in dimensions between MPS VI and heterozygote/normal cats at most ages examined. The exceptions to this were nose to occiput length at most ages measured, tibial length at 3 months which almost attained statistical significance (males $p < 0.067$; females $p < 0.059$), and L5 vertebral width at 6 months (males) and 11 months (females). Bone dimensions were not affected to the same degree in each bone measured, with the dimensions of the remaining parameters in MPS VI cats being either higher or lower than in heterozygote/normal cats (Figure 3.6). The bone with the most abnormal dimensions in MPS VI cats was the C3 vertebra which only attained 64 to 70% of the normal length at 11 months, but had a greatly increased width of 122 to 146% at 6 and 11 months. Interestingly, the L5 vertebral dimensions were not as severely affected as the C3 vertebral dimensions, which is also very obvious radiographically. Longitudinal growth was much less affected in the tibia, which attained 92 to 93% of the normal length at 11 months.

Bone dimensions also appeared to alter with increasing age (see Table 3.3). Percentage of normal for most parameters tended to decrease with increasing age in both MPS VI males and females. As seen in bodyweight changes discussed in Section 3.3.4.2 and also presented in Table 3.3, this trend with increasing age suggested a decrease in growth rate compared with normal. However, the exception was patella length, which appeared to increase as a percentage of normal with increasing age.

The tibial corticomedullary ratio also decreased between 3 and 6 months, but by 11 months appeared to be higher than the mean value observed at 3 months, and was only just not statistically different between MPS VI and heterozygote/normal females ($p < 0.062$). This

may be due to the low numbers of animals observed at 11 months. Progressive cortical thinning is observed radiographically with increasing age in MPS VI cats.

For the majority of parameters examined, the standard deviations for each measurement were greater in MPS VI cats than heterozygote/normal cats (data not shown), suggesting increased variability in the MPS VI animals. However, using the Levene test for equality of variance to compare variability between MPS VI and heterozygote/normal cats of the same sex, statistically significant differences were present for only some measurements (see Table 3.3). Some of the variability observed for C3 length and width measurements was probably due to the increasing difficulty of making accurate measurements due to the very irregular epiphyses with increased age. It was also interesting that in 3 and 5 month old heterozygote/normal males, the variability in the tibial corticomedullary ratio was greater than in the MPS VI males, probably reflecting increased growth rate and corresponding bone turnover. Some increased variability was also probably present in the female heterozygote/normal group due to the larger number of animals for most ages, which may have reduced statistical differences in variance between heterozygote/normal and MPS VI females.

Table 3.3: Mean bone dimensions and bodyweights of male and female MPS VI cats at different ages, expressed as a percentage of mean values from male and female heterozygote/normal cats.

Parameter	Age (months)	Male MPS VI (% heterozygote/ normal $\pm$ sd)	Female MPS VI (% heterozygote/ normal $\pm$ sd)
Third cervical vertebral width	3	126 $\pm$ 9 ^b	124 $\pm$ 14 ^{d,e}
	5	121 $\pm$ 12 ^{b,e}	121 $\pm$ 15 ^{d,e}
	6	122 $\pm$ 13 ^{b,e}	123 $\pm$ 16 ^{d,e}
	11	143 [†]	146 $\pm$ 18 ^{c,e}
Fifth lumbar vertebral width	3	116 $\pm$ 10 ^b	119 $\pm$ 14 ^{d,e}
	5	116 $\pm$ 15 ^a	115 $\pm$ 22 ^c
	6	112 $\pm$ 18	111 $\pm$ 14 ^c
	11	109	123 $\pm$ 14
Nose to occipit	3	101 $\pm$ 3	100 $\pm$ 3
	5	98 $\pm$ 3	98 $\pm$ 2
	6	97 $\pm$ 2 ^a	98 $\pm$ 2 ^d
	11	96	100 $\pm$ 2
Tibial length	3	94 $\pm$ 8	97 $\pm$ 5
	5	91 $\pm$ 7 ^{b,e}	92 $\pm$ 5 ^d
	6	90 $\pm$ 7 ^{b,e}	91 $\pm$ 4 ^d
	11	93	92 $\pm$ 2 ^d
Fifth lumbar vertebra length	3	81 $\pm$ 10 ^{b,e}	80 $\pm$ 10 ^{d,e}
	5	75 $\pm$ 7 ^{b,e}	74 $\pm$ 7 ^d
	6	74 $\pm$ 7 ^b	75 $\pm$ 6 ^d
	11	69	74 $\pm$ 3 ^d
Patella length	3	61 $\pm$ 16 ^b	68 $\pm$ 14 ^d
	5	77 $\pm$ 9 ^b	80 $\pm$ 7 ^d
	6	76 $\pm$ 10 ^b	80 $\pm$ 7 ^d
	11	80	82 $\pm$ 3 ^d
Third cervical vertebral length	3	70 $\pm$ 9 ^{b,e}	75 $\pm$ 6 ^d
	5	62 $\pm$ 8 ^{b,e}	66 $\pm$ 8 ^d
	6	61 $\pm$ 8 ^{b,e}	66 $\pm$ 8 ^d
	11	64	70 $\pm$ 4 ^d
Tibial corticomedullary ratio	3	56 $\pm$ 11 ^{b,f}	74 $\pm$ 21 ^d
	5	47 $\pm$ 6 ^{b,f}	52 $\pm$ 15 ^d
	6	46 $\pm$ 6 ^b	51 $\pm$ 12 ^d
	11	73	77 $\pm$ 13
Bodyweight	3	85 $\pm$ 13 ^b	86 $\pm$ 14 ^d
	5	71 $\pm$ 9 ^b	71 $\pm$ 11 ^d
	6	67 $\pm$ 8 ^b	69 $\pm$ 8 ^d
	11	59	75 $\pm$ 5 ^d

Numbers of animals in each group at each time point was 9-11, with the exception of only 3 MPS VI females and 1 MPS VI male being measured at 11 months of age.

† statistical analysis for heterozygote/normal male vs MPS VI male not performed for all 11 month values as only one animal in MPS VI male group.

a indicates $p < 0.05$, b indicates $p < 0.01$, two-tailed t-test assuming unequal variance, heterozygote/normal male vs MPS VI male.

c indicates $p < 0.05$, d indicates $p < 0.01$, two-tailed t-test assuming unequal variance, heterozygote/normal female vs MPS VI female.

e indicates $p < 0.05$, Levene test for equality of variances, MPS VI vs heterozygote/normal (males and females compared separately). Variance greater in MPS VI than heterozygote/normal.

f indicates $p < 0.05$, Levene test for equality of variances, MPS VI vs heterozygote/normal (males and females compared separately). Variance greater in heterozygote/normal than MPS VI.

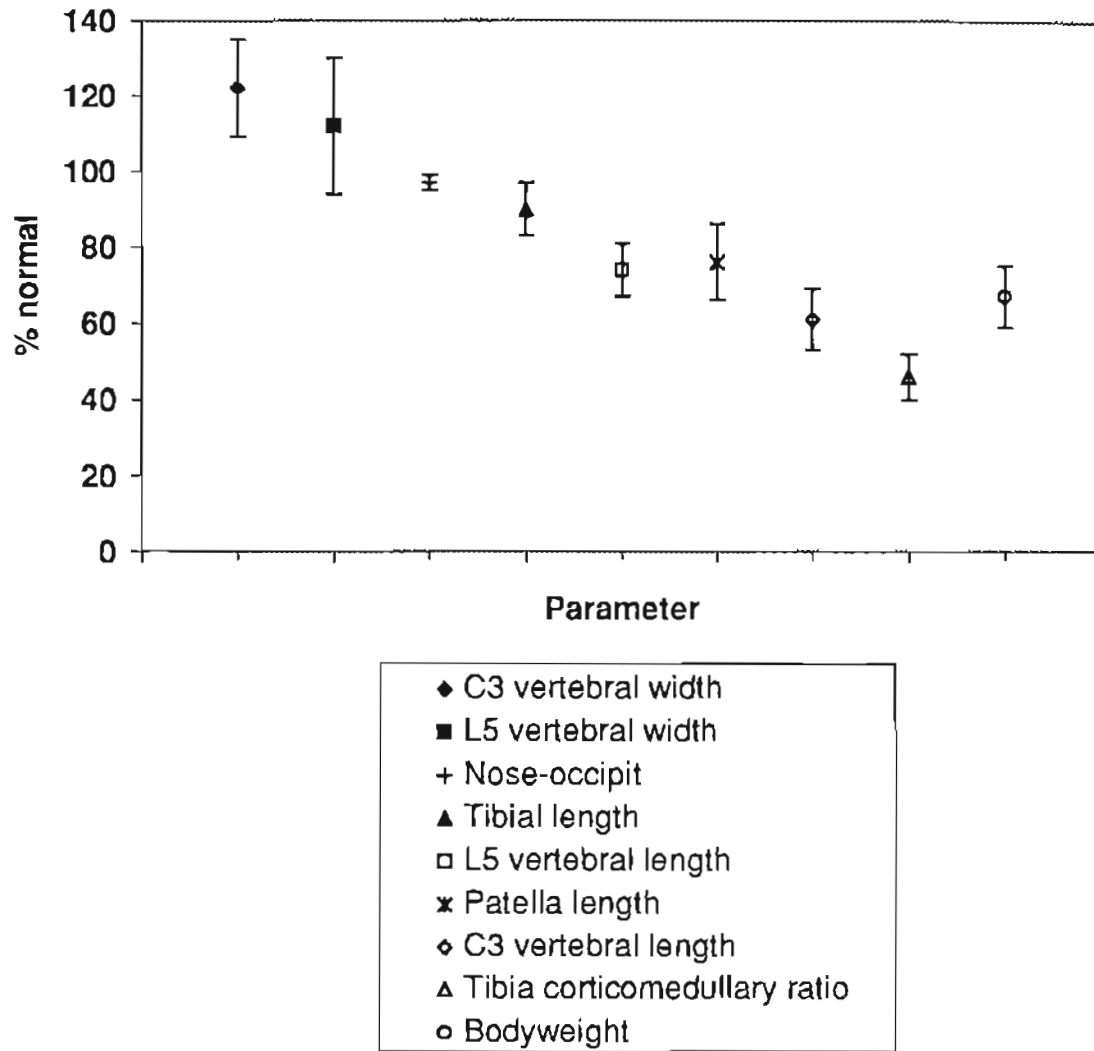


Figure 3.6: Mean bone dimensions (percentage of normal) in male MPS VI cats at 6 months: variable severity in different bones.

Comparison of mean dimensions in different bones expressed as a percentage of normal from male MPS VI cats at 6 month of age (n=9). Mean bodyweight is also included. Vertebral widths are increased compared with normal and C3 vertebral length is the most reduced length. Error bars = 1 SD.

3.3.5 Gross pathology

3.3.5.1 *General postmortem observations*

The majority of MPS VI cats were euthanased at 6 months of age, with four examined at 11 months, and one at 30 months of age. The 30 month old cat was not euthanased at a younger age because she maintained the ability to walk at all times without any neurological deficits. This may have suggested that this cat was less severely affected than the majority of other MPS VI cats, however based on all other parameters, disease appeared to progress and increase in severity with increasing age. The major differences observed at postmortem with increasing age were associated with degenerative joint changes and marginally increased thickening of the heart valves.

There were no noticeable abnormalities in gross appearance of the abdominal viscera, with liver and spleen appearing normal in size. In the thoracic cavity, lungs grossly appeared normal, however tracheal cartilage rings were irregular in shape, so that the tracheal shape was bumpy and not uniform in sections. Once removed from the thoracic cavity, the thoracic portion of the aorta in normal cats was thin and collapsible for most of its length. In MPS VI cats, it was rubbery and maintained its tubular shape and the vessel walls subjectively appeared thicker. The heart externally appeared normal, however usually the free margin of the mitral valve, and rarely the tricuspid valve, appeared thickened and bumpy. The degree of thickening was variable between different cats.

In one 6 month old MPS VI cat with a very loud uncharacterised heart murmur (238f), half of the mitral valve was dysplastic, leaving a large hole in one of the valve leaflets. Four MPS VI kittens had very loud “machinery” heart murmurs, as well as very poor growth rates compared with littermates. Euthanasia was necessary before 35 days due to cyanosis and impending death due to the stress of handling in 1 case, and poor growth. Patent ductus arteriosus was present at postmortem in all four kittens. It is not possible to determine the true incidence of these defects in the colony as a whole, as many heterozygote/normal kittens were euthanased without cardiac auscultation or postmortem, including several that had poor growth rates.

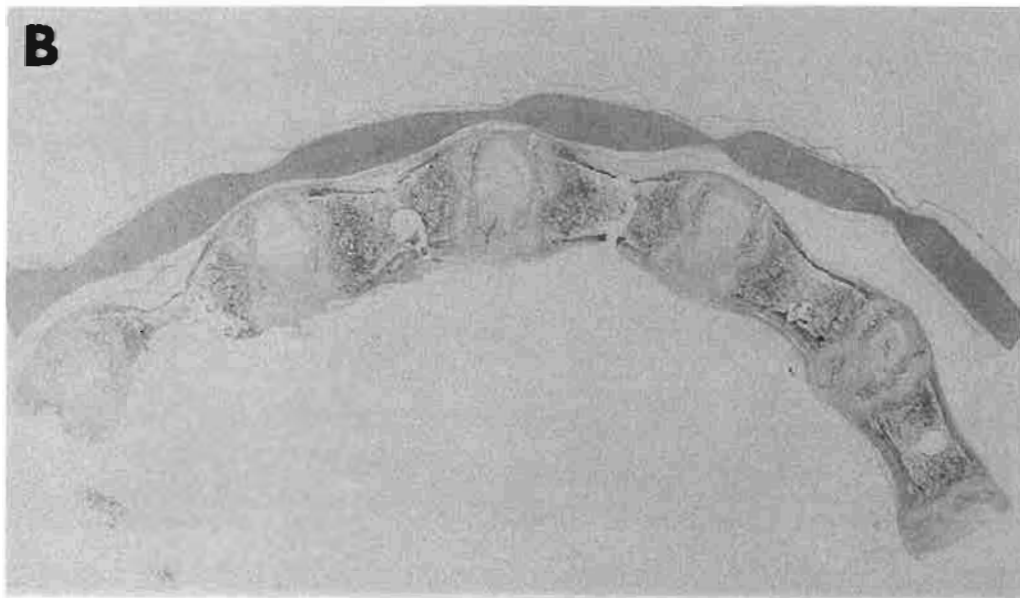
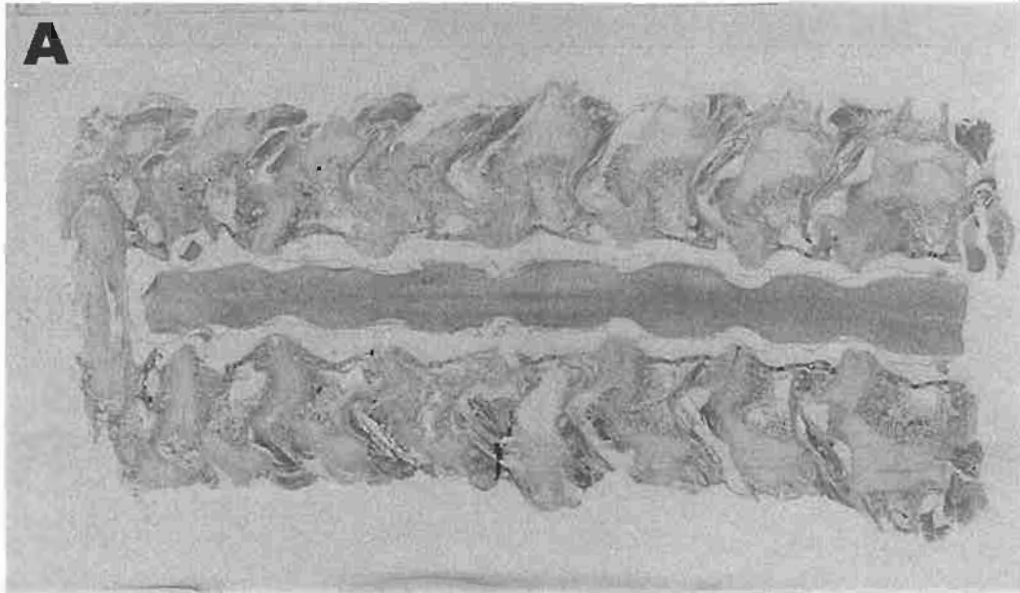
Exposure of the spinal cord by removal of the dorsal vertebral arches from C4 - L4 revealed cord compression at multiple levels in MPS VI cats. The presence of compression correlated well with the presence of neurological deficits just prior to euthanasia, however it was not possible to correlate the location and severity of cord involvement with severity of neurological impairment. Only 2 out of 21 6 month old cats had no obvious cord compression. One of these cats was physically and radiographically probably the most severely affected MPS VI cat (106f), while the other was one of the mildest (135f). The regions most commonly affected by compression were T2-T9 and T13-L4, with the most severe lesions being found at T2-T5 and T13-L3. Overall, lesions were observed in the C7/T1 to T9/10 region and T11/12 to L3/4 region. Histological sections of spinal cord *in situ* showed that cord compression was due to narrowing of the spinal canal at vertebral junctions due to bony/cartilagenous flaring of the epiphyses and dorsal articular facets, and some bulging or hyperplasia of the dorsal region of the anulus fibrosus (Figure 3.7 A and B). In some cases, the thoracic spinal cord was dorsolaterally compressed to almost a pear shape when viewed in cross section. No cord compression was observed in the upper cervical spine (C1-C4) on sections of the cord *in situ*.

In two 11 month old MPS VI cats in which some reversal in severity of hindlimb neurological abnormalities was observed (see footnotes in Table 3.2 above), severe cord compression was found in one animal (223m) due to an osteophyte impinging on the cord at L3-L4. Elsewhere this cat had very mild compression in the thoracic region (T2-4, T5-8). Only mild compression was evident from T2-T9 and L1-L3 in the second cat (222f). No cord compression was present in any heterozygote/normal cats examined. During removal of the dorsal vertebral arches, thickening around the articular facet joints was often obvious in MPS VI cats. This was interpreted to be degenerative joint changes similar to processes occurring in the major joints examined in the limbs.

Enlarged lateral ventricles in the brain have been reported in MPS VI cats (Haskins *et al.*, 1980). This was initially found to be very difficult to assess, and was not examined further.

Figure 3.7: Histological sections of spinal cord *in situ* in a 6 month old MPS VI cat.

Spinal cord *in situ* from a 6 month old MPS VI cat demonstrating spinal cord compression at multiple levels at vertebral junctions. (A) transverse sections of thoracic spinal cord (T1-T8); (B) median sections of thoracolumbar spinal cord (T13-L4).(H & E).



3.3.5.2 *Postmortem joint examination*

The shoulder, elbow, hip and stifle joints were examined in 6 month (n = 17), 11 month (n = 4) and 30 month (n = 1) old MPS VI cats. Normal control cats (for joint examination only), were restricted to the D520N/normal genotype (see Chapter 6). Degenerative joint changes were observed in these joints in MPS VI cats, increasing in severity with increasing age. Changes were also variable between different age-matched MPS VI cats. Erosive lesions in the articular cartilage were generally found in specific locations within each major joint, and these were thought to correspond to either high load bearing regions or to regions with very thickened cartilage.

Thickness of cartilage was assessed using the degree of whiteness of cartilage, i.e. very thick cartilage appeared white, whereas thin cartilage was pinkish purple due to the colour of the subchondral bone directly underneath the articular cartilage. Thickness of cartilage was also assessed by shaving pieces off for electron microscopy. In normal cats, cartilage thickness decreased with increasing age, with joints increasing in pinkish colour intensity and cartilage becoming very difficult to shave off (Figure 3.8 A and B). There was variability in cartilage thickness in different MPS VI cats, with a tendency of smaller “more severe” MPS VI cats to have thicker cartilage and smaller joints, indicative of more poorly developed epiphyses. In addition, those with thicker cartilage usually had more obvious irregularities in subchondral bone, giving a spotty appearance underneath the articular surface. In general, synovial hyperplasia with increased synovial fluid was observed in most joints, even in those lacking obvious lesions in the articular cartilage surface (Figure 3.8 C and D).

In the shoulder joints of 6 month old MPS VI cats, increased cartilage thickness, was evident particularly over the ball of the proximal humerus, with irregular subchondral bone visible in other regions with thinner cartilage. In 20 out of 34 joints examined in 6 month old cats, erosions in the cartilage surface were present where the biceps tendon passes over the joint into the intertubercular groove, in the cranial aspect of the joint (Figure 5.17 C and D). These lesions varied greatly in size and depth, some being up to ~2 mm deep. In normal cats, the articular cartilage was very thin in this region. Larger erosive lesions in the same

region were generally observed in the 11 month old cats, although no erosions were present in one cat (222f). Additional cartilage fibrillation and erosions over the ball of the proximal humerus were also observed in one 11 month old cat (223m)(Figure 5.17 D). The most severe lesions were observed in the 30 month old MPS VI cat. This cat exhibited severe erosions and extensive loss of articular cartilage exposing subchondral bone in one shoulder which was also severely remodelled (see Figure 6.8 G). In 6 and 11 month old cats, the articular cartilage in the glenoid of the scapula was also thickened. All cats had a central dark discolouration in the subchondral bone/cartilage, which in some cases was an actual fissure, thought to be the site of the growth plate for the glenoid tuberosity.

It was hypothesised that erosive lesions were being caused by overuse or increased loads in the shoulders, however no obvious correlation in 6 and 11 month old cats between the presence of lesions in the shoulder and paralysis in the hindlegs was observed.

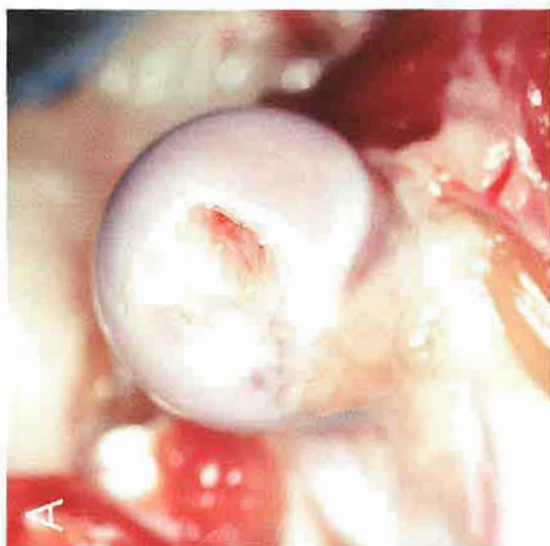
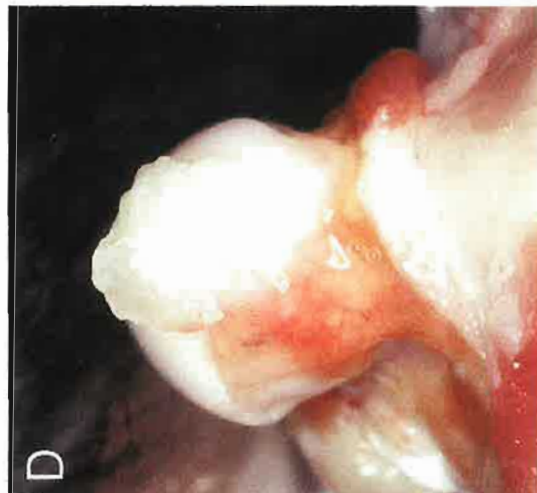
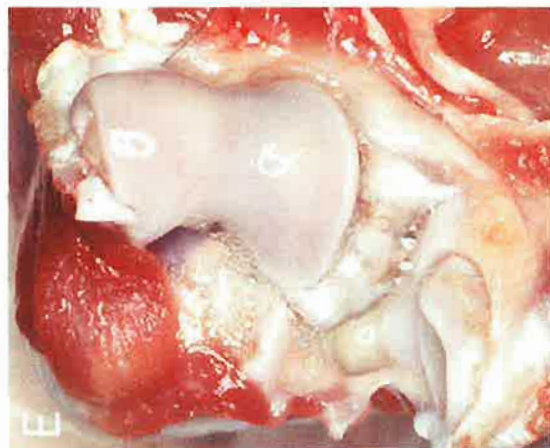
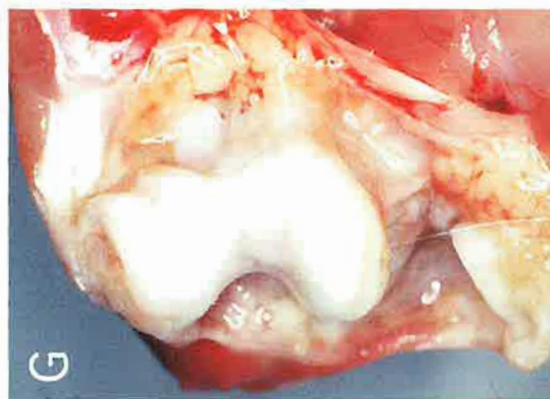
Degenerative joint changes in the hip (coxofemoral joint) were not apparent in most 6 month old MPS VI cats compared with the changes observed in other joints (Figure 3.8 C). Overall the femoral heads were smaller and flattened, and were relatively loose in the acetabulum. Thickness of cartilage increased but was quite variable over different regions of the articular surface. In many cats, synovium on the ventromedial aspect of the joint was reddened and/or hyperplastic(C). Several 11 month old cats had slight erosion or “receding” of the articular cartilage in the same region (Figure 3.8 D). In the 30 month old cat, the femoral head was small with extensive remodelling, loss of articular cartilage and osteophyte development.

The articular cartilage in the MPS VI elbow joints was also thicker than normal (Figure 3.8 G and H). Articular cartilage separated to form blisters then flaps typically on the cranial aspect of the lateral distal humeral condyle. These lesions were observed in only several 6 month old cats, but were present in all 11 month cats (Figure 3.8 H). In normal control cats, the articular cartilage in the same region was slightly whiter. In the 30 month old cat, the whole joint was abnormal, with almost complete loss of normal cartilage and osteophyte development. Cartilage was very thickened on the medial rim of the medial condyle and by 11 months of age, erosions or flaps had developed in this region (Figure 3.8 H).

Degenerative changes in the stifle (femorotibial) joint were not obvious in most cats at 6 months of age, however erosions adjacent to the cruciate ligaments were present in the 11 month and 30 month old cats. Osteophytes were also present in the older cats, seen as thickening of the trochlear ridges and also on the most proximal aspect of the trochlea. Thick white cartilage was present in the trochlea of many 6 month old cats, however others had thinner cartilage in this region. The subchondral bone in the trochlea was also spotty and irregular in some cats. Cartilage was very thick in all the cats at all ages on the ventral aspect of the femoral condyles. When the cartilage was removed from the condyles, the subchondral bone was very irregular with whitish and red spicules interdigitating with cartilage, reminiscent of the irregular subchondral bone contours and coarse trabecular pattern seen on radiographs. In normal cats, cartilage on the ventral aspect of the condyles appeared slightly whiter, although it was still thin and difficult to remove. One MPS VI cat also had a fractured patella (221f).

Figure 3.8: Gross appearance of the hip and elbow joints in MPS VI cats compared with normal control cats at 6 and 11 months of age.

The hip joint (A-D, femoral head; ventromedial aspect) and elbow joint (E-H, distal humeral condyle in the top half of each figure, with the lateral condyle uppermost, and the proximal radius/ulna in the bottom half) at postmortem. Views A, C, E, G are from 6 month old cats, and views B, D, F, H are from 11 month old cats. In normal control cats, cartilage is thin with the pinkish purple subchondral bone clearly visible in both joints at both 6 and 11 months (A, B, E, F). The femoral head is rounded (A, B) and fits tightly in the acetabulum. In MPS VI cats, the articular cartilage is thick and white in both joints at 6 and 11 months (C, D, G, H), and some remodelling changes in the shape of the joints is apparent, particularly in the femoral head, increasing in severity between 6 months (C) and 11 months (D). Mild synovial hyperplasia is apparent on the ventromedial aspect of the femoral head at 6 months (C), with erosion of articular cartilage at the joint margins and increased synovial hyperplasia evident at 11 months (D). In the elbow joints in MPS VI cats, at 6 months of age the predominant feature is thickened cartilage, but by 11 months, blister formation and erosion of the cartilage is apparent on the lateral and medial distal humeral condyles respectively.



3.3.5.3 Bone histomorphometry

Compared with normal cats at all ages examined, MPS VI cats had greatly reduced bone mineral volume indicating a marked osteopaenia. This was also reflected in the reduced trabecular thickness and number, and increased trabecular separation. Both within normal and MPS VI groups, values for each parameter at different ages appeared similar (Table 3.4). At 6 months of age there were no statistical differences between males and females in both the normal and MPS VI groups (data not shown), therefore data from male and female cats was combined for each group.

Table 3.4: Static bone histomorphometric data for normal and MPS VI cats at different ages.

Age (months)	n	BV/TV	Tb. Th.	Tb. Sp.	Tb. N.
Normal* 6m	10	19.64 ± 5.30 [†]	81 ± 12	0.35 ± 0.08	2.38 ± 0.39
Normal 11m	3	17.41 ± 2.18	79 ± 3	0.38 ± 0.06	2.20 ± 0.28
MPS VI 6m	15	4.10 ± 1.11	43 ± 9	1.05 ± 0.25	0.97 ± 0.28
MPS VI 11m	4	2.36 ± 1.09	43 ± 4	2.03 ± 0.81	0.54 ± 0.21

* Normal control L5 vertebrae were only collected from D520N/normal genotypes (see Chapter 6).

† Results are expressed as the mean ± 1 standard deviation where sufficient numbers were analysed. Data for males and females is combined.

BV/TV (%) = bone mineral volume; Tb. Th. (µm) = trabecular thickness;

Tb. Sp. (mm) = trabecular separation; Tb. N. (per mm) = trabecular number;

3.3.6 Light and electron microscopy

3.3.6.1 Development of pathology with age

Lysosomal storage was present in all tissues examined in the mid-term foetus and increased rapidly in severity and extent with increasing age. Severe lysosomal storage was observed in most tissues by 35 days after birth (Table 3.5). The pattern of initial lysosomal storage in the foetus was different in various tissues. In some tissues, lysosomal inclusions were present in virtually all of the affected cell type, with these cells increasing in size and distension with increasing age (aorta, liver), whereas in some other tissues, lysosomal inclusions were present in an increasing proportion of cells with increasing age (heart valve, skin, cerebrum).

Table 3.5: Development of pathology in MPS VI cats with increasing age.

Tissue/cell type	Foetal (n=1)	0 days (n=2)	35 days (n=2)	53 days (n=1)	≥ 175 days (n≥3)
Heart-valve fibroblasts	1+	±-2+	3+	NA	3+
Aorta-smooth muscle	±-1+	1+-2+	3+	3+	3+
Skin-fibroblasts	1+	1+-2+	3+	3+	3+
Cornea-keratocytes	±	1+*	3+*	3+	3+
Cerebrum-perivascular cells	NA	±-1+	2+	2+	3+
Liver-Kupffer cells	1+	2+-3+	3+*	3+	3+
Cartilage-chondrocytes	1+	2+-3+	3+*	NA	3+

*n=1; NA not available; 0, no lysosomal vacuolation; ±, very mild vacuolation; 1+, mild; 2+, moderate; 3+, severe lysosomal vacuolation.

3.3.6.2 General observations

Slides were generally examined blind and arbitrarily grouped according to the grading system. Macrophages are present throughout various connective tissues, and both fibroblasts and macrophages are distended with storage vacuoles in MPS VI. No attempt

was made to distinguish between these two cell types when grading the severity of lysosomal storage. Mild vacuolation in some cells in heart valve was also observed in rapidly fixed tissues from normal cats (various ages) not bred within the colony. It is possible that the vacuolated cells were active tissue macrophages. In skin, care was taken to distinguish between foamy cells normally observed in sebaceous glands and cells with lysosomal storage.

With the exception of cats less than 2 months of age, the pattern of storage was uniform throughout most tissues. In the cornea however, more severe keratocyte storage was observed in the most superficial and deepest layers. It was not possible to determine if severity of storage changed in different regions of the cornea due to technical difficulties (in the centre compared with the limbus). However no mention of any differences were made in a study involving reciprocal corneal transplantation in MPS VI and normal cats (Aguirre *et al.*, 1992). Lysosomal storage was present in all layers in dura mater but was not uniform, although tissue from only small numbers of animals was examined.

Some mild variation in the extent of lysosomal storage was observed between different age-matched cats. For example, there appeared to be variation in size of distension of corneal keratocytes between different cats at the same age, and storage in skin sections in one 11 month old cat was not quite as severe as observed in some 6 month old cats. Some of these differences were due to poor tissue fixation or embedding, resulting in compression and fragmentation of sections, particularly those with dense connective tissue such as skin and hip joint capsule. Poorly sectioned tissues were therefore not evaluated further. Other reasons for these differences may be due to non-standardised sampling locations of tissues.

Unfortunately, a standardised sampling location in aorta was instituted only towards the end of this study. It was therefore not possible to standardise measurements to evaluate tunica media thickness. In one MPS VI cat examined, significant variation was observed in overall diameter, and also tunica media thickness in different regions of the aorta.

Kidney sections in 6 month old cats were evaluated but not graded for the presence of lysosomal storage. Vacuolation was obvious in interstitial cells, but varied greatly between

cats. There was also intermittent extensive vacuolation in tubules thought to be proximal convoluted tubules, although cell type was difficult to identify as the morphology was greatly altered from normal.

Kupffer cells were inconspicuous in normal control cats. Ito cells containing several large lipid droplets were evident in most MPS VI and heterozygote/normal cats. Vacuolated fibroblasts were observed occasionally in MPS VI cats in connective tissue around bile ducts. Lysosomal storage in hepatocytes was not evaluated.

3.4 Discussion

3.4.1 Diagnosis of MPS VI homozygotes and heterozygotes

Development of methods to enable diagnosis of MPS VI kittens at birth or soon after was essential in order to be able to start ERT as early as possible, since significant lysosomal storage was present in most tissues at birth. Initial urine screening of young kittens demonstrated that total urine GAG was greatly elevated in MPS VI kittens less than 40 days of age using the ABC method, clearly distinguishing them from heterozygote/normal littermates. Qualitative urinalysis by HRE enabled the detection of DS, confirming the diagnosis of MPS VI at all ages. Examination of stained blood films was complicated by the presence of abnormal basophilic neutrophil granulation in the majority of kittens, however, MPS VI kittens were rapidly and accurately identified by the presence of non-staining eosinophils. White cell morphology is discussed in greater detail in Chapter 6.

In older cats, once sufficient blood could be sampled, diagnosis of MPS VI and heterozygotes was complicated by the abnormally low 4S/ β hex ratios in apparently normal cats. The presence of abnormal basophilic neutrophil granulation in many of these cats, and in some cases, the lack of MPS VI kittens born to parents with very low 4S/ β hex ratios all pointed to the possibility of an additional mutation within the colony. This was investigated further and is discussed in Chapter 6. PCR based mutation analysis was not available until towards the end of this study (September, 1995). Subsequently breeding efficiency of the colony greatly improved as a result of accurate identification of heterozygotes.

3.4.2 Clinical findings

Presentation and progression of disease in the MPS VI cats was essentially the same as described previously (Jezyk *et al.*, 1977; Haskins *et al.*, 1979b, 1980, 1983b; Konde *et al.*, 1987). The exception to this was the very high incidence of hindlimb neurological problems associated with spinal cord compression within our colony. Ninety percent of 6 month old MPS VI cats had some sort of hindlimb neurological deficits ranging from mild deficits to paralysis, compared with previous observations of only approximately 25% (Haskins *et al.*, 1983b).

The spinal cord compression observed at postmortem in the majority of MPS VI cats was due to bony compression of the cord, as described previously (Haskins *et al.*, 1983b). However, cord compression was much more extensive, and included the upper thoracic region, whereas compression had only previously been observed from T12 to L2, although mild distortion of outline and Wallerian degeneration was observed in the cervical spinal cord in 2 cats (Haskins *et al.*, 1983b). These differences are difficult to explain, and cannot be due to smaller numbers (n=6) evaluated in this previous study by Haskins *et al.* (1983b) as almost all the MPS VI cats observed in this work had upper thoracic cord involvement, although only mildly in some cases. No compression was observed in the upper cervical spine, which in contrast, is a common site of pathology in human MPS VI patients due to dural thickening (Tamaki *et al.*, 1987). Bony compression in the thoracolumbar region has been reported in one human MPS VI patient (Wald and Schmidek, 1984).

In addition, partial reversal of hindlimb paralysis to severe paresis was observed in several cats, whereas the paralysis was previously reported to be stable 2 to 4 weeks after onset (Haskins *et al.*, 1983b). Haskins *et al.* (1983b) proposed that the lack of onset of hindlimb neurological dysfunction after 8 months of age was related to the time of vertebral epiphyseal closure at approximately 9 months of age. One explanation for the partial reversal of hindlimb paralysis is that remodelling of the vertebral metaphysis/epiphysis took place at sites of spinal cord compression, with subsequent remyelination of damaged nerve fibres and some reversal of neurological signs. Although not apparent from average bone dimensions, metaphyseal flaring was quite obvious in some 2-3 month old MPS VI cats and

was reduced overall with increasing age. Remodelling of bones, particularly long bones also became apparent in older MPS VI cats (11 and 30 months). In cats 222f and 223m, the mild or very mild cord compression observed at postmortem (apart from severe compression due to an osteophyte at one level in cat 223m) despite a history of paralysis would support this hypothesis. It is not possible to ascertain if the compression was more severe in these cats at a younger age.

There were no obvious differences between MPS VI and heterozygote/normal kittens in morbidity or mortality due to infectious diseases. Although incidence in heterozygote/normal kittens was not carefully evaluated, there appeared to be a possible association between MPS VI and the presence of patent ductus arteriosus in 7% of the MPS VI kittens born (4 out of 55). This abnormality or a ductus diverticulum was also noted in some MPS VII dogs (Haskins *et al.*, 1991a) and one MPS VI baby (Fong *et al.*, 1987) suggesting a possible role of GAGs in vessel development. Development may have been disrupted in the presence of elevated GAG levels, perhaps through proteoglycan association with collagen, or through the ability of small proteoglycans to bind to the growth factor TGF- β (Yamaguchi *et al.*, 1990; Hildebrand *et al.*, 1994). Alternatively, genes associated with the patent ductus may be linked to the mutations in MPS VI cats and humans, and MPS VII dogs, but this would appear very unlikely.

The reason for consistent observations of a shorter duration of anaesthesia in MPS VI cats, even when several drugs were administered together both at higher doses, compared with heterozygote/normal cats is unknown. One possibility is poorer absorption of the drugs in MPS VI cats when injected subcutaneously, however intravenous and inhalational routes were not evaluated to support this. Premedication resistance has also been observed in human MPS patients (King *et al.*, 1984; Whitley, 1993).

3.4.3 Skeletal pathology

The skeleton is one of the major sites of pathology in MPS VI, and the pattern of skeletal disease in human and feline MPS VI is similar. Impaired endochondral ossification at multiple sites (dysostosis multiplex) causing dwarfism, and altered intramembranous

ossification leading to abnormal skull growth and facial dysmorphia is observed in both. The radiographic changes and histomorphometric values observed in MPS VI cats were no different from those described previously (Konde *et al.*, 1987; Norrdin *et al.*, 1993). In summary, impaired endochondral ossification in MPS VI cats resulted in smaller abnormal epiphyses, reduced bone growth, reduced bone trabecular thickness and trabecular number, with a resulting generalised osteopaenia, particularly evident radiographically in older cats.

Variability in physical appearance, hindlimb neurology and bone dimensions was very obvious in different MPS VI cats, and in some cases between siblings in successive litters. In general, those with the smallest stature had the most obvious reduction in bone length and smallest epiphyses, and also had more pronounced flaring of some metaphyses. However, surprisingly, the variability observed in MPS VI cats for the majority of objective parameters was statistically no different from the variability observed in heterozygote/normal littermates. The objective data generated from these skeletal measurements provide an important baseline to later compare with data from MPS VI cats that underwent ERT from birth (see Chapter 5).

It was also notable that for almost all skeletal measurements, male and female heterozygote/normal cats were significantly different from each other. This meant that it was important to take this into consideration when later examining efficacy of ERT in groups of animals containing both sexes, even though for the majority of parameters measured in MPS VI cats, there were no significant differences between males and females.

The effects of MPS VI on bone growth was not uniform, with cervical vertebral length most severely affected, followed by patellar and lumbar vertebral length, then tibial length. Despite inaccuracies in measurements of the C3 vertebra due to the severe changes at the epiphyses, increased pathology was apparent in the cervical vertebrae compared with the lumbar vertebrae, seen both from measurements and radiographs. Although only measured in the vertebrae, the greatest increase in width was also observed in the cervical vertebra, corresponding with the most severely affected length. In general, the lower limb long bones in MPS patients appear to be less severely affected than those in the upper limbs (Whitley,

1993), however the extent of these differences are difficult to assess, particularly due to the heterogeneity of mutations causing disease, and hence heterogeneous phenotypes.

For most bones measured, with increasing age, the decreasing lengths as a percentage of normal, reflected impaired endochondral ossification. As observed on radiographs in heterozygote/normal and MPS VI kittens at birth, primary centres of ossification were similar in length, but with increasing age differences gradually became more obvious, indicating an impaired ability to maintain a normal bone growth rate in MPS VI kittens. Curiously the patella length appeared to initially lag behind when first measured at 3 months, and later increased more towards normal in older MPS VI cats. The patella is a sesamoid bone and becomes ossified in a similar way to secondary centres of ossification (epiphyses) compared with the vertebrae and tibia which consist of a primary centre of ossification with epiphyses at either end. In both normal and MPS VI cats, secondary centres of ossification were not present at birth, with different centres gradually appearing at different times after birth (see Berman, 1986). Therefore in MPS VI cats, patella ossification was initially delayed, but perhaps because the overall length of the patella was quickly achieved in the normal cat (because of the relatively small amount of bone growth needed due to its small size), the MPS VI cat was able to “catch up” at later ages. However the final length was still reduced from normal.

It is unknown why growth in different growth plates in the MPS VI cat would be affected to different degrees, with the least reduction in length observed in the longest bone measured, the tibia. In normal bone growth, a complex relationship between factors including proteoglycan levels, vascularization and growth factors must regulate the rate of growth in different growth plates. In the human foetus, decorin was faintly detectable in the primary growth plate matrix in the limb long bones, whereas the same zones stained prominently in the more immature metacarpal, phalangeal and vertebral growth plates, which are also much smaller bones. These growth plates were also poorly vascularized or uninvaded (Bianco *et al.*, 1990). Mutations in several members of the transforming growth factor- β (TGF- β) superfamily have been observed in mice, and cause specific skeletal changes such as altering length and number of bones in the limbs but not affecting axial skeletal growth. This suggests that action of different growth factors may regulate skeletal

growth at different sites (Storm *et al.*, 1994). In normal cartilage, TGF- β may be sequestered in the extracellular matrix by the proteoglycans decorin, biglycan and fibromodulin, perhaps playing a role in regulating its activity (Hildebrand *et al.*, 1994).

The obvious osteopaenia observed in the older MPS VI cats radiographically and at all ages histomorphometrically, may be caused by either a reduced bone formation rate, increased rate of bone resorption, or a combination of both mechanisms. Similar histomorphometric results were also observed in iliac crest biopsies in MPS VI cats 13 to 18 months of age (Norrdin *et al.*, 1993). Histologically, disorganized columnar architecture of the proliferative and hypertrophic zones was observed in the MPS VI feline growth plate. The calcification zone was disorganised and there was excessive calcified cartilage retained in the primary spongiosa (Abreu *et al.*, 1995). This suggests impaired calcification of trabeculae emerging from the growth plate which may be due to reduced bone formation rate. Bone formation rate normalised to bone surface area was reduced in 6 month old MPS VI cats compared with normal controls (Byers *et al.*, 1997a), however there was no difference observed in older MPS VI cats (Norrdin *et al.*, 1993). There was also reduced osteoclast specific tartrate resistant acid phosphatase staining in the lower hypertrophic and primary spongiosa regions (Abreu *et al.*, 1995), suggesting a reduced osteoclast number or possibly a reduced ability to stain histochemically. However, static and dynamic histomorphometric analysis of trabecular bone in the primary ossification centre of the L5 vertebra, together with morphologic identification of osteoclasts, indicated that there were the same number of osteoclasts for the same amount of bone surface in MPS VI compared with normal cats (Byers *et al.*, 1997a). In addition, the proximal tibial growth plate in some MPS VI cats, appeared well organised and no different from normal (Sharon Byers, unpublished observation). It therefore appears that different growth plates may be affected to varying degrees which may result in conflicting observations. It is possible that these differences may correlate with the variable reduction in bone growth in different bones, however this was not examined.

3.4.4 Gross pathology and histopathology

Variable degrees of degenerative joint disease in MPS VI cats has previously been observed radiographically, with increasing severity with age (Konde *et al.*, 1987), however gross joint changes at postmortem have not previously been well described. The degenerative joint changes suggest an inability of either articular cartilage or subchondral bone, or both, to withstand normal load bearing and wear. This is discussed further in Chapter 6.

In the MPS VI cats at postmortem, there was mild mitral valve thickening, and occasionally tricuspid valve thickening. Aortic valves did not appear thickened. Valve thickening did not obviously increase with age, however only 4 cats older than 6 months of age were examined. Histologically there was no obvious excessive collagen deposition or fibrosis however this was not evaluated objectively. Mitral and aortic valves are severely affected in human MPS VI patients (Tan *et al.*, 1992; Wippermann *et al.*, 1995). This difference in location and severity of valve involvement may be due to insufficient time for pathology to develop in the MPS VI cats, however overall it would appear that cardiac valve involvement in the cats is not as severe as in humans. Because of the severity of disease observed at this site in humans, it may have been useful to have measured heart valve collagen content in the MPS VI cats. The collagen specific anionic dye Sirius Red (Picrosirius) (Junqueira *et al.*, 1979) may be useful to examine this in future studies.

The histological changes observed in MPS VI cats have been described more extensively by Haskins *et al.* (1980). As observed previously, the cell types affected were mesodermal in origin and predominantly fibroblast and macrophage cell types. Some mild variability in severity of lysosomal storage was observed in various tissues between age-matched MPS VI cats. Some of this may have been due to different sampling location, however most variability was encountered in samples that had poor preservation or embedding, emphasising the importance of this for accurate evaluation.

Lysosomal storage in liver, spleen, heart valve, skin, brain and dura has been observed histologically in human MPS I and II fetuses as early as 20 to 22 weeks of age (Wiesmann *et al.*, 1980; Crow *et al.*, 1983). Similar observations were made in newborn murine MPS VII pups, with mild storage also observed in corneal keratocytes (Vogler *et al.*, 1990). Our

observations of foetal chondrocyte and aortic smooth muscle cell pathology add to the cell types examined, and also demonstrate widespread *in utero* pathology of feline MPS VI foetuses. The rapid increase in extent of pathology after birth emphasises the need for early therapeutic intervention to halt the pathological processes.

3.5 Summary and conclusions

Improved diagnostic methods were developed which enabled diagnosis of MPS VI kittens at birth by examination of leukocyte morphology on blood films. In addition reflex urination at less than 30 days allowed collection of urine in young animals. MPS VI kittens had elevated urine GAG levels and were clearly distinguishable from unaffected littermates at 40 days or less.

Disease in MPS VI cats was overall as described previously, except for an unexplained increased incidence of hindlimb neurological deficits in our colony compared with a previous report. Systematic observation of clinical features, radiographic, postmortem and histological changes at specific ages in both MPS VI and heterozygote/normal controls was used to develop baseline values for a number of objective and subjective parameters up to 11 months of age. Sex differences in skeletal dimensions were very apparent between male and female heterozygote/normal cats, but these differences were minimal between male and female MPS VI cats. The effects of MPS VI disease on skeletal growth was not uniform, with dimensions of different bones affected to different degrees compared with normal. Differences for most dimensions in MPS VI cats compared with heterozygote/normal became more significant with increasing age. Variability in skeletal dimensions between age matched animals within heterozygote/normal and within MPS VI groups was not significantly different, despite the widely differing physical and radiographic appearance observed in some MPS VI cats.

These baseline observations provide an important comparison for evaluation of response to ERT examined in Chapters 4 and 5. In addition, pathology present in the mid-term MPS VI foetus and the rapid increase in extent and severity after birth, suggests that early onset of therapy will be important to maximise response to any therapy.

In the following chapter, distribution and fate of rh4S at two dose rates is evaluated in normal cats as a preliminary to ERT in MPS VI cats. Following this, low dose ERT was undertaken in several young and adult MPS VI cats, in order to determine viability and efficacy of long term ERT, prior to further ERT studies from birth described in Chapter 5.

4. Preliminary enzyme replacement therapy studies

4.1 Introduction

The close comparison of disease characteristics between the feline model of MPS VI and MPS VI in humans, as discussed in the review chapter, illustrates the potential of feline MPS VI for evaluation of new therapies. In addition, collection of data from MPS VI and normal control cats described in the previous chapter enables objective evaluation of efficacy of these new therapies. At the outset of our studies, ERT in MPS animal models had only just begun, with short term ERT in an MPS VII mouse model being the first to be evaluated (Vogler *et al.*, 1993). Hence longer term ERT studies in larger animal models of MPS disorders were warranted.

The two major aims of experiments described in this chapter were to determine the distribution and fate of recombinant human 4S (rh4S) in normal cats, and then to perform a preliminary ERT trial with rh4S in MPS VI cats to evaluate the viability and efficacy of long term therapy. Different carbohydrate residues on glucocerebrosidase have previously been shown to be able to alter targeting to different cell types in rat liver (Furbish *et al.*, 1981), hence the availability of three rh4S forms with different carbohydrate targeting sequences (CHO, LEC and LEC-P) warranted evaluation in these initial experiments. However, due to the difficulty of large scale production and purification of these enzymes, very limited amounts of these rh4S forms were available for distribution and enzyme replacement studies. Hence the numbers of animals in experimental groups was unfortunately small, and therefore results must be interpreted with some caution.

For the distribution studies following intravenous administration, the experimental plan was to evaluate the plasma clearance, plasma half-life and tissue distribution of the three rh4S forms at two different dose rates, and then to determine the half-life of one of these rh4S

forms in various tissues. Rh4S levels were compared with feline 4S (f4S) levels found in normal cats. Rh4S and f4S activities were detected using an immunobinding assay.

To evaluate efficacy of ERT in three MPS VI cats, clinical appearance, radiographic skeletal appearance, urinary GAG levels, plasma anti-rh4S antibody titre, postmortem changes, tissue histopathology and bone histomorphometric values were assessed.

Together, these findings were then used as a baseline for choice of optimum dose rate and dose frequency for further ERT studies outlined in Chapter 5.

4.2 Specific methods

4.2.1 Enzyme production and purification

Michael Brogan and Peter Clements (Department of Chemical Pathology, WCH) purified enzyme using a four column procedure for use in the distribution studies. Enzyme dephosphorylation was performed by Jill Durrant (Department of Chemical Pathology, WCH).

For distribution studies and preliminary ERT, rh4S was expressed in two different cell lines to produce two different rh4S enzyme forms. CHO and LEC-1 cells expressed CHO rh4S and LEC rh4S respectively. The LEC-1 cell line (American Type Culture Collection, Maryland, USA) is a CHO cell derived mutant which lacks N-acetylglucosamine-transferase activity (Stanley *et al.*, 1975) altering the N-linked glycosylation of the rh4S. The monoclonal antibody column method (see Section 2.2.9) was used to purify both rh4S forms to be used in ERT in the MPS VI cats. The mean specific activity of CHO rh4S was $26\,220 \pm 6\,025$ nmol/min/mg (n=32) and for LEC rh4S it was $29\,930 \pm 7\,250$ nmol/min/mg (n=15).

Enzyme used for distribution studies in normal cats was purified using a four column procedure using Reactive Blue A-Agarose (Amicon), Heparin Sepharose (Pharmacia) and Decyl Agarose (Sigma)(unpublished method, Michael Brogan and Peter Clements) to the

same degree of purification as the monoclonal antibody column purified enzyme. Purity was ensured by running each preparation on SDS-PAGE under reducing conditions as outlined in Section 2.2.9.

A portion (5 mg) of the purified LEC rh4S had the terminal phosphate of the carbohydrate chain removed (denoted by LEC-P) by coincubation with 11 mg bovine intestinal alkaline phosphatase (EC 3.1.3.1, Type VII-N, Sigma Chemical Co., St. Louis, USA) in dimethylglutarate buffer at pH 6 for 16 hours at 37 °C. This was then run on a F58.3 monoclonal antibody column as above and dialysed into PBS and assayed for activity ready for injection (Section 2.2.9).

4.2.2 Enzyme administration in MPS VI cats

Three MPS VI cats were injected intravenously with two forms of rh4S (CHO and LEC) with therapy starting at between 2 and 12 months, and in two cats with variable dose rates at different times (Table 4.1).

Table 4.1: Age at onset of ERT, dose regime and rh4S form used in three MPS VI cats, Alf, Barney and Duncan.

Cat ID	Alf		Alf		Barney		Barney		Duncan	
Enzyme form	CHO rh4S		CHO rh4S		LEC rh4S		LEC rh4S		CHO rh4S	
Dose	0.8	mg/kg	1.5	mg/kg	0.5	mg/kg	1.4	mg/kg	0.8	mg/kg
	per 14 days		per 7 days		per 14 days		per 7 days		per 14 days	
Age at dose (months)	7* - 22		22 - 27		12* - 23		23 - 27		2* - 15	

* Age at onset of ERT

In the last 4 months of therapy, premedication with 2 mg/cat chlorpheniramine maleate (Niramine ®, Jurox Pty Ltd, Silverwater, NSW) was given to all cats subcutaneously prophylactically. Enzyme was infused in a volume of 2 - 10 mls (diluted with PBS) into the cephalic vein over a period of 5 - 20 minutes. If signs suggestive of developing anaphylaxis occurred (see Section 2.2.10), enzyme infusion was stopped and reinstituted within 30 minutes.

4.2.3 Distribution studies in normal and MPS VI cats

Assistance with arterial cannulation was kindly provided by Tim Kuchel (Institute of Medical and Veterinary Science, Adelaide), and processing of blood samples and assistance with handling tissues was done by Viv Muller and Chris Boulter (Department of Chemical Pathology, WCH).

Seven normal cats (12 - 24 months old) were sedated with 0.25ml Anamav ® then anaesthesia was induced by intravenous Saffan ® at ~9 mg/kg and then maintained on halothane. The femoral artery was cannulated using polyethylene tubing (PE 50, Dural Plastics and Engineering, Auburn, NSW) attached to a three-way tap filled with heparinised saline. Purified rh4S at a dose of 1 mg/kg (CHO, LEC and LEC-P) or ~7.5 mg/kg (CHO at 7.3 mg/kg and LEC at 7.95 mg/kg) was infused into the cephalic vein in a volume of 7ml over 40 seconds, with time zero taken from 20 seconds. Serial 1ml heparinised blood samples were collected from the femoral artery for up to 2 hours after enzyme infusion. Samples were centrifuged (1600 g for 3 minutes) at 20°C within 2 - 5 minutes of sampling, plasma collected and the buffy layer was removed and immediately washed by resuspending in saline. The buffy layer samples were then recentrifuged (as above) and then separated from any contaminating red blood cells using dextran sedimentation (see Section 2.2.7) within 2 - 4 hours of sampling. Buffy layer and plasma samples were then stored at -20°C until assayed. Polyethylene tubing and three-way taps were flushed with heparinised saline between sampling times. After 4 hours, cats were euthanased using an overdose of intravenous barbiturate. Tissues were then collected (whole organs where possible) and weighed, then frozen at -20°C until assayed for rh4S activity. Distribution of LEC-P at the ~7.5 mg/kg dose was not possible due to limited supply of this enzyme.

Four normal cats (8 - 66 months old) were injected intravenously with 7ml CHO rh4S: three at 1 mg/kg at 2, 4 and 7 days prior to euthanasia, and the fourth at 7.4 mg/kg 7 days prior to euthanasia. A fifth normal cat was injected subcutaneously with 2.7ml CHO rh4S at 1 mg/kg, 2 days prior to euthanasia. Two additional normal cats (one bred within the colony, and one from outside the colony) were used for detection of f4S levels in tissues and were not exposed to rh4S. Euthanasia and tissue collection was performed as above.

MPS VI cats Alf and Barney were euthanased at 27 months, 7 days after last enzyme injection, and Duncan at 15 months, 4 days after last enzyme injection, using an overdose of intravenous barbiturate. Euthanasia and tissue collection was performed as above.

4.2.4 Distribution study tissue analysis

Tissue preparation and analysis using the immunobinding assay was performed by Barb King and Chris Boulter (Department of Chemical Pathology, WCH).

Preparation of tissue extracts, detection of rh4S activity in feline tissues, white cells and plasma, and detection of f4S activity in normal control cat tissues was according to methods described previously (Brooks *et al.*, 1991a, 1994).

Briefly, this involved binding sheep anti-mouse immunoglobulin to a 96-well polyvinylchloride plate, then adding a second layer of either a human specific monoclonal antibody (F66) to detect rh4S, or a polyclonal anti-h4S antibody (that cross reacts with f4S) to detect f4S. Tissue extracts were then applied to the plates and 4-methylumbelliferyl sulphate (4MUS) substrate used to determine rh4S or f4S activity (nmol/min/gram tissue) fluorometrically. To compare relative amounts of rh4S due to different doses of rh4S, rh4S activities for each tissue were divided by the total enzyme dose (mg/kg) to correct all values to 1 mg/kg.

Plasma half-life of the different rh4S forms was estimated by measuring the time required for the plasma concentration in the linear elimination phase to decrease by 50%.

4.2.5 Clinical and biochemical evaluation of ERT treated MPS VI cats

A clinical and neurological examination was done on a monthly basis, and slit lamp examinations of the corneas were performed in the conscious animals on three occasions (at 19, 20 and 23 months in Alf and Barney and at 7, 8 and 11 months in Duncan)(Section 2.2.11).

Standardised radiographic views were taken at 3 monthly intervals under general anaesthesia (see Section 2.2.13), and standardised measurements of bones were taken directly from the radiographs (Section 2.2.14).

Blood samples for determination of plasma antibody titre to rh4S were collected on a monthly basis (Section 2.2.21). On one occasion, plasma samples were collected from Alf before rh4S infusion, then at 30 and 60 minutes post infusion.

Urine samples were collected by manual bladder expression with xylazine sedation (0.5 mg/kg intravenously or 1 - 2 mg/kg subcutaneously) or general anaesthesia while undergoing radiology procedures. Total urine GAG content was quantitated using the ABC method (Section 2.2.16) and then HRE was performed on the same samples (Section 2.2.17). Densitometric estimations of the approximate relative proportions of dermatan sulphate (DS1+DS2) was determined from the cellulose acetate strips (Section 2.2.17).

4.2.6 Pathology in the MPS VI cats

Skin biopsies were taken from Alf and Barney at 9 months of age under general anaesthesia. This was 7 weeks after onset of ERT in Alf. Skin biopsies were repeated in both cats at 12 months of age before the onset of ERT in Barney.

Skin biopsies and tissues collected at postmortem were processed for light and electron microscopy (Section 2.2.22), using 1% (v/v) glutaraldehyde/4% (w/v) paraformaldehyde fixative for electron microscopy. Fluorescent antibody staining of frozen kidney sections to

detect glomerular immune complexes was also performed (Section 2.2.24). The L5 vertebra was removed for histomorphometric analysis and processed and analysed as described in Section 2.2.25.

4.3 Results

4.3.1 Plasma clearance of rh4S forms in normal cats

Three forms (CHO, LEC and LEC-P) of rh4S with different glycosylation structures were evaluated for plasma half-life and tissue distribution. At 1 mg/kg, all rh4S forms were cleared from circulation by approximately 30 minutes (Figure 4.1). At ~7.5 mg/kg there was a dose dependent clearance of both CHO and LEC rh4S, with rh4S activity in plasma detectable for up to 120 minutes (Figure 4.1). Plasma half-life for all forms of rh4S at 1 mg/kg was 11.1 ± 0.6 minutes (11.3, 10.4 and 11.6 for LEC, CHO and LEC-P respectively), and at ~7.5 mg/kg was ~25 minutes for both LEC and CHO rh4S. At either dose, and for all three forms, rh4S could not be detected in peripheral blood leukocytes.

4.3.2 Tissue distribution and half-life of rh4S forms in normal cats

Four hours after administration of 1 mg/kg rh4S, the tissue distribution of all rh4S forms was similar (Table 4.2) with the majority of the infused rh4S found in the liver. Significant levels of rh4S were also observed in spleen, lung, kidney, heart, skin, aorta, cerebrum, cerebellum and lymph node compared with f4S levels in a normal control (Table 4.2 & Table 4.3). Increased dose resulted in a proportional increase in levels of rh4S in all tissues, however when all values were corrected to 1 mg/kg, similar rh4S activities were found in most tissues between the 1 and ~7.5 mg/kg dose rates for both CHO and LEC rh4S.

Minor differences were noted in the distribution pattern for CHO and LEC rh4S. Proportionally more CHO rh4S was detected in lung (9-fold), whereas LEC rh4S activity was detectable in cartilage and cornea at both dose rates (Table 4.2). Dephosphorylation of rh4S (LEC-P) resulted in lower enzyme activities in the majority of tissues, however the pattern of distribution remained consistent (Table 4.2 & Table 4.3).

F4S activities in the colony bred normal control cat were consistently lower in all tissues tested compared with the normal cat obtained from outside the colony. The genotype of the colony bred normal cat was unknown, however the leukocyte 4S/ β hex ratio was 3.3, which suggested either a normal or heterozygote genotype (see Table 6.1).

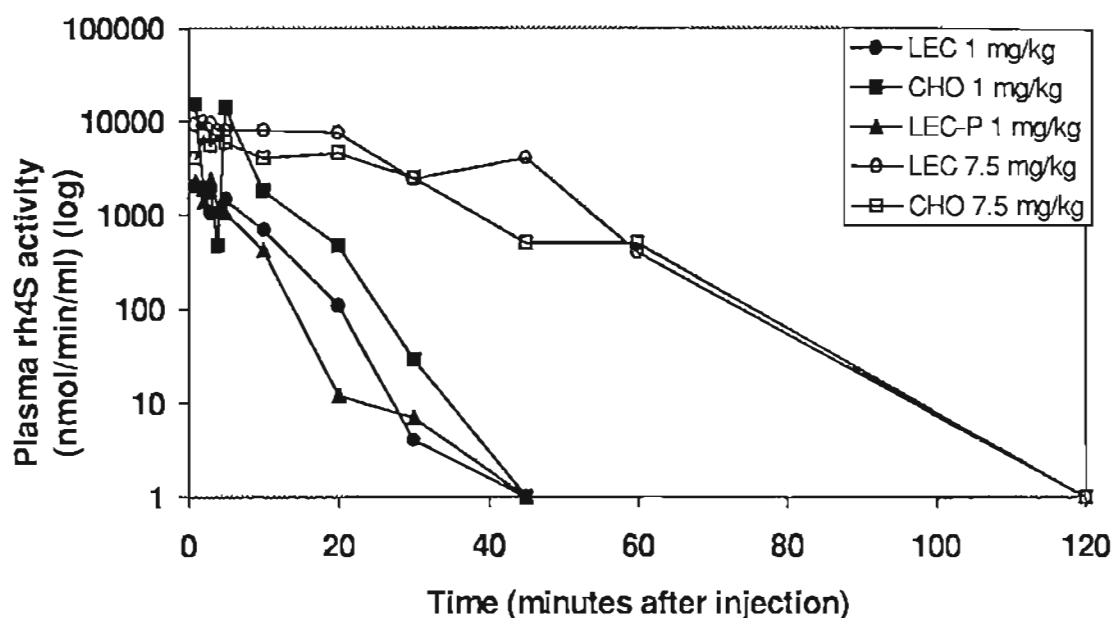


Figure 4.1: Plasma clearance of three rh4S forms in normal cats at 2 dose rates.

Plasma clearance of rh4S in normal anaesthetised cats in serial arterial blood samples after intravenous administration of 1 mg/kg LEC (●), CHO (■) and LEC-P (▲) rh4S and ~7.5 mg/kg LEC (○) and CHO rh4S (□).

Table 4.2: Tissue enzyme activity (nmol/min/gram tissue) of CHO, LEC and LEC-P rh4S at 2 dose rates, 4 hours after intravenous infusion, compared with f4S activity in normal control cats.

rh4S type	CHO	CHO	LEC	LEC	LEC-P	f4S [†]	f4S [†]
	n=3	n=1	n=1	n=1	n=1	n=1	n=1
Dose	1 mg/kg (mean ± sd)	7.3mg/kg*	1 mg/kg	7.95mg/kg*	1 mg/kg	(no dose)	(no dose)
						COLONY NORMAL	OUTSIDE NORMAL
Liver [‡]	2526 ± 1435	1229	1407	1691	701	5	31
Spleen	61 ± 20	15.4	45.7	80.5	10.5	10.2	55.3
Lung	29.0 ± 14.7	15.4	1.8	5.9	3.2	1.3	N.T.
Kidney	14.5 ± 6.6	15.4	17.6	23.1	12.9	1.3	8.2
Heart	2.6 ± 2.2	1.6	0.9	0.4	3.2	0.6	N.T.
Skin	1.2 [§]	0.7	0.7	0.3	0.5	0.04	N.T.
Aorta	1.2 ± 1.0	0.3	3.1	0.2	2.2	0.3	3.5
Cerebrum	0.2 [§]	2.2	0.4	0.1	0	6.4	N.T.
Cerebellum	0.5 [§]	0.2	1.5	0.1	0	10.2	N.T.
Cartilage	0 ^{**}	0.01	0.09	0.01	0	N.T. [¶]	1.1
Cornea	0 [§]	1.29	0.22	0.05	0	0.1	N.T.
Lymph node	26 ± 43	176	21	19	1.1	1.3	N.T.
Bone marrow	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

* values corrected to 1 mg/kg by dividing each tissue activity by the total enzyme dose;

† f4S activities; ‡ Note: for liver values only, an average of 337% of total enzyme injected was recovered (range 140-740%) when calculated from original organ weights. The reason for this is not fully understood, but factors include variable specific activity measurements of the original enzyme, and altered enzyme activity due to dilution in liver tissue extract.

§ n=2 ; ¶ N.T. = not tested; ** Limits of detection for the assay were 4.5×10^{-3} nmol/min.

Table 4.3: Ratio of tissue activity of CHO, LEC and LEC-P rh4S at 1 mg/kg, 4 hours after infusion (from Table 4.2), to f4S activity in a (colony) normal control cat.

	CHO	LEC	LEC-P
	Ratio	Ratio	Ratio
TISSUE	rh4S/f4S	rh4S/f4S	rh4S/f4S
Liver	495.3	275.9	137.4
Spleen	6.0	4.5	1.0
Lung	22.3	1.4	2.5
Kidney	11.2	13.8	10.0
Heart	4.3	1.5	5.3
Skin	31.3	17.5	12.5
Aorta	3.9	10.3	7.3
Cerebrum	0.03	0.06	-
Cerebellum	0.05	0.15	-
Cornea	- *	2.0	-
Lymph node	19.8	16.2	0.8

* Ratio not calculable due to value of numerator being equal to zero.

Tissue half-life evaluated for CHO rh4S at 1 mg/kg only, was estimated to be 2 - 4 days in liver, spleen, lung, kidney and heart (Figure 4.2). Tissue enzyme activities decreased as the injection to euthanasia interval increased, except for an initial increase observed in the spleen (Figure 4.2 B). Enzyme activity was also detectable in skin and cornea at 2 days after injection (Table 4.4), and in aorta, cerebrum, cerebellum, lymph node and bone marrow at both 2 and 4 days after injection (Table 4.4 and Table 4.5). At 1 mg/kg 7 days after injection, low but detectable levels of CHO rh4S were observed in liver, spleen, lung, kidney (Figure 4.2 A-C, and Table 4.5) and bone marrow (Table 4.5), but rh4S was not detectable in heart, skin, aorta, cerebrum and cerebellum (Table 4.5). However, at ~7.5 mg/kg 7 days after infusion, rh4S was still detectable in skin, aorta, cerebrum and cerebellum, and it was also detectable in cartilage and lymph node (Table 4.5).

Two days following subcutaneous administration of 1 mg/kg CHO rh4S, significant enzyme levels were detectable in liver, spleen, lung, kidney, aorta, and lymph node, compared with the same dose of CHO rh4S 2 days after intravenous infusion, and with f4S activity in normal (Table 4.4). Skin and subcutaneous tissues were assayed at the injection site, and high levels of enzyme activity was still present at this site.

4.3.3 Tissue distribution of rh4S forms in MPS VI cats

The distribution of rh4S in MPS VI cats was similar to that seen in normal cats, with highest enzyme levels present in the liver (Table 4.5). However in Alf, CHO rh4S activity was not detectable in the remaining tissues, compared with detectable activity in spleen, lung and kidney in the three remaining cats (MPS VI and normal) analysed 7 days after enzyme infusion. In Barney, compared with the 4 hour 1 mg/kg LEC rh4S distribution (Table 4.2) and the 7 day 1 mg/kg CHO rh4S distribution, significant levels of enzyme activity were still present in heart, aorta, cerebrum and cerebellum.

Figure 4.2: Fate of rh4S in normal cat tissues, 4 hours to 7 days after intravenous administration of 1 mg/kg.

Fate of CHO rh4S in: (A) liver ($t_{1/2}=3.4$ days); (B) spleen ($t_{1/2}=4.3$ days); (C) lung ($t_{1/2}=2.3$ days), kidney ($t_{1/2}=2.6$ days) and heart ($t_{1/2}=3.8$ days) of normal cats up to 7 days after intravenous administration of 1 mg/kg. For all points $n=1$, except $n=3$ for the 4 hour time point.

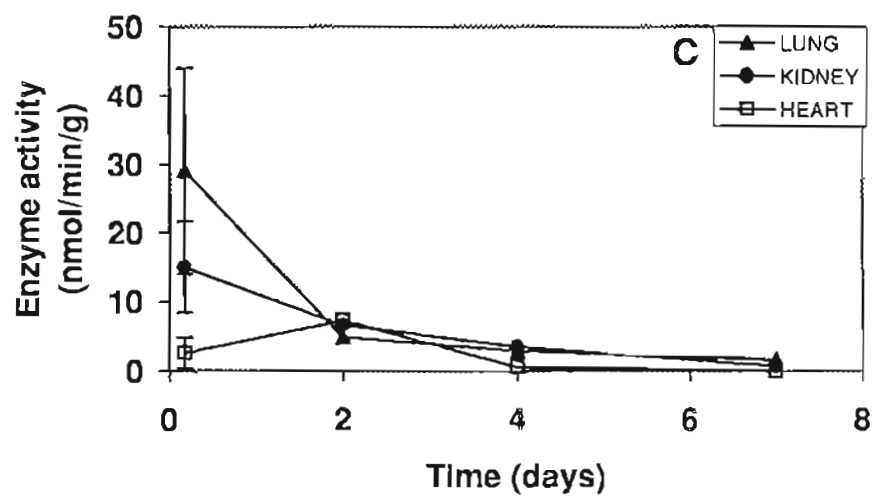
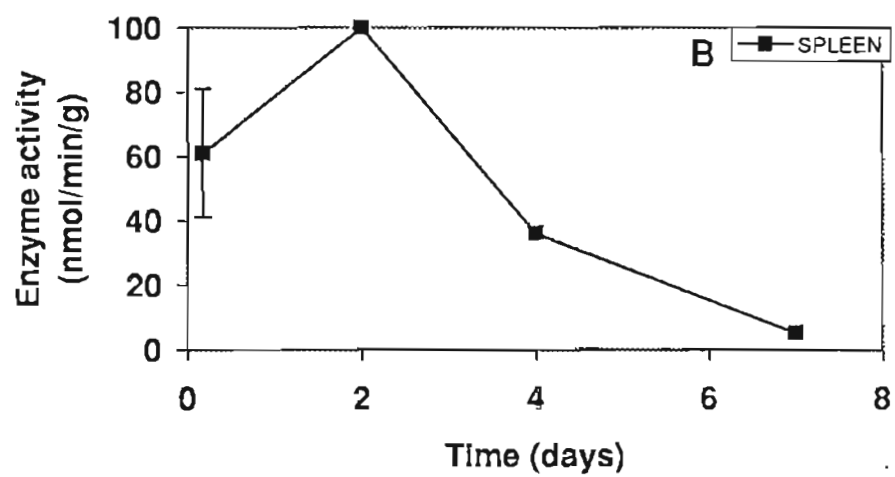
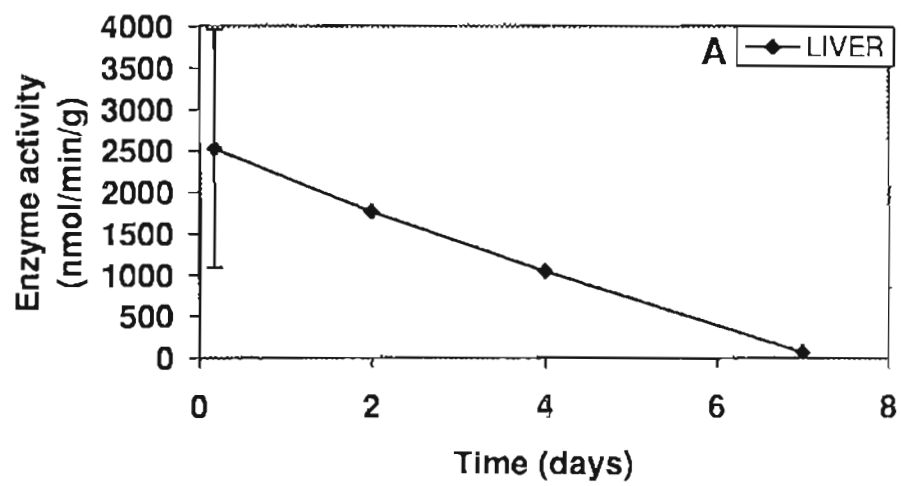


Table 4.4: Comparison of tissue enzyme activity (nmol/min/g tissue) 2 days after intravenous and subcutaneous administration of 1 mg/kg CHO rh4S in normal cats.

Route	Intravenous 1 mg/kg (n=1)	Subcutaneous 1 mg/kg (n=1)
Liver	1766	243
Spleen	100.0	13.2
Lung	4.8	0.6
Kidney	6.6	2.2
Heart	7.2	0.07
Skin	0.1	0
Aorta	0.6	0.6
Cerebellum	0.5	0
Cartilage	N.T.*	0
Cornea	0.6	N.T.
Lymph node	12.0	0.6
Bone marrow	152.0	4.6
Injection site	N.T.	3891

* N.T. not tested;

Table 4.5: Comparison of tissue enzyme activities (nmol/min/g tissue) in normal and MPS VI cats, 4-7 days after intravenous infusion of two forms of rh4S. Values corrected to 1 mg/kg.

rh4S type	CHO	CHO	CHO	CHO	CHO	LEC
& dose	1 mg/kg	0.8mg/kg	1 mg/kg	7.4mg/kg	1.5mg/kg	1.4mg/kg
	Normal	MPS VI	Normal	Normal	MPS VI	MPS VI
		Duncan			Alf	Barney
Interval	4 days	4 days	7 days	7 days	7 days	7 days
Liver	1047	908	67	134	399	51
Spleen	36.0	30.0	5.2	5.5	0	8.6
Lung	2.9	20.0	1.6	1.2	0	2.1
Kidney	3.4	4.5	0.7	2.4	0	0.4
Heart	0.6	2.5	0	N.T.	0	0.3
Skin	0	0	0	0.03	0	0
Aorta	0.27	0	0	0.07	0	0.29
Cerebrum	0.18	0	0	0.03	0	0.14
Cerebellum	0.18	0.62	0	0.04	0	0.14
Cartilage	N.T.*	0	N.T.	0.01	0	0
Cornea	0	N.T.	0	0	0	0
Lymph node	0.7	18.8	0	2.4	0	0.7
Bone marrow	113.4	28.1	3.2	N.T.	0	3.6

* N.T. = not tested

4.3.4 Disease progression in MPS VI cats undergoing ERT

Three MPS VI cats (Alf, Barney and Duncan) were treated with two forms of rh4S, with the onset of therapy at different ages according to the protocol in Table 4.1.

Alf, Barney and Duncan all developed physical features consistent with untreated MPS VI cats observed in the colony (see Section 3.3.4). Abdominal distension was prominent in Alf and Duncan due to the shortened body length and marked thoracolumbar spine kyphosis in Alf. Alf, Barney and Duncan weighed 2.8, 3.0 and 2.5 kg respectively at euthanasia compared with a normal adult male weight range of 4 - 7 kg.

In Alf, mild hindlimb paresis was evident by 4 months which progressed to almost complete hindlimb paralysis with hypertonicity and extension of the hindlimbs by 7 months. Localisation of the upper motor neurone lesion was not performed. ERT was instituted at 7 months as an alternative to euthanasia. After 4 weeks, Alf was very mobile and was walking using the hindlimbs, although no neurological examination was performed. By 10 months, Alf deteriorated exhibiting progressive hindlimb paresis eventuating in absence of extensor postural thrust. At 16 months mobility and proprioception was unexpectedly improved in the hindlimbs, with presence of extensor postural thrust, patellar hyperreflexia, delayed proprioceptive positioning and a staggy unsteady gait. In subsequent months, use of his hindlimbs and presence of extensor postural thrust was intermittent/variable but improved mobility with greatly increased activity occurred at 22 months after increasing the enzyme dose to 1.5 mg/kg.

Barney did not appear to develop any neurological deficits, although gait and mobility were altered due to progression of skeletal disease. No obvious changes in physical activity or mobility occurred with the increased ERT dose at 23 months of age. Barney remained the most mobile of all three cats.

ERT started at 2 months of age in Duncan, at which time only mild features of MPS VI were evident. Very mild hindlimb paresis was first noticed at 6 months and progressed marginally such that the extensor postural thrust was slightly slower than normal. By 9

months until euthanasia at 15 months, Duncan was usually sedentary and the most inactive of the three, but was still able to walk reasonably well with moderate hindlimb paresis.

Slit lamp examination of the corneas of all three cats showed a mild degree of corneal opacity which did not appear to change during therapy.

There were subtle radiographic improvements in bone density of all three treated cats following ERT compared with four 11 month and one 30 month old untreated MPS VI cats. This was seen particularly in the distal femoral epiphysis and in the lumbar vertebrae. Otherwise, radiographic changes in all 3 cats were similar to the changes described previously in MPS VI cats (see Section 3.3.4.6, and Konde *et al.*, 1987), with reduced vertebral length with irregular endplates, particularly in the cervical spine, hypoplasia and fragmentation of the dens, small deformed long bone epiphyses with evidence of degenerative joint changes such as remodelling and osteophyte formation, coxofemoral subluxation with remodelling of the acetabulae and femoral heads, pectus excavatum, and reduced cortical thickness of long bones. Moderate to severe kyphosis of the lumbar spine occurred in all three cats with severe degenerative changes in the vertebral articular facet joints. All lesions progressed with age.

No real differences in bone dimensions compared with the ranges of values observed in four 11 month old and one 30 month old untreated MPS VI cats (Table 4.6) supported the observations of minimal improvements in skeletal appearance.

Table 4.6: Bone dimensions in ERT MPS VI cats (Alf, Barney and Duncan) compared with 11 and 30 month old untreated MPS VI and 12 month or older heterozygote/normal cats.

Cat ID & Age	C3 length	C3 width	L5 length	L5 width	Tibial length	Patella length	Tibial Corticomedullary ratio
11m* MPS VI no ERT	0.68-0.76	0.48-0.61	1.45-1.56	0.50-0.62	9.81-11.04	1.02-1.17	0.68-0.90
30m† MPS VI no ERT	0.60	0.36	1.47	0.64	9.72	1.10	0.57
Alf (27m)	0.82	0.46	1.45	0.42	10.58	1.08	0.85
Barney (27m)	0.70	0.42	1.62	0.58	11.04	1.12	0.63
Duncan (15m)	0.68	0.40	1.48	0.44	10.96	1.08	0.82
Het/normal males >12m‡	1.15-1.30	0.40-0.52	2.10-2.36	0.47-0.62	10.92-12.38	1.38-1.60	n.d.

* n=4 : 3 females and 1 male; except for greater tibial and patella length, bone dimensions for the 11 month old male were in the middle of the range of measurements from the 11 month old females.

† n=1 : female; ‡ n=9; n.d. not done

4.3.5 Bone histomorphometry

Most values for each parameter in the ERT treated MPS VI cats were different from the untreated MPS VI cat, but were not normalised (Table 4.7). There was a trend towards more normal values correlating with the age of onset of ERT, with the most improved values seen in Duncan (onset of ERT at 2 months), then in Alf (onset of ERT at 7 months). This trend was seen most clearly with bone mineral volume measurements (BV/TV), with values in Duncan being closest to normal, followed by Alf. Bone mineral volume in Barney, in which therapy was initiated after skeletal maturity (onset of ERT at 12 months), was no different from the untreated MPS VI cat (Table 4.7). Values for trabecular thickness (Tb. Th.) and trabecular separation (Tb. Sp.) followed the same trend. Trabecular number (Tb. N.) was improved in all the treated cats and showed the same trend associated with the age of onset of ERT, but values were still less than the normal control.

Table 4.7: Histomorphometric analysis of the fifth lumbar vertebra in MPS VI cats undergoing ERT (Alf, Barney and Duncan) compared with normal and untreated MPS VI control cats.

Cat ID	BV/TV*	Tb. Th.	Tb. Sp.	Tb. N.	Age (months)
Normal (n=1)	23.8	110	0.89	2.2	30
Alf	9.6	60	0.94	1.6	27
Barney	5.0	40	0.96	1.2	27
Duncan	12.2	62	0.94	2.0	15
MPS VI no ERT (n=1)	5.5	56	0.96	1.0	30

*BV/TV (%) = bone mineral volume; Tb. Th. (μm) = trabecular thickness;

Tb. Sp. (mm) = trabecular separation; Tb. N. (per mm) = trabecular number;

4.3.6 Antibody response

All cats exhibited restlessness, trembling and increased respiratory rate to varying extents, indicating possible development of hypersensitivity, but none developed typical anaphylactic reactions during therapy. Antibody titres tended to increase after the initiation of ERT, and ranged up to 1,024,000 in Alf, 64,000 in Barney, and 64,000 in Duncan. Titres fluctuated after reaching high levels, and did not appear to correlate with clinical appearance of the treated cats, such as improved or reduced mobility. Values in age matched untreated normal control cats ranged from 4,096 - 32,000. Therefore only Alf showed antibody titres significantly above the control.

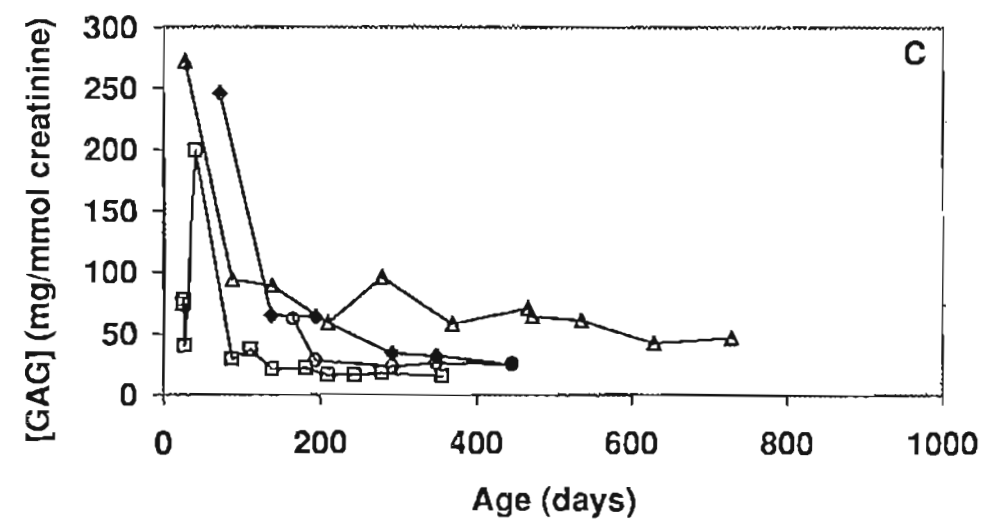
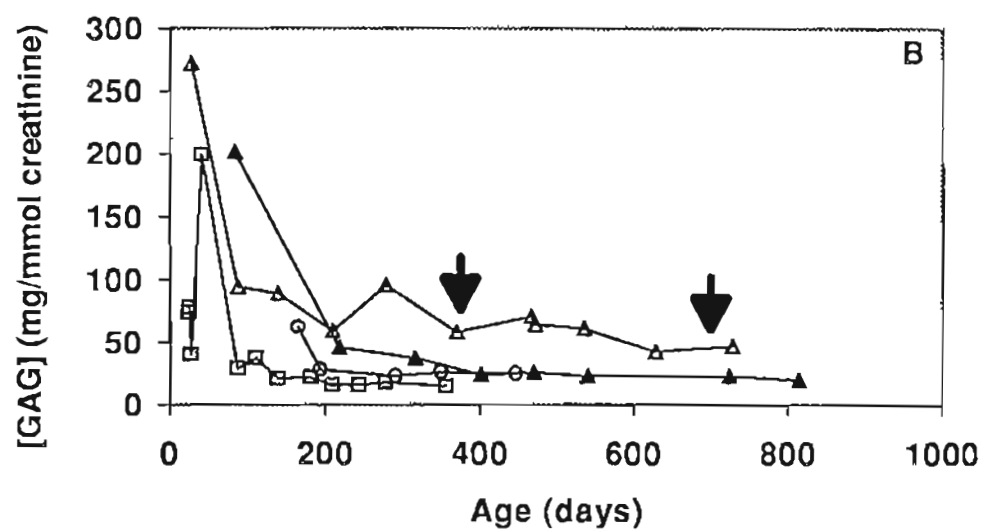
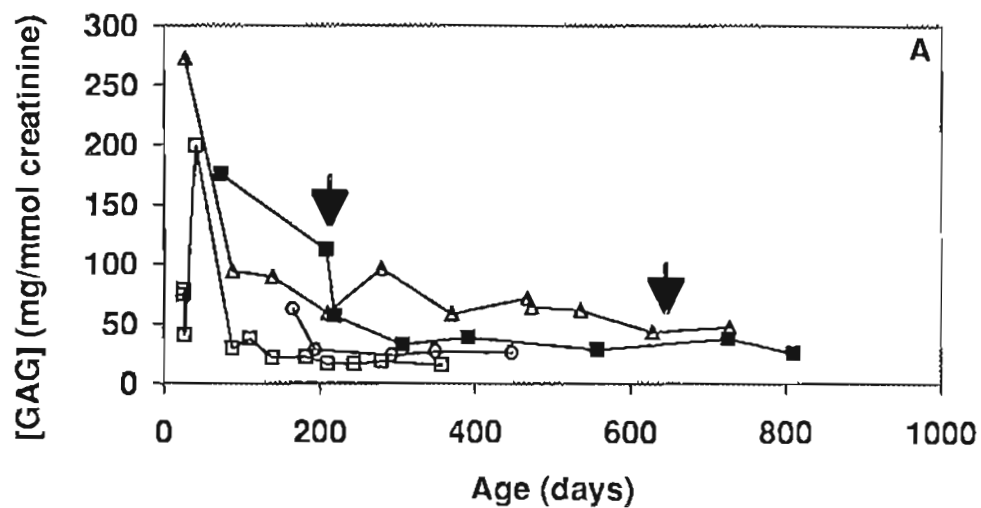
In an experiment 8 months after onset of therapy, administration of rh4S to Alf resulted in a reduction in antibody titre from >512,000 before rh4S infusion, to 2,048 by 30 minutes and 1,024 by 60 minutes after rh4S infusion.

4.3.7 Urinary glycosaminoglycans

As observed previously (see Figure 3.2), between 0 and ~100 days of age, total urine GAG decreased with increasing age in both MPS VI (treated and untreated) and normal cats. Following onset of ERT in Alf (208 days of age), total urine GAG dropped dramatically, however no change was obvious following the new increased dose at 656 days (Figure 4.3 A). Similarly, in Barney, a slight reduction in urine GAG was evident after the onset of ERT (373 days), however no change was apparent following the new increased dose at 702 days (Figure 4.3 B). Initially, total urine GAG values for Duncan appeared no different from the untreated MPS VI cat, however after 200 days of age, the values levelled out below the untreated MPS VI control (Figure 4.3 C). At 400 days of age, the total GAG content in the urine Alf was approximately midway between the normal control cats and the untreated MPS VI cat (Figure 4.3 A), and in Barney and Duncan, the total GAG content was approximately equal to the normal control cats (Figure 4.3 B & C).

Figure 4.3: Total urinary GAG excretion in Alf, Barney and Duncan.

Total urinary glycosaminoglycan excretion in A) Alf (■), B) Barney (▲), and C) Duncan (◆), undergoing ERT compared with a normal female (□), normal male (○) and female untreated MPS VI cat (Δ). The black arrow indicates onset of ERT and the new dose regime (Alf and Barney only).



Despite reduction of total urinary GAG to nearly normal levels, DS was still present in the urine in significant amounts in all three ERT treated cats. From densitometric scans of the HRE plates to determine the relative proportions of the different GAG species present in the urine, there was no apparent change in the %DS (DS1 + DS2) following onset of ERT or changes in dose, with a wide range in values observed in all cats: Alf, 36-70%; Barney, 44-66%, Duncan 43-55%. Values in the untreated MPS VI control and the normal control were 51-84% and 1-8% respectively. The proportion of DS2 was approximately one to twofold higher than the level of DS1 in the MPS VI cats with or without ERT.

4.3.8 Pathology

4.3.8.1 *Postmortem*

The abdominal organs of all three cats appeared normal. The mitral heart valve was mildly thickened in Alf and Barney, and appeared normal in Duncan compared with normal control cats. There was diffuse or irregular whiteness of articular cartilage due to variable cartilage thickness in the femorotibial, coxofemoral and shoulder joints. Cartilage also appeared loosely attached and was easily removed from the subchondral bone in Alf and Barney. In Alf, there was evidence of cartilage flap formation in one femoral head and in one humeral head. Underlying subchondral bone appeared white and avascular and in many areas, plugs of cartilage remained in subchondral bone. The articular surface of the proximal humerus also had severe focal erosion of cartilage down to subchondral bone. The distal femur had undergone moderate remodelling in Barney and Duncan. The spinal cord was exposed from the C2 to L4 vertebral level and mild compression was present only in Duncan in the C7/T1 to T2/3 region.

4.3.8.2 *Light and electron microscopy*

As described in Section 2.2.22.3, well orientated thick sections from resin embedded tissues and electron microscopy of the same samples were the optimum for evaluation of tissues for pathology. Tissues from the MPS VI cats described in this chapter were collected for electron microscopy prior to being aware of the importance of tissue orientation, and also prior to optimum fixation and embedding. Therefore unfortunately, only limited tissues were available for analysis. However paraffin embedded light microscopy sections provided some

information about the pattern of overall storage in some tissues. Because of these limitations, grading of the degree of lysosomal storage as described in Section 2.2.23 was not performed, and the results were interpreted with some caution. For comparison with results from ERT treated MPS VI cats below, the presence of lysosomal inclusions in untreated MPS VI cats is outlined in Table 3.5.

No lysosomal storage was observed in liver Kupffer cells in all three ERT treated MPS VI cats (Figure 4.4). Similarly, in Barney and Duncan, lysosomal inclusions were almost absent in renal interstitial fibroblasts, however mild inclusions were present in this cell type in Alf (Figure 4.5). The glomerular basement membranes showed no evidence of immune complex deposition in all three cats. In skin fibroblasts from Alf, a progressive reduction in the degree of lysosomal storage was observed with increasing duration of therapy (2, 5 and 20 months after onset of ERT)(Figure 4.6). Minimal lysosomal storage was observed in skin fibroblasts from all 3 cats at euthanasia.

Chondrocytes from articular and tracheal cartilage from Alf, Barney and Duncan were distended with numerous membrane bound inclusions and were indistinguishable from an untreated MPS VI cat. No reduction in the degree of storage was observed in cartilage samples taken from Alf at 2 months and 20 months after onset of ERT (Figure 4.7).

Resin embedded samples from aorta were poorly embedded and were not orientated, however moderate smooth muscle cell vacuolation was present in Alf, and no vacuolation in Barney and Duncan. Similar changes were present in paraffin embedded sections in Alf and Duncan, however some mild lysosomal vacuolation appeared to be present in Barney. It is unknown if this difference in Barney could be due to sampling from different regions of the aorta or due to tissue orientation.

4.3.8.3 Renal glomerular immunofluorescence

No positive staining for immunoglobulin was observed in the glomerular basement membranes in Alf and Barney (samples from Duncan were unavailable). No positive control was available for immunofluorescence, however evaluation was made by an experienced technician (Mary Carli).

Figure 4.4: Electron microscopy of Kupffer cells in the liver following ERT.

Electron microscopy of Kupffer cells in liver showing (A) numerous enlarged lysosomes in a 15 month old untreated control MPS VI cat, (B) normalisation of morphology in Duncan, and (C) lipid vacuolation and absence of enlarged lysosomes in Barney. Bar, 2 μ m.

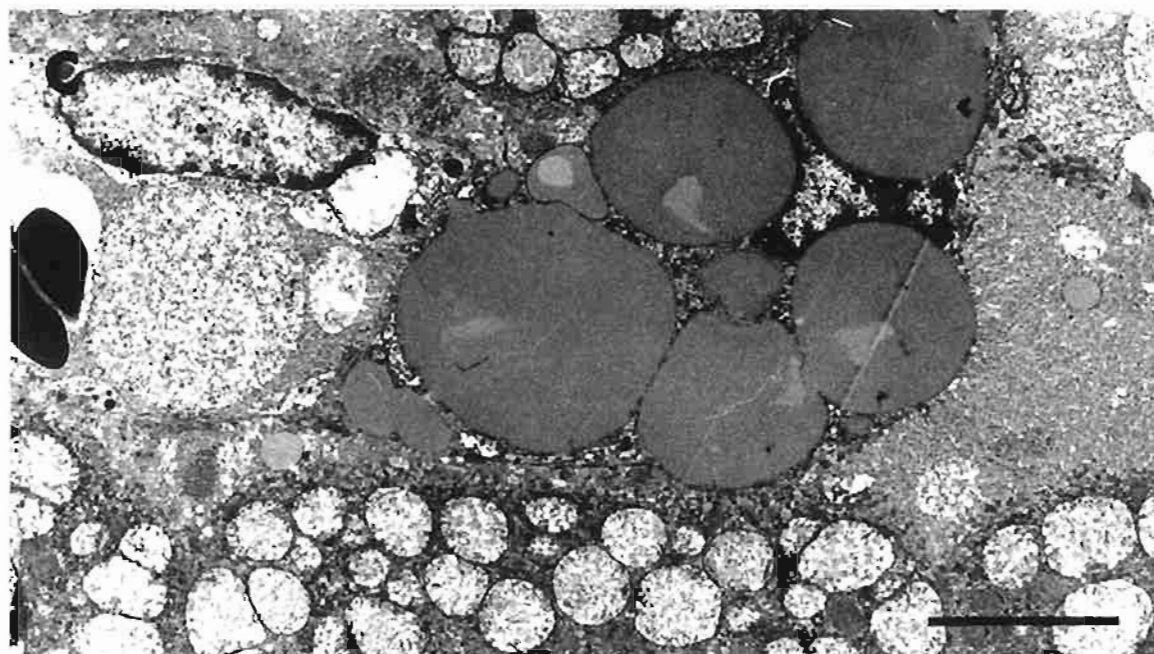
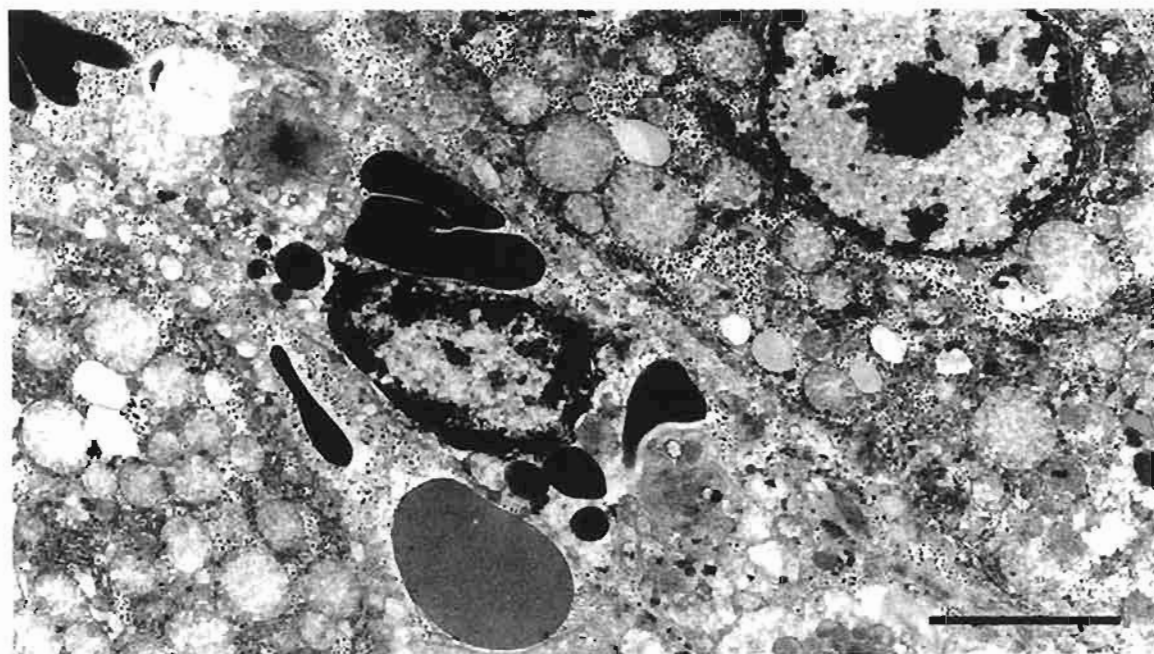
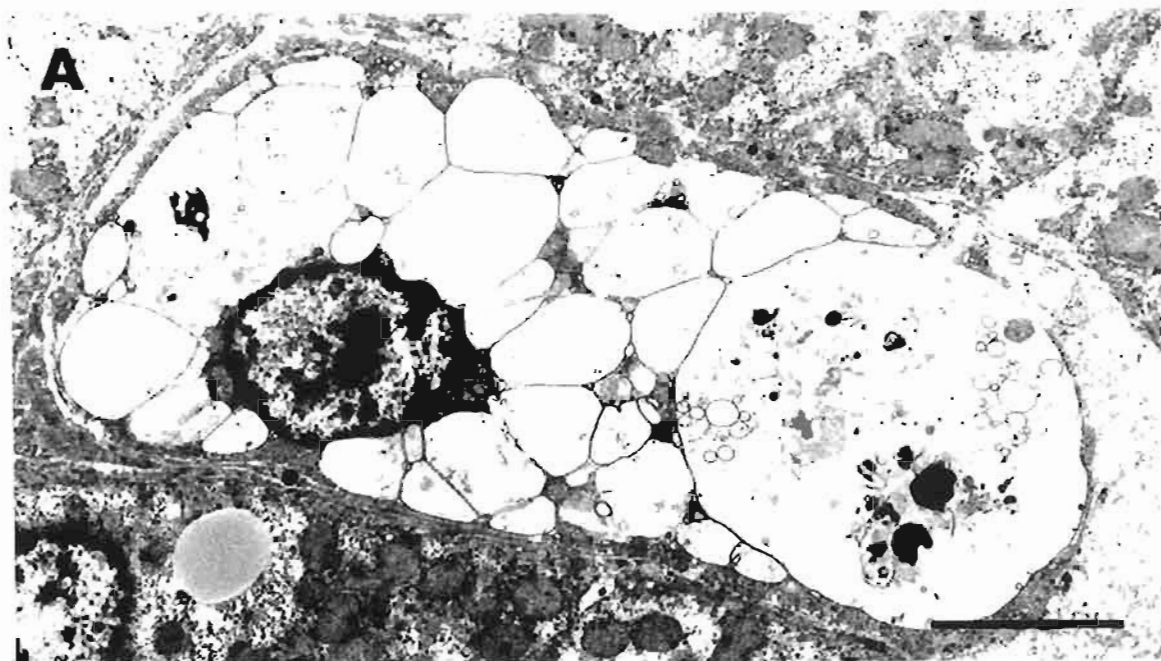
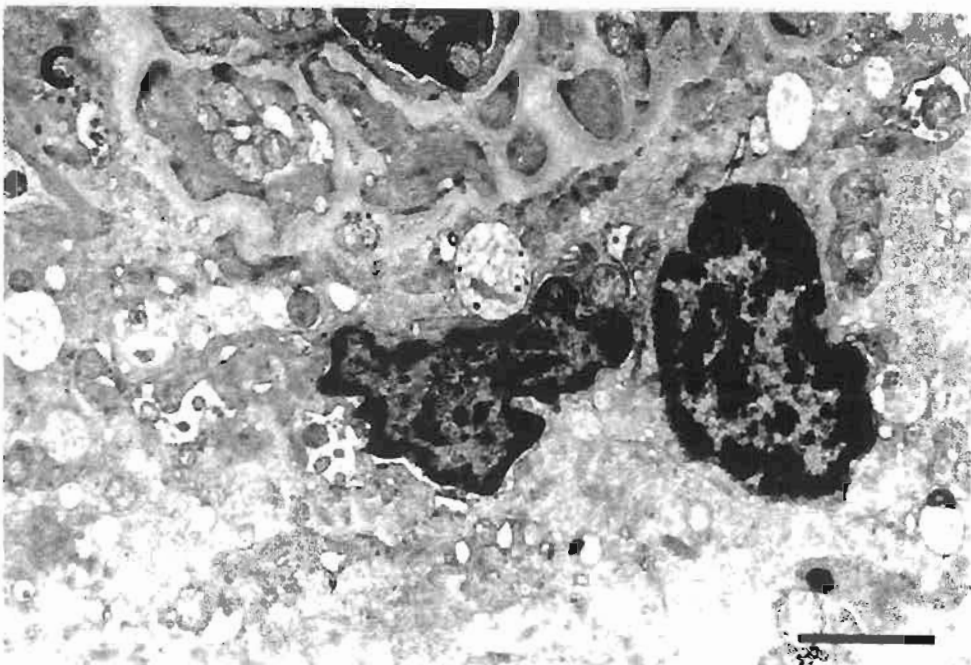
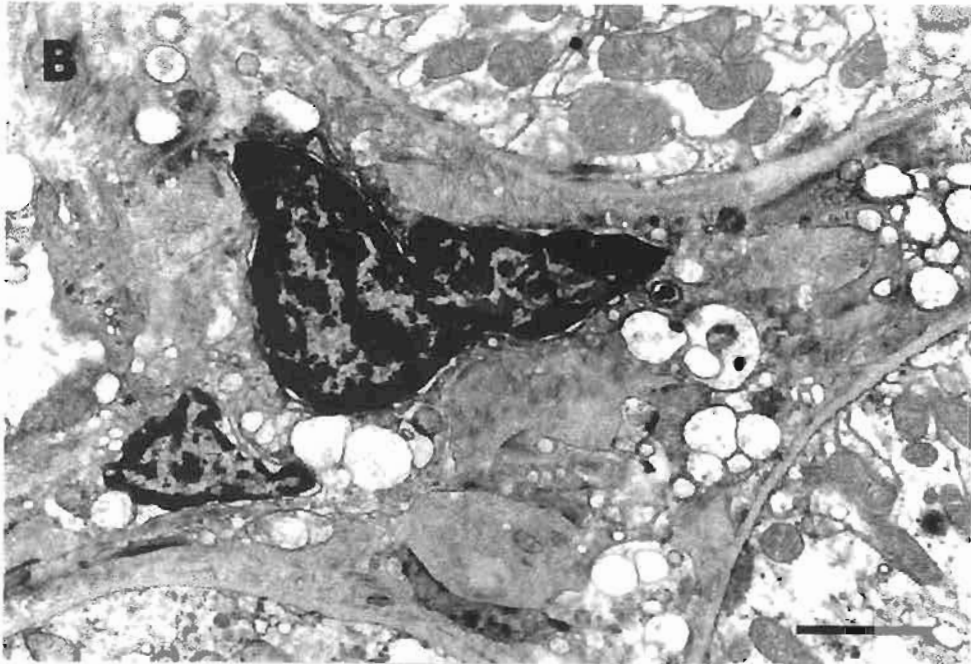
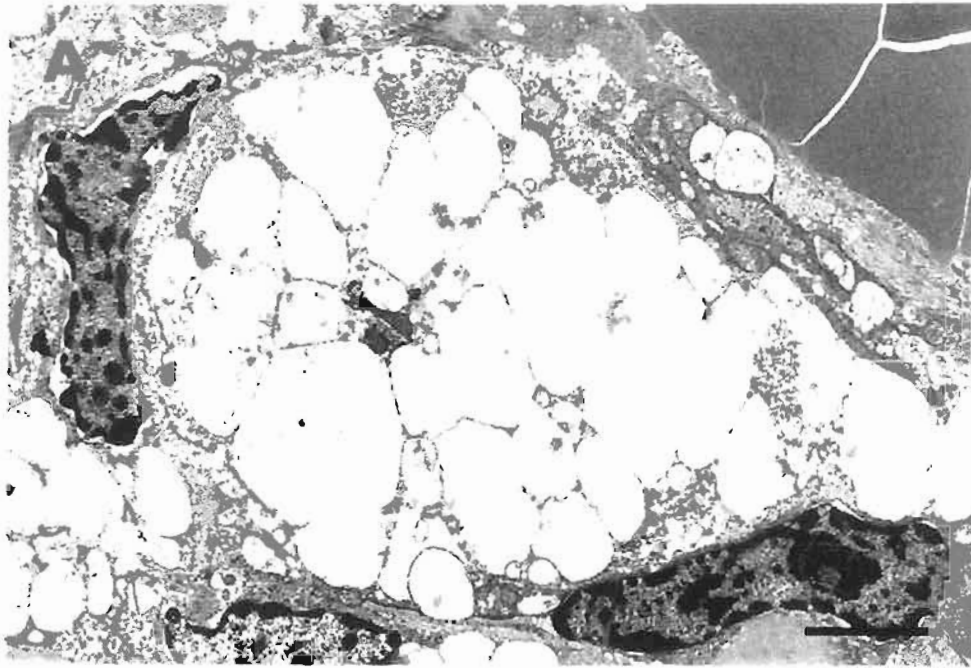


Figure 4.5: Electron microscopy of renal interstitial fibroblasts following ERT.

Electron microscopy of renal interstitial fibroblasts in (A) a 6 month old untreated control MPS VI cat with extensive lysosomal vacuolation, (B) reduced numbers of storage vacuoles in Alf, (C) almost complete normalisation in Duncan. Bar, 2 μm .

Figure 4.6: Electron microscopy of skin fibroblasts from skin biopsies following ERT.

Electron microscopy of fibroblasts from skin biopsies in (A) Alf, 2 months after onset of therapy with numerous enlarged lysosomes, (B) & (C) Alf, 5 months after onset of therapy with greatly reduced degree of lysosomal vacuolation. Bar, 2 μm .



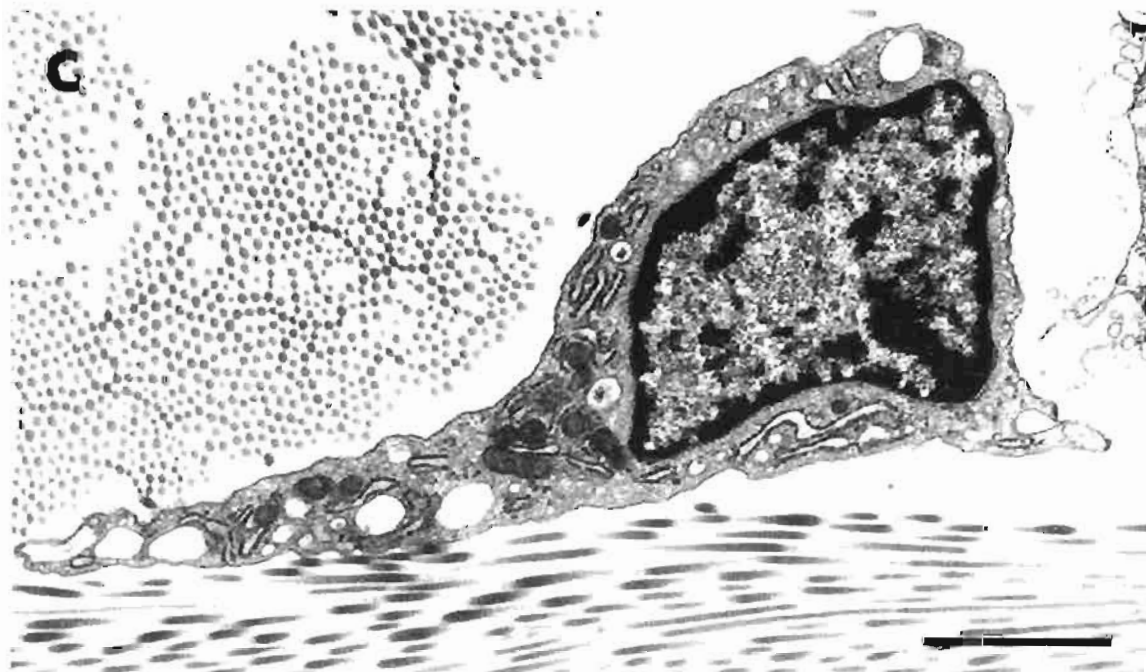
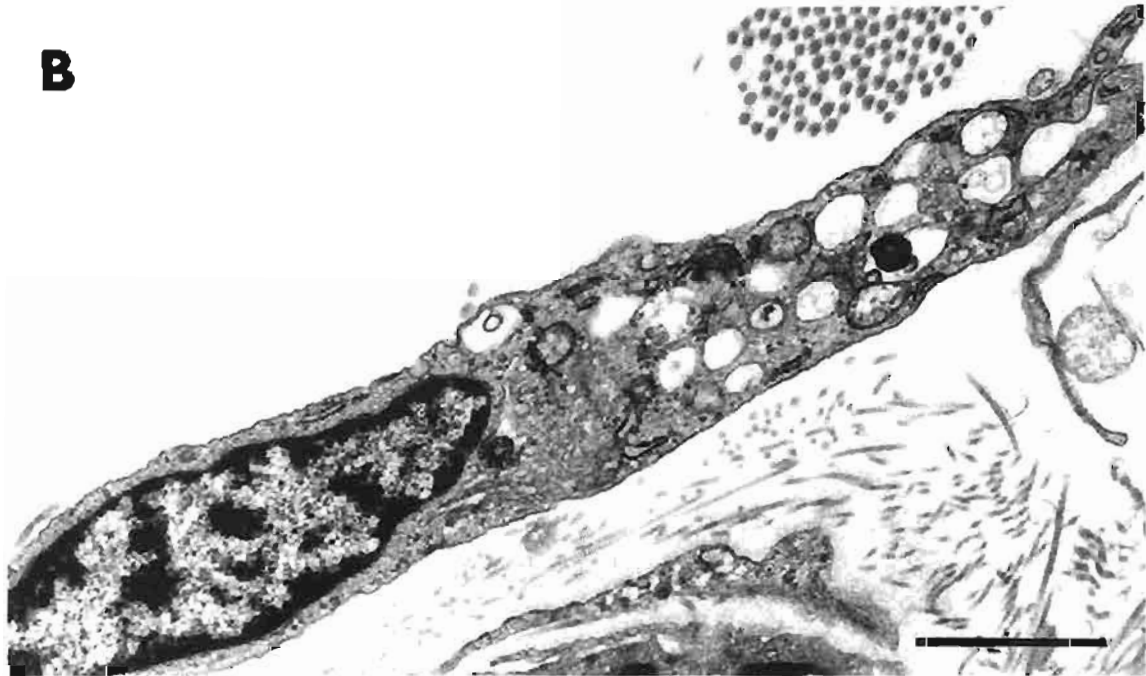
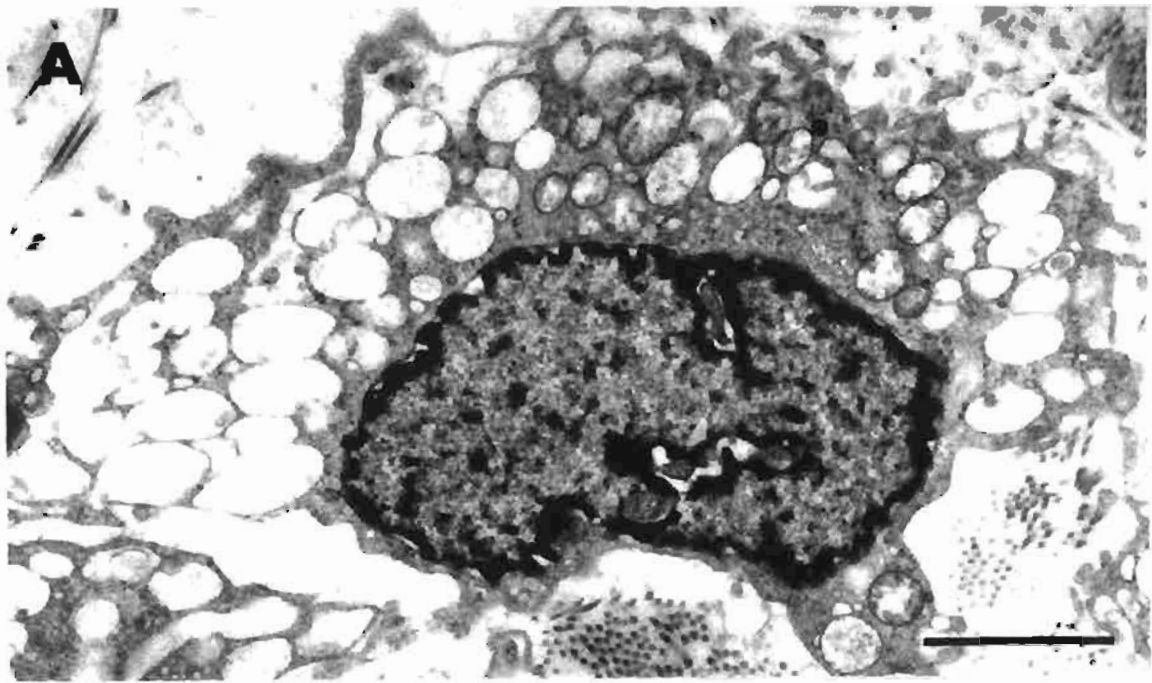
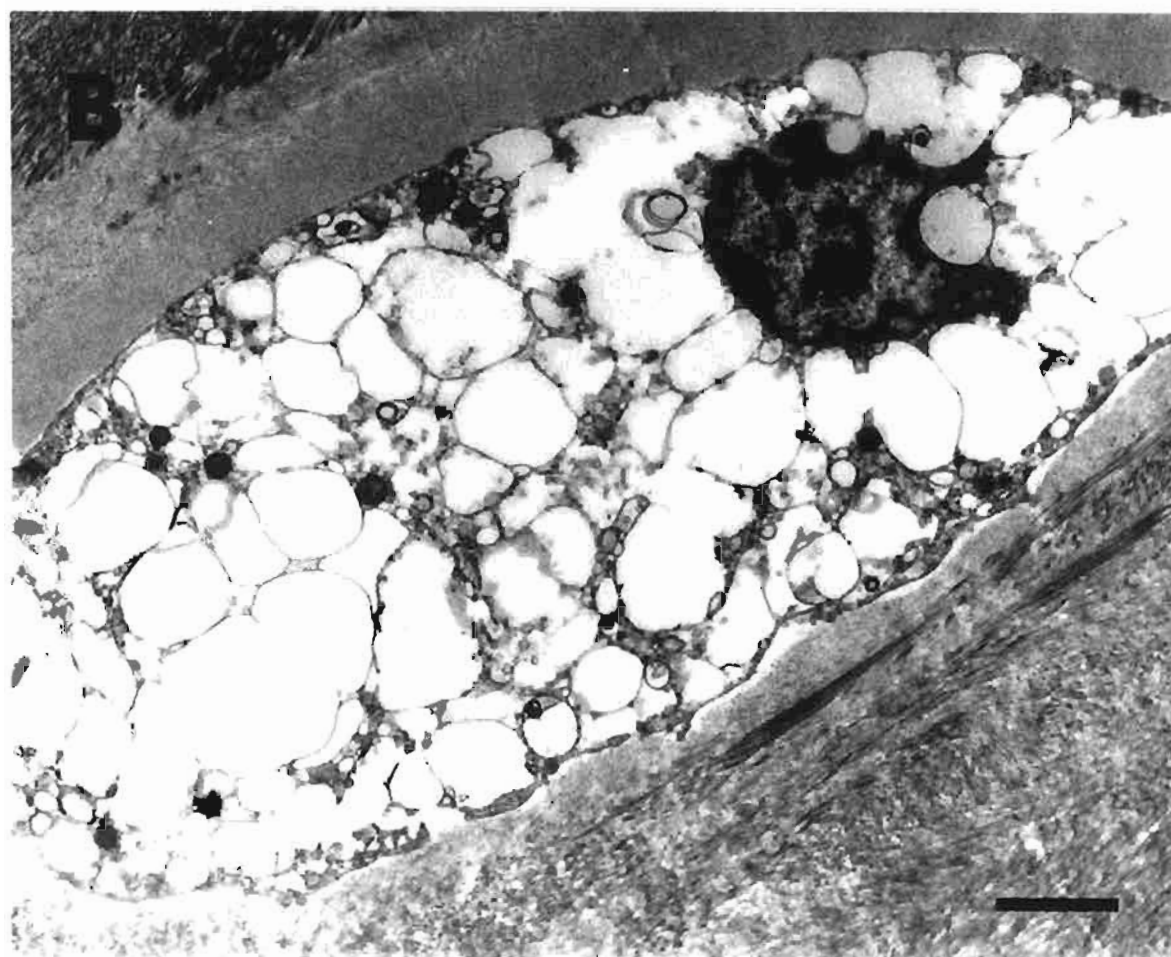
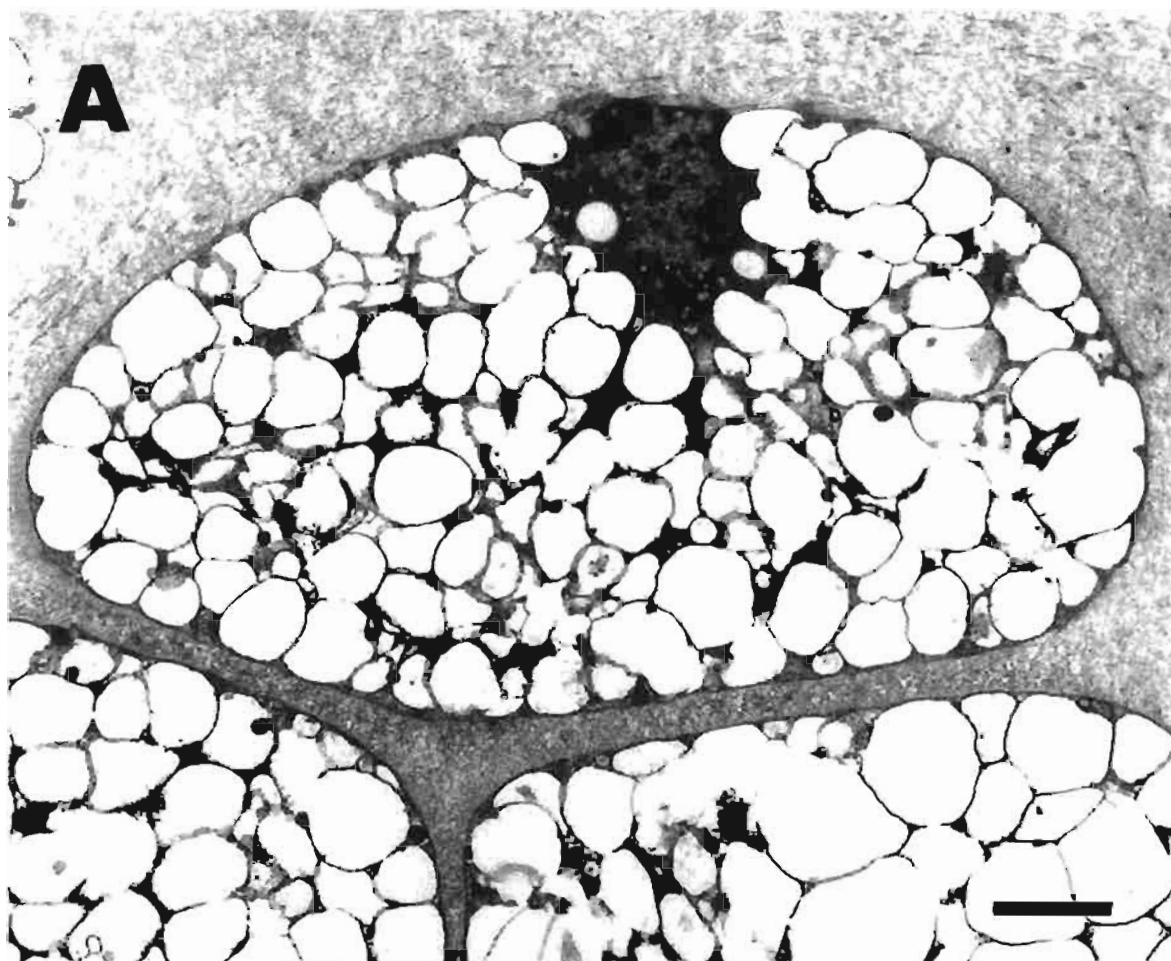


Figure 4.7: Electron microscopy of chondrocytes following ERT.

Electron microscopy of chondrocytes in (A) Alf, from an iliac crest biopsy 2 months after onset of therapy showing numerous large empty lysosomal vacuoles, (B) Alf , in tracheal cartilage 20 months after onset of therapy showing no reduction in degree of vacuolation.

Bar, 2 μ m.



4.4 Discussion

As part of preliminary studies, the aim of the experiments in this chapter was to evaluate distribution and fate of three variably glycosylated forms of rh4S in normal cats, and to determine the viability and efficacy of long term ERT with two of these forms in MPS VI cats.

4.4.1 Distribution studies

Rapid plasma clearance of enzyme largely by liver is considered to be a significant problem in ERT causing reduced availability of enzyme to other tissues (Rattazzi and Dobrenis, 1991). Short plasma half-life of enzymes predominantly due to liver clearance has been observed previously in distribution studies with a number of different lysosomal enzymes (Achérd *et al.*, 1977; Rattazzi *et al.*, 1979b; Koster *et al.*, 1994; Shull *et al.*, 1994) with the dose dependent plasma clearance observed for the rh4S forms appearing very similar to that observed in normal cats infused with human placental β -hexosaminidase (Rattazzi *et al.*, 1979b). At the highest doses of β -hexosaminidase, the clearance curve approached zero-order kinetics indicating that plasma clearance was mediated by a saturable receptor mechanism. A similar clearance curve was obtained at the ~ 7.5 mg/kg dose rate for both the CHO and LEC rh4S forms, suggesting a comparable process taking place. After 4 hours, it was equivocal whether increased circulating amounts of enzyme for a longer period seen in the higher dose rate had lead to increased relative amounts of rh4S activity in some tissues (CHO rh4S: cartilage, cornea, lymph node; LEC rh4S: spleen, lung). However, after 7 days, trace amounts of enzyme activity were present in a number of tissues in the ~ 7.5 mg/kg animal, with no activity in the comparable tissues in the 1 mg/kg animal, suggested that increased relative amounts of rh4S had been distributed to these tissues in the high-dose animal. Greater numbers of animals in each dose group are needed to confirm this result.

It has been suggested that as little as 5% of normal levels of enzyme activity may be sufficient to correct lysosomal storage in cells (Hopwood and Morris, 1990). Four hours after infusion of 1 mg/kg CHO rh4S, widespread tissue uptake of enzyme had occurred, with highest enzyme activity in the liver followed by spleen, lung and kidney, with activity of rh4S in these organs higher than the activity of f4S in a normal control cat. These organs

have significant levels of perfusion *in vivo* compared with the remaining organs and it is therefore not surprising that they contain higher enzyme activities. Trends observed in tissue distribution were comparable to those found in β -D-glucuronidase infused MPS VII mice (Sands *et al.*, 1994), β -D-hexosaminidase infusion into normal cats (Rattazzi *et al.*, 1981), and α -L-iduronidase infusion into MPS I dogs (Shull *et al.*, 1994; Kakkis *et al.*, 1996). These other studies showed highest levels of infused lysosomal enzymes in the liver followed generally by spleen, lung or kidney, and heart.

Although the glycosylation structures were not characterised, CHO rh4S is thought to have undergone complex oligosaccharide modifications and phosphorylation of terminal mannose structures. Conversely, high mannose structures of LEC rh4S have undergone phosphorylation but no complex oligosaccharide modifications due to lack of N-acetylglucosamine-transferase activity (Stanley *et al.*, 1975). In uptake experiments in rat lung macrophages LEC and CHO rh4S were taken up by different receptors and it was hypothesised that different uptake mechanisms reflected altered carbohydrate structures on each rh4S form utilising different cell surface receptors (Fuller *et al.*, 1998). Studies in M, 46 000 mannose 6-phosphate receptor deficient mice showed that uptake of lysosomal enzymes could take place by at least several different carbohydrate specific receptors (Koster *et al.*, 1994) thought to be due to the presence of different oligosaccharide residues on lysosomal enzymes (von Figura and Hasilik, 1986).

In these studies, CHO, LEC and LEC-P rh4S produced only minor differences in uptake, with the overall pattern of distribution being similar for each form. Greater numbers of animals in each group are needed to determine if these minor differences are significant. Cell uptake of CHO and LEC rh4S may have been by the mannose-6-phosphate receptor which is found on most cell types and which recognises phosphomannosyl terminating oligosaccharides (Koster *et al.*, 1993). However, other receptors may also be involved. Dephosphorylation of LEC rh4S to produce LEC-P rh4S should completely inhibit its uptake by mannose-6-phosphate receptors and lead to its uptake by other receptor systems. The lower enzyme activities in some tissues following the LEC-P rh4S 4 hour distribution supports this hypothesis.

The selectively impermeable blood-brain barrier prevents most proteins entering the central nervous system (CNS). Because of the CNS pathology observed in a number of the MPS types, targeting of enzyme through this barrier is of therapeutic value. Low but detectable levels of enzyme activity in brain samples of normal cats up to 7 days after injection of CHO rh4S were of interest, but it is not possible from the tissue analysis to determine if the enzyme activity detected in the samples was due to enzyme present within the brain parenchyma itself or in the capillary endothelium. Fractionation of samples by centrifugation to remove the capillary fraction would enable this distinction. Histochemical staining of brain sections from newborn MPS VII mouse pups, given intravenous enzyme one hour before euthanasia, demonstrated enzyme activity in the vessels and meninges, but not in CNS neurons (Vogler *et al.*, 1993). ERT studies in other MPS animal models have shown detectable enzyme levels in whole brain samples (Sands *et al.*, 1994; Shull *et al.*, 1994) and also subsequent correction of some neuronal storage (Sands *et al.*, 1994; Vogler *et al.*, 1996), however improved methods to target higher enzyme concentrations to the CNS are still needed.

The difference in f4S activity in various tissues between a colony bred normal cat, and an outside bred normal cat are interesting (Table 4.2). The consistently higher activities in the outside normal cat tissues suggests that the colony normal cat was quite possibly a heterozygote resulting in reduced tissue f4S activities, although subsequent genotype analysis was not possible. Lower white cell f4S activities are observed in heterozygotes, and this and other observations are presented in Chapter 6. The range of f4S activities in "outside" normal cat tissues is also unknown and no other animals were analysed for f4S activity to determine if this was a consistent observation in normal cats from different origins.

The estimated half-life of human 4S in cultured fibroblasts was 7 - 14 days (Taylor *et al.*, 1990) and this value was used to decide on the initial dose frequency in all three treated MPS VI cats. However the half-life of CHO rh4S ranged from 2 - 4 days in selected tissues, suggesting that the 7 day dose interval in Alf and Barney was more appropriate, than the two week interval used initially in Alf and Barney, and for the duration of ERT in Duncan. Similar tissue half-lives have been reported for bovine testes acid α -glucosidase in mice (2-4

days) (Van der Ploeg *et al.*, 1991) and for recombinant β -D-glucuronidase in MPS VII mice (1.4-4.5 days) (Vogler *et al.*, 1993).

Surprisingly, significant enzyme activity was observed in liver, spleen, kidney, and aorta 2 days after subcutaneous injection of 1 mg/kg CHO rh4S. It is unknown how much of this enzyme was absorbed into the peripheral circulation, and it is possible that enzyme activities may also reflect macrophage uptake at the site of injection and transfer to other tissues. However large amounts of enzyme remaining at the injection site, and lower enzyme activity in tissues compared with intravenous infusion indicate that the subcutaneous route is not optimal for enzyme replacement therapy.

When the total enzyme recovered in the 4 hour distribution studies was estimated using the enzyme specific activity and organ weights, greatly increased levels of enzyme recovery was calculated in the liver (up to 740 % of the total amount injected). Using an alternative method with radiolabelled rh4S, comparable trends in distribution were observed in cats (Sharon Byers, unpublished observations) and also in rats (Brooks *et al.*, 1997), however much lower values were obtained in the liver (50% of the total injected in the cats). The reasons for the elevated liver levels using the immunobinding assay are unknown, however variable specific activity measurements of the original enzyme, and altered enzyme activity due to dilution in liver tissue extract are possible factors. Direct assay of rh4S in homogenised liver extracts is not possible due to the presence of a non-dialyzable inhibitor (Brooks *et al.*, 1994).

Tissue distribution of rh4S was very similar in normal and MPS VI cats. This was also observed with distribution of human β -hexosaminidase in a GM₂ affected kitten (Rattazzi *et al.*, 1979a), indicating that the presence of lysosomal storage did not inhibit enzyme uptake.

4.4.2 Efficacy of enzyme replacement therapy in MPS VI cats

Interpretation of results from the preliminary ERT studies discussed in this chapter were overall limited by the small numbers of ERT animals and age-matched untreated MPS VI controls evaluated. Lack of obvious differences among the ERT cats in clinical appearance and progression of disease, despite different ages of onset of therapy, probably largely

reflects the initial low dose rate and dose frequency in all three ERT cats, and also the clinical heterogeneity observed in MPS VI cats within the colony (see Chapter 3).

Therapy was instituted in Alf and Barney after skeletal pathology was well established, and radiographically, at the dose rates and frequencies used, long term ERT was unable to significantly alter the appearance of skeletal disease in all three cats. However, subtle improvements in bone density in all three cats, and a trend towards increased fifth lumbar vertebral bone mineral volume (BV/TV) and trabecular number (Tb. N.) in Duncan, suggested that ERT may have had some effect on skeletal disease, and that earlier onset of therapy may have been an important factor in this process. At the dose rates used, ERT was unable to improve bone volume once skeletal maturity was reached, as seen in Barney. Obviously these results needed to be evaluated in more ERT treated animals. A relatively high level of enzyme was present in the bone marrow in distribution studies (Table 4.4 and Table 4.5), which would suggest that rh4S was available to cells surrounding bone trabeculae emerging from the growth plate, and possibly also to the growth plate itself, resulting in the improved histomorphometric parameters in the growing cats.

Rats and mice have open growth plates throughout life, compared with growth plate closure on maturity in larger animals such as cats, dogs and humans. This suggests that there may be important differences in growth plate regulation between these animal groups, therefore studies of skeletal pathology in the MPSs and their response to therapy may be more appropriate in larger animal models with similar growth regulation to humans.

These studies clearly indicated that long term ERT with rh4S in MPS VI cats completely removed and prevented further accumulation of lysosomal storage in liver, and had some effect on the degree of lysosomal storage in some other soft connective tissues. In the limited number of tissues analysed, the minor differences in tissue distribution between CHO and LEC rh4S did not appear to significantly alter the degree of lysosomal vacuolation in various tissues.

Normalisation of morphology in Kupffer cells has been demonstrated in other animal models undergoing ERT (Sands *et al.*, 1994; Shull *et al.*, 1994), which is not surprising considering the large amounts of enzyme found in the liver following infusion. The significant reduction

in storage vacuoles in renal interstitial fibroblasts was not observed in MPS I dogs that underwent ERT (Shull *et al.*, 1994). This may have been due to the lower dose rates used in the MPS I dogs. Our study suggests that skin biopsies may be a useful way of monitoring response to ERT over time. Reduction in lysosomal storage in dermal fibroblasts was also observed by Navarro *et al.* (1991) in an MPS I patient who had undergone BMT 21 months earlier. The reduction of total urinary GAG in MPS VI cats undergoing ERT, although not completely normalised, correlated with the reduction in vacuolation seen in most tissues examined.

Lack of correction of storage seen clinically in the corneas and demonstrated by electron microscopy in the chondrocytes, correlates with the lack of or negligible levels of enzyme activity in these tissues in the distribution studies, as well as with ERT studies in other animal models (Sands *et al.*, 1994; Shull *et al.*, 1994). The degenerative joint disease observed in the ERT treated MPS VI cats was slightly milder than that observed in a 30 month old untreated MPS VI cat (see Section 3.3.5.2), however this was not considered to be a significant difference, as notable variation in severity of degenerative joint changes was observed in 11 month old untreated MPS VI cats.

All cats tolerated enzyme administration with minimal side effects, indicating the potential viability of long term ERT at the doses rates used, at least in the feline model of MPS VI. It was not possible to correlate development of antibody titre and subsequent fluctuations with clinical well-being of the cats in this study. The dramatic reduction in plasma antibody titre in Alf after enzyme administration indicates antibody binding to the rh4S enzyme. A dose related inactivation of rh4S was also observed *in vitro* with the addition of the high titre plasma to rh4S (Crawley *et al.*, 1996). In high titre rats immunised against rh4S, altered intracellular routing and more rapid turnover of rh4S was observed, with reduced levels of rh4S activity in the lysosomal compartment (Brooks *et al.*, 1997). Analysis of rh4S activity in tissues taken 7 days after enzyme infusion in Alf and Barney showed that Barney had rh4S detectable in most tissues, whereas Alf only had activity detectable in liver. This supports the hypothesis of reduced activity of rh4S in the presence of high titre antibody as seen in Alf, however these differences may also be due to enzyme form, although tissue activities in Barney (LEC rh4S) were comparable to the 7 day CHO rh4S distribution studies.

Although reduced efficacy of ERT was not obvious clinically in Alf compared with Barney and Duncan, there was an impression of slightly more lysosomal storage in connective tissues in Alf. This may possibly reflect the hypothesis of increased enzyme degradation and altered intracellular targeting in the presence of a high antibody titre. However these subtle differences may also be due to tissue sampling, as distribution of lysosomal storage in ERT treated MPS VI cats was later observed to be non-uniform in some tissues (see Section 5.3.9). A third possibility is that the differences in pathology between Alf and Barney (both euthanased 7 days after last injection) were due to differences in enzyme form.

At the time, the apparent reversal of hindlimb paralysis in Alf following onset of ERT was thought to be in response to ERT. However subsequent observations of the same phenomenon in several untreated MPS VI cats at similar ages (see Section 3.3.4.4) suggests that the fluctuation in use of the hindlimbs in Alf was probably incidental, although subjective improvement in mobility in Alf after increased dose and frequency of dosing may have been as a result of a reduction in joint capsule storage. Improved mobility has been reported in MPS VI patients and MPS VI cats following bone marrow transplantation (Gasper *et al.*, 1984; McGovern *et al.*, 1986) and in an MPS I dog undergoing long-term low dose ERT (Kakkis *et al.*, 1996) supporting the possibility that improved mobility in Alf may have been partially or fully in response to therapy. The lack of spinal cord compression in Alf despite a history of hindlimb paresis/paralysis was possibly due to subsequent remodelling of the vertebrae/spinal canal, despite a lack of significant radiographic improvement in overall skeletal disease. This conflict between change in hindlimb neurology clinically, and lack of cord compression at postmortem was not observed in any untreated MPS VI cats. However only mild compression was observed in some cats despite a history of severe hindlimb paresis/paralysis followed by some reversal of neurological deficits, supporting the possibility of remodelling of the bone surrounding the spinal canal. Alternatively the spinal cord compression may have been located higher than C4 or lower than L4, however this is not consistent with the neurological findings.

4.5 Summary and conclusions

As observed in previous distribution studies with lysosomal enzymes, all rh4S forms were rapidly cleared from circulation, with the majority of enzyme activity found in the liver, although significant enzyme activity was detectable in a number of other tissues. Minimal differences in the pattern of distribution to different tissues between the rh4S forms indicated alternative receptor mechanisms other than the mannose-6-phosphate receptor were able to mediate enzyme uptake. Low or undetectable enzyme levels were found in important sites of pathology such as cartilage and cornea with no histological correction in these tissues, however long-term ERT was effective in removal of lysosomal storage in liver, and in kidney and skin fibroblasts. There was no obvious reversal of skeletal changes radiographically, however there was a trend towards normalisation of some bone histomorphometric parameters in vertebral bone samples, correlating with earlier onset of therapy suggesting that therapy from birth may be the most efficacious. Minimal side effects to therapy were observed, however the presence of an elevated antibody titre in one cat possibly reduced efficacy of therapy. The estimated half-life for CHO rh4S of 2-4 days in various tissues indicated that a dose interval of 7 days may be more appropriate than 14 days.

In the next chapter, larger numbers of MPS VI cats treated from birth using increased dose rates and frequency were evaluated. Enzyme modifications to improve enzyme penetration into cartilage were also evaluated in animals undergoing the same protocol.

5. Enzyme replacement therapy from birth

5.1 Introduction

In the previous chapter, distribution studies in normal cats demonstrated widespread tissue uptake of rh4S and a tissue half life of approximately 2-4 days. Low or negligible enzyme activity was detected in cartilage and cornea, and no histological or clinical correction was observed in these tissues in the ERT treated cats. Although low dose ERT in MPS VI cats was unable to alter overall progression of clinical disease, results indicated a trend towards improved bone histomorphometric parameters following earlier onset of ERT.

The two major aims of experiments described in this chapter were firstly, to evaluate the efficacy of weekly ERT from birth in MPS VI cats for a duration of 5-11 months at three different dose rates. And secondly, to compare these findings with the efficacy of ERT using a twice weekly dose regime and also using modified enzyme to improve penetration of enzyme to cartilage.

To evaluate efficacy of ERT, clinical appearance, radiographic skeletal appearance, urinary GAG levels, plasma anti-rh4S antibody titre, postmortem changes, tissue histopathology and bone histomorphometric values were assessed.

5.2 Specific methods

5.2.1 Enzyme production and purification

Enzyme coupled with ethylene diamine or poly-L-lysine was kindly provided by Sharon Byers (Department of Chemical Pathology, WCH).

Only CHO rh4S was used in the experiments described in this chapter. Rh4S was produced and purified ready for injection using the monoclonal antibody column method and assayed for activity using 4MUS (Section 2.2.9). An increased enzyme specific activity of 55 000 nmol/min was used as the equivalent of 1 mg of enzyme compared with the specific activity of enzyme used in Chapter 4 due to changing the diluent in the final assay to 0.05% (v/v) Tween. Injection dose was calculated based on the amount of active enzyme.

Rh4S was also coupled with either ethylene diamine dihydrochloride (ED; Sigma Chemical Co.) or poly-L-lysine hydrobromide (PL; Sigma chemical Co.; average molecular weight = 3350 to 4000 Da) to change the pI of the enzyme to a net positive charge at neutral pH. Cationization of proteins is believed to enhance cellular uptake by adsorption to the negatively charged groups on the plasma membrane with subsequent endocytosis of the protein (Rattazzi and Dobrenis, 1991). Due to the highly negatively charged cartilage matrix, molecules are excluded from cartilage on the basis of size and charge (Maroudas, 1976). Therefore increasing the pI of rh4S would theoretically lead to improved enzyme diffusion into the cartilage matrix.

Briefly, the carboxyl groups on rh4S were coupled with either ED or PL according to previously published methods, using the water soluble crosslinker EDC (N-ethyl, N'(3-dimethylaminopropyl) carbodiimide hydrochloride; Sigma Chemical Co.)(Hoare and Koshland, 1967; Griffen and Giffels, 1982) and enhancing the reaction by the addition of NHS (N-hydroxysulphosuccinimide, Pierce, Rockford, Illinois, USA) (Staros *et al.*, 1986). Aliquots were run on an isoelectric focussing gel to confirm a change in pI. Activity of the coupled enzyme was then assayed routinely using 4MUS. The pI of unmodified rh4S was between 5.5 and 6.5, whereas coupling to ED resulted in an increase to 6.8-7.0, and coupling to PL resulted in a pI of >9 (Sharon Byers, unpublished observations). Coupling to ED and PL resulted in a decrease in the original enzyme activity by 65% and 48% respectively (Sharon Byers, unpublished observations). Injection doses for coupled enzyme were calculated based on the amount of active enzyme, with 55 000 nmol/min activity being used as the equivalent of 1 mg of enzyme.

5.2.2 Enzyme administration in MPS VI cats

MPS VI kittens were identified at birth by examination of a blood film (see Section 6.3.4), and diagnosis was subsequently confirmed by mutation analysis (Section 2.2.6). Normal cats were selected on the basis of a lack of neutrophil granulation on blood films. Retrospective mutation analysis has shown all of these cats to be D520N/normal heterozygotes. Where specified, additional control cats with different genotypes were sometimes used to increase control group size for some data. Different genotypes are compared and discussed in detail in Chapter 6.

MPS VI kittens were injected weekly or twice weekly with intravenous (IV) unmodified rh4S, or PL or ED modified rh4S (denoted rh4S, PL4S and ED4S respectively) at variable dose rates starting at between 14 and 58 hours after birth, for a duration of five, six (175 ± 3 days) and eleven months (323 ± 13 days) (Table 5.1). Rh4S at 1 mg/kg was also administered to one cat subcutaneously (SC). The enormous task and cost of enzyme production and purification ultimately limited the number of animals that could be treated by ERT. The dose rates were selected based on a combination of results from the distribution and preliminary ERT studies in Chapter 4, the estimated mg/kg dose used for ERT in Gaucher patients with Ceredase® ($60 \text{ IU/kg} \approx 1.8 \text{ mg/kg}$) (Murray *et al.*, 1991), and also according to the anticipated dose rate to be used in future human MPS VI ERT trials.

For ease of understanding, the animals fall broadly into two groups which in general will be referred to as the:

1. Primary dose group
2. Enzyme/dose modification group

Table 5.1: Numbers of MPS VI cats for each dose rate undergoing intravenous weekly* ERT from birth with unmodified rh4S, or undergoing ERT with modified enzyme and/or dose frequencies, and total duration of ERT for these groups.

	6 months	11 months
<u>Primary dose group*</u>	duration	duration
0.2 mg/kg rh4S	1m	-
1 mg/kg rh4S	2m, 2f	1f
5 mg/kg rh4S	2m [†]	1m
<u>Enzyme/dose modification group</u>		
1 mg/kg PL4S	1m, 1f	-
1 mg/kg ED4S	1m, 1f	-
0.5 mg/kg rh4S twice weekly	1m, 2f	-
0.5 mg/kg ED4S twice weekly	1m, 2f	-
1 mg/kg rh4S SUBCUTANEOUSLY	1f	-

* weekly dose frequency administered IV unless otherwise stated

† 5 months duration due to reactions in 1 cat (see Section 5.3.1) and also due to limited enzyme supply and the very large doses required with increasing bodyweight.

m, male; f, female.

All cats undergoing ERT were given oral premedication from six weeks of age with 1mg cyproheptadine and 1mg chlorpheniramine orally at least 30 minutes prior to injection. Premedication dose was increased to 2mg of each, once bodyweight exceeded 2000 g. A maximum enzyme volume of approximately 0.2ml/100g bodyweight was infused into newborn kittens, with a maximum infusion rate of 0.15ml/100g/minute (90ml/kg/hour). In older kittens, enzyme was infused in volumes of up to 6 mls into the cephalic vein over a period of 5 to 10 minutes. Where the animal had a history of possible mild anaphylactic reactions to infusions, enzyme was diluted with PBS and infusions were extended for a duration of 15 to 45 minutes. If signs suggestive of developing anaphylaxis occurred, enzyme infusion was stopped and reinstituted within 60 minutes. Several enzyme

preparations were tested negative for the presence of pyrogens using the Limulus lysate assay (*performed by Lakshmi Kumaratilake, Department of Paediatrics, WCH*).

5.2.3 Clinical and biochemical evaluation of ERT treated MPS VI cats

A clinical, neurological (Section 2.2.11) and standardised radiological examination under general anaesthesia (Section 2.2.13) was done at 3, 5 and 6 months of age, and slit lamp examinations of the corneas were performed in conscious animals at 4.5 to 6 months of age (Section 2.2.11). In cats that were being observed for 11 months duration, clinical, neurological and radiological examinations were done at 3, 5, 7, 9 and 11 months, and slit lamp examinations at 5 and 10 months.

Radiographs taken at 5 to 6 months of age were evaluated “blind” for evidence of skeletal changes in response to therapy, compared with characteristic features of MPS VI in untreated controls (Section 3.3.4.6). Standardised measurements of bones were taken directly from the radiographs (Section 2.2.14).

Urine, direct blood smears and heparinised plasma were collected under general anaesthesia whilst undergoing radiology procedures, and renal function parameters (Section 2.2.15) and antibody titres (Section 2.2.21) were measured from urine and plasma samples respectively. Blood films were stained routinely (Section 2.2.8) and examined for characteristic morphological changes found in MPS VI as described in Section 6.3.4.

Total urine GAG content in approximately half of the urine samples was quantitated by both the ABC method (Section 2.2.16) and by the DEAE method (Section 2.2.18), and results obtained from both methods were compared. High resolution electrophoresis (HRE) was also performed on the same samples (Section 2.2.17). Several urine samples were purified by both CPC precipitation (Section 2.2.17) and the DEAE method and then electrophoresed on a gradient gel (Section 2.2.20). The uronic acid content (μg uronic acid/mg creatinine) of the total GAG purified by both CPC precipitation and the DEAE method from the same urine samples was also compared.

5.2.4 Pathology in the ERT treated MPS VI cats

Cats undergoing ERT were euthanased at 5, 6 or 11 months (see Table 5.1), 4 days after the last enzyme injection, using an overdose of IV barbiturate. Tissues were collected using a standard postmortem procedure and were processed for light and electron microscopy as outlined in Sections 2.2.22. Paraffin embedded tissues were evaluated by light microscopy for any pathological changes associated with therapy. Fluorescent antibody staining of frozen kidney sections to detect glomerular immune complexes was also performed and is outlined in Section 2.2.24.

Taking note of certain orientation of particular tissues (Section 2.2.22.3), 1µm thick resin embedded sections were cut and stained with Toluidine Blue and were evaluated for the presence of lysosomal storage as described in Sections 2.2.23 and 3.3.6.2. Selected samples were evaluated further under electron microscopy, including examination of renal glomeruli for the presence of immunoglobulin deposits in 5 mg/kg dose, in high antibody titre and in PL4S and ED4S treated animals. The L5 vertebra was removed for histomorphometric analysis and processed and analysed as described in Section 2.2.25.

5.3 Results

5.3.1 Enzyme administration

In animals which had recurrent reactions to enzyme infusions (see Section 2.2.10), symptoms were observed as early as 4 weeks of age, but usually began at between 6-8 weeks of age. Most cats given 1 mg/kg rh4S exhibited mild to moderate reactions on occasions throughout therapy. More frequent reactions were observed in two out of the three cats at the 5 mg/kg dose rate, however the third cat only exhibited several very mild reactions. None of the cats at 0.2 mg/kg, 1 mg/kg ED4S, 0.5 mg/kg rh4S twice weekly, 0.5 mg/kg ED4S twice weekly and 1 mg/kg SC exhibited any obvious signs of reactions to enzyme infusions on any occasion. Both cats given 1 mg/kg PL4S reacted repeatedly from 7-8 weeks of age. Reactions were usually well controlled by diluting the enzyme dose with PBS and increasing the time of infusion, as well as temporarily stopping infusion once a

reaction had started. On rare occasions, adrenalin was used to treat more severe reactions such as vomiting and increased respiratory rate (see Section 2.2.10).

One cat (125m, 5 mg/kg) developed moderate reactions to infusions on repeated occasions after nearly 5 months of therapy, including vomiting, trembling, increased respiratory rate and pyrexia. Enzyme preparations used in this animal were negative for bacterial culture and were pyrogen free. Two cats given either no premedication (176f, after 6 months ERT at 1 mg/kg PL4S) or only one of the two antihistamines (112f, after 10 months ERT at 1 mg/kg rh4S) both showed moderate reactions after enzyme infusion, with vomiting, trembling, increased respiratory rate and lethargy. This reaction was not present in 112f on subsequent injections when given the full premedication (no further injections were given to 176f as the reaction occurred on the final enzyme injection for the trial).

Three additional MPS VI kittens undergoing ERT were not included in Table 5.1 as they died at 12 to 14 weeks of age from parvoviral enteritis diagnosed histologically. These kittens were undergoing ERT at 1 mg/kg and 5 mg/kg rh4S weekly, and 0.5 mg/kg rh4S twice weekly. The two cats at 1 mg/kg and 5 mg/kg rh4S had both exhibited signs reactions following enzyme infusion, with severe reactions observed in the 5 mg/kg kitten (118m) from 4 weeks of age, and mild to moderate reactions observed in the 1 mg/kg kitten (119m) from 7 weeks of age. No reactions were observed in the third kitten.

There was no obvious correlation between severity or frequency with which reactions to enzyme infusions occurred and antibody titre against rh4S (see Section 5.3.2).

5.3.2 Antibody titres

The highest titres recorded during the study for each animal in the MPS VI no ERT, MPS VI + ERT and normal control groups are presented in Figure 5.1. No statistically significant differences were observed between untreated MPS VI, MPS VI + ERT and normal control groups (ANOVA; $p < 0.66$). No differences in titres were observed in the MPS VI + ERT animals between the primary dose group and enzyme/dose modification group, except the cat given SC rh4S injections which had a persistently elevated titre (at both 5 and 6 months)

at 256 000. In addition, an MPS VI + ERT animal (1 mg/kg rh4S, 119m) which died from parvoviral enteritis 8 days after the plasma sample was taken, had a very elevated titre of 1 024 000. A littermate (118m, 5 mg/kg) which also died from parvovirus 5 days earlier, had a titre of 32 000, and a surviving normal control littermate also had a titre of 32 000.

Generally there were only small fluctuations observed in antibody titres against rh4S from plasma taken at 3, 5 and 6 months of age, and also in the MPS VI cats treated to 11 months of age. High titres were sometimes followed by low titres in subsequent samples in several cats. These samples were reassayed and gave the same results. In some cases these animals had concurrent illnesses at the time of sampling (e.g. suspected subclinical feline parvoviral enteritis, mild to moderate upper respiratory tract infection probably due to herpesvirus), however other animals also undergoing ERT and untreated controls with similar symptoms never presented with elevated titres. There was also no obvious correlation between anti-rh4S titre and any changes in vaccination regime against infectious diseases amongst different cats, or the time between last vaccination and plasma sample collection.

Several cats including MPS VI + ERT cats were sampled at less than 3 months of age, and lower titres (128-1024) were observed.

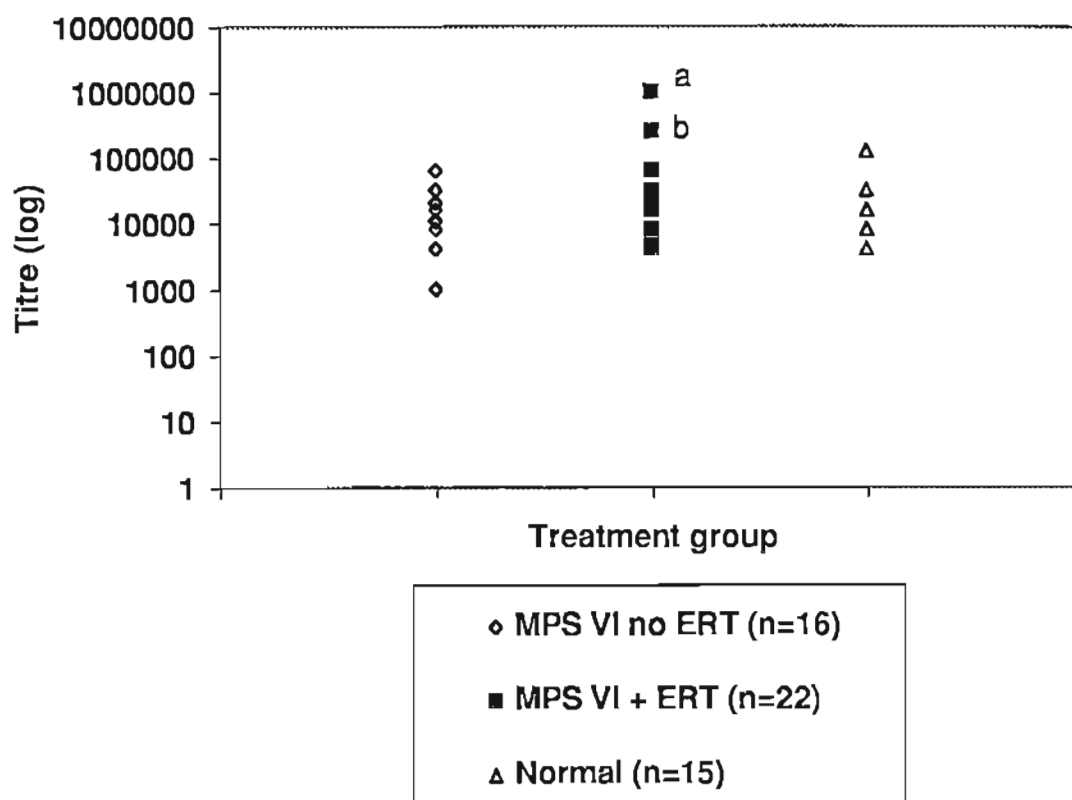


Figure 5.1: Antibody titres to rh4S in MPS VI cats +/- ERT and normal control cats.

Antibody titres to rh4S in MPS VI untreated cats, MPS VI cats undergoing ERT at all dose regimes, and normal control cats. Cats were sampled at 3, 5 and 6 months of age, and the highest titre recorded for each cat is shown. Very high antibody titres were found in (a) one cat undergoing ERT at 1 mg/kg rh4S which died from feline parvoviral enteritis 8 days after sampling (titre = 1 024 000); and (b) one cat given 1 mg/kg rh4S SC (titre = 256 000).

5.3.3 Disease progression in MPS VI cats undergoing ERT: clinical examination

All MPS VI cats showed mild degrees of corneal clouding, and cats undergoing ERT showed no changes in degree of clouding by slit lamp examination. Treated MPS VI cats were generally noticeably larger and heavier than untreated MPS VI controls, but weighed less than the normal controls (Table 5.2). There was an apparent trend between increasing dose rate and increasing bodyweight in the primary dose group, however in the enzyme/dose modification group, there was great variability in bodyweight particularly amongst males undergoing different dose regimes. As noted previously for untreated MPS VI cats, variability in physical appearance and disease severity was noticeable between ERT treated MPS VI animals in the same dose groups and also between ERT treated MPS VI littermates in different dose groups (see footnotes Table 5.2). All ERT treated cats retained features of MPS VI, however there was variable resolution of facial dysmorphia and thoracolumbar spinal kyphosis, near normalisation of ear size, and increased body and neck length in individuals at most dose rates except for 0.2 mg/kg and 1 mg/kg SC.

Considerable variability in physical appearance and disease progression was observed in animals from the enzyme/dose modification group, particularly with the 1 mg/kg PL4S and ED4S dose rates. Both of these dose groups contained one male and one female cat, and at both of these dose rates, the female cats appeared to respond more favourably to therapy than the male cats. This was thought to be incidental, reflecting the wide variability observed in disease severity in untreated MPS VI cats described previously (Chapter 3). However one cat (213m, 1 mg/kg PL4S) was also found to have a very fibrosed kidney at postmortem, probably as a result of a urinary tract infection detected at 8 days old which was subsequently treated with antibiotics. Overall this cat responded less well to therapy than the female counterpart on the same dose (176f), with slightly less cervical flexibility, mild hindlimb neurological deficits, and lower radiographic bone dimensions and bone histomorphometric parameters. Chronic infection may have affected the overall growth rate in this cat, but it is impossible to determine to what extent. However bodyweight in this cat was not the lowest of the ERT treated male cats.

Table 5.2: Mean bodyweights (grams) in 5 and 6 month old MPS VI cats undergoing ERT, and untreated MPS VI and normal controls.

	MALE		FEMALE		MALE		FEMALE	
Dose rate	n	5 months old	n	5 months old	n	6 months old	n	6 months old
MPS VI no ERT controls	10	2114 ± 259*	11	1867 ± 293	9	2305 ± 292	10	2032 ± 232
MPS VI 0.2 mg/kg	1	2391	-	-	1	2657	-	-
MPS VI 1 mg/kg	2	2658	3	2241 ± 244	2	2836	3	2302 ± 183
MPS VI 5 mg/kg	3	2744 ± 341	-	-	1	2740	-	-
<u>Enzyme or dose modifications:</u>								
MPS VI 0.5 mg/kg twice weekly	1	2933†	2	2242	1	3179	2	2348
MPS VI 1 mg/kg PL 4S	1	2430‡	1	2292	1	2628‡	1	2442
MPS VI 1 mg/kg ED 4S	1	2726	1	2148	1	2863	1	2370
MPS VI 0.5 mg/kg ED 4S twice weekly	1	2281†	2	2216	1	2490	2	2318
MPS VI 1 mg/kg SC	-	-	1	1970	-	-	1	2045
Normal controls	3	2936 ± 70	8	2460 ± 308	3	3279 ± 204	7	2891 ± 335

* mean ± 1 SD; † littermates undergoing different dose regimes; ‡ cat 213 male;

Figure 5.2: Clinical appearance of an ERT treated MPS VI cat at 5 months of age.

MPS VI cat 125m was given weekly intravenous rh4S at 5 mg/kg from birth. Compared with untreated MPS VI controls, cat 125m exhibited increased bodyweight and size, greater cervical spine flexibility, increased body and neck length, increased ear size, partial resolution of facial dysmorphism, and no hindlimb neurological deficits. A certain degree of thoracolumbar spinal kyphosis was still present in this cat, although was not present in some other ERT treated MPS VI cats. Corneal clouding was unchanged.



From the start of the ERT trial in September 1993 to its end in April 1996 there were intermittent outbreaks of infectious diseases within the colony. This introduced variable environmental factors which affected different litters of kittens to different degrees. Feline parvoviral enteritis and feline viral rhinotracheitis (herpesvirus) were both positively diagnosed, with parvovirus causing deaths in several ERT treated cats (see Section 5.3.1) and in at least one non-trial kitten. The vaccine regime following some of these outbreaks was modified to try and reduce morbidity. Persistent upper respiratory tract infections, conjunctivitis and diarrhoea from undetermined causes also affected trial and non-trial cats intermittently. All affected animals were treated symptomatically, accordingly.

Untreated MPS VI cats showed moderate reduction in cervical spine flexibility allowing only $\sim 70^\circ$ lateral movement at 5 months, $\sim 50^\circ$ at 6 months and $\sim 45^\circ$ by 11 months (see Section 3.3.4.3). The reduction in flexibility in the 0.2 mg/kg treated cat and the 1 mg/kg SC treated cat was no different from the untreated MPS VI cats. However greatly improved flexibility was observed at the 5 mg/kg dose, with cats allowing $\sim 180^\circ$ lateral movement at 5 months and $\sim 160^\circ$ at 11 months (normal is $\sim 180^\circ$). Similarly, cervical flexibility in the 1 mg/kg rh4S cats was improved with only slight reductions at 5 and 6 months (~ 130 - 160°), but flexibility was reduced to $\sim 90^\circ$ by 11 months.

In animals from the enzyme/dose modification group, great variability was observed, with similar or slightly less cervical flexibility in most animals from the 1 mg/kg PL4S and 0.5 mg/kg rh4S twice weekly groups compared with the 1 mg/kg rh4S group at 5 and 6 months of age. Most animals in the 1 mg/kg ED4S and 0.5 mg/kg ED4S twice weekly groups had reduced cervical spine flexibility to between the range observed in untreated MPS VI cats and MPS VI cats treated at 1 mg/kg rh4S.

Extension of the coxofemoral joint was very variable and unreliable for evaluating flexibility.

Table 5.3: Hindlimb neurological status in 6 month old MPS VI cats undergoing ERT and untreated MPS VI controls.

Dose/control groups	normal	mild deficits	mild-moderate paresis	paralysis
MPS VI no ERT	2/21*	2/21	10/21	7/21
0.2 mg/kg	-	1/1	-	-
1 mg/kg	3/4	1/4	-	-
5 mg/kg [†]	3/3	-	-	-
1 mg/kg PL4S	1/2	1/2	-	-
1 mg/kg ED4S	-	1/2	1/2	-
0.5 mg/kg twice weekly	3/3	-	-	-
0.5 mg/kg ED4S twice weekly	2/3	-	-	1/3
1 mg/kg SC	-	-	1/1	-

* denominator denotes the total number of animals examined

† 5 months old

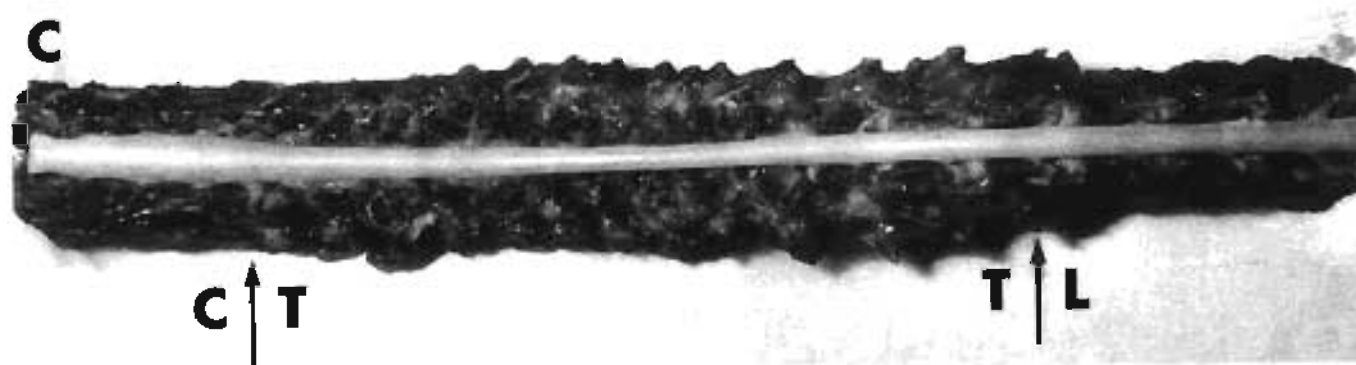
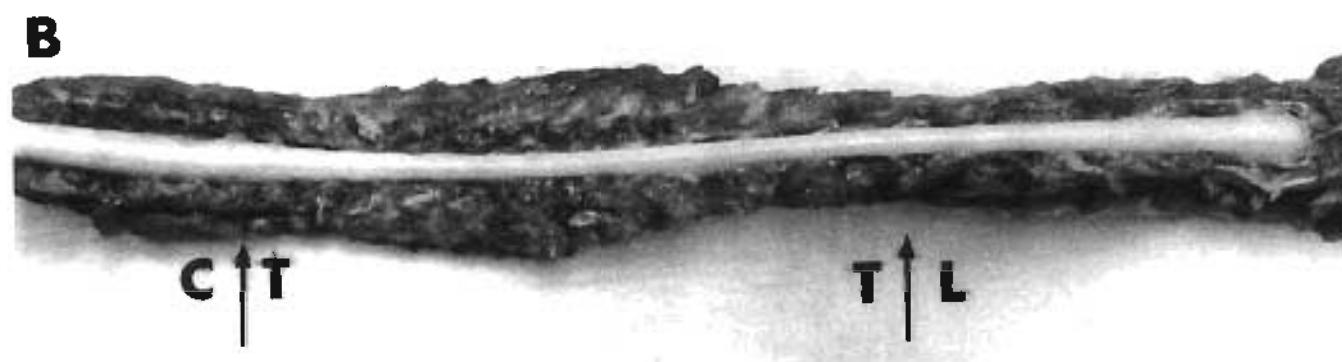
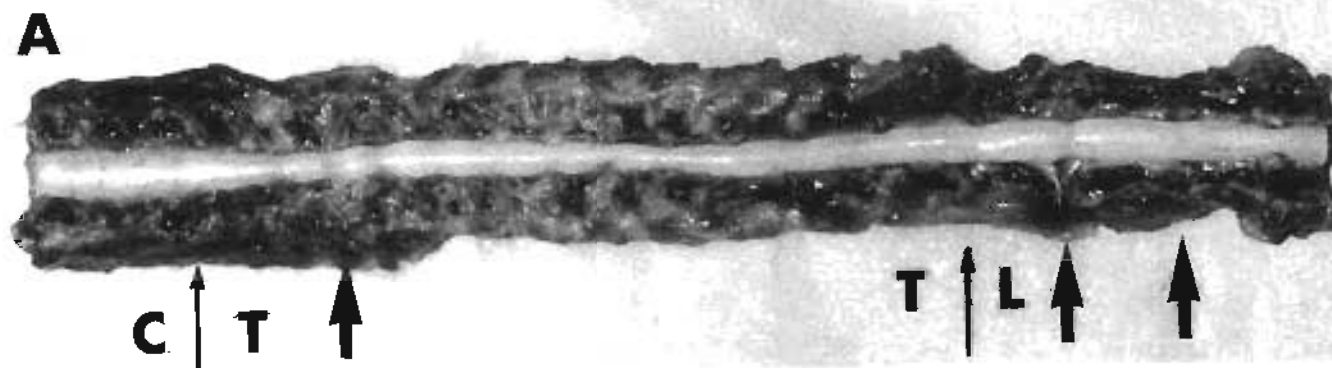
By 6 months of age, the majority of untreated MPS VI cats had either hindlimb paresis or paralysis due to spinal cord compression at multiple levels (Table 5.3). In contrast, the majority of ERT treated cats in both the primary dose group and enzyme/dose modification group were either neurologically normal or had only mild neurological deficits in the hindlimbs (Table 5.3). One treated cat had complete hindlimb paralysis (233f, 0.5 mg/kg ED4S twice weekly).

The two cats treated to 11 months at 1 and 5 mg/kg showed no hindlimb neurological deficits. The 5 mg/kg treated MPS VI cat (male) was housed together with a number of females, and mated successfully at 11 months of age with 5 live kittens born 2 months later, indicating reasonable flexibility and normal hindlimb neurology. However this is not unprecedented, as an untreated male MPS VI cat has successfully reproduced previously (Haskins *et al.*, 1982).

The incidence of spinal cord compression at postmortem was greatly reduced in MPS VI cats undergoing ERT (Figure 5.3). The presence of hindlimb neurological deficits correlated well with the presence of spinal cord compression in all but two of the treated cats at 6 months of age (see Table 5.3). One of these cats had no obvious neurological deficits but very mild upper thoracic cord compression, and the other had very mild neurological deficits but no obvious cord compression. Both of the 1 mg/kg and 5 mg/kg cats treated to 11 months had no cord compression compared with the four untreated MPS VI controls which had mild to severe compression at 11 months of age. In those ERT treated cats with cord compression, no differences were observed in the location of compression compared with untreated MPS VI cats, with compression found at T1-T9 and T11-L4 spinal cord levels.

Figure 5.3: Spinal cord compression in untreated and ERT treated MPS VI cats.

Spinal cord *in situ* after removal of the dorsal vertebral arches from C4 to L4: (A) an untreated MPS VI cat showing severe cord compression at multiple levels in the upper thoracic and thoracolumbar regions (bold arrows); (B) MPS VI at 5 mg/kg ERT with no cord compression; and (C) normal control cat. Photographs not standardised for magnification. Thin arrows indicate junctions of cervical (C) and thoracic (T), and thoracic (T) and lumbar (L) levels.



5.3.4 Radiological examination

At 3 months of age, the lumbar vertebrae of the ERT MPS VI cats were overall more normal in shape than the untreated MPS VI controls. However variability was already noticeable by this age in both groups. In the primary dose group, only 5/8 of the cats at 1 and 5 mg/kg had clearly longer and narrower lumbar vertebrae compared with two untreated MPS VI controls with the mildest skeletal changes at this age. The remaining 3/8 1 and 5 mg/kg cats and the 0.2 mg/kg cat were difficult to distinguish from the two mild untreated MPS VI controls. Similarly in the enzyme/dose modification group, 2/10 of the IV treated MPS VI cats had a similar skeletal appearance to the mild untreated MPS VI controls, and the 1 mg/kg SC treated MPS VI cat had more severe skeletal changes comparable with the majority of untreated MPS VI cats. No obvious differences in cervical vertebral length and bone density/quality were observed between untreated and treated MPS VI cats at this age.

At 5-6 months, it was possible to distinguish MPS VI cats undergoing ERT at all dose rates from untreated MPS VI controls, with the exception of the 0.2 mg/kg IV and 1 mg/kg SC dose rates, on the basis of improved bone quality, density and dimensions. At all other dose rates apart from 0.2 mg/kg IV and 1 mg/kg SC, more uniform bone density between lumbar vertebral epiphyses and vertebral bodies was apparent, compared with sclerotic, irregular epiphyses and radiopaque (osteopaenic) vertebral bodies seen in untreated MPS VI cats (Figure 5.4 A-D). In addition, the lumbar vertebrae and pedicles of the lumbar dorsal vertebral arches were longer and narrower compared with the short and broad vertebrae and pedicles in untreated cats, and there were milder degenerative changes seen in the lumbar vertebral articular facets (Figure 5.4 A-D, and Figure 5.5 A-C). Cervical vertebrae were also longer and cervical vertebral epiphyses were larger, and were generally more regular in these treated cats, more so at the 5 mg/kg dose (Figure 5.6 A-C). Trabecular pattern was distinctly more uniform in the distal femoral epiphysis and generally in the proximal humeral epiphyses compared with a coarse trabecular pattern in untreated MPS VI cats. The subchondral bone surfaces in these epiphyses were also smoother, compared with untreated cats which showed irregular bone surface contours (Figure 5.7 A-D). Overall, these epiphyses were larger, however several individuals still had similar sized epiphyses compared with some of the untreated MPS VI cats. These features were also seen in the

femoral head, and generally the head was rounder with less remodelling changes, however all cats still showed some degree of bilateral hip subluxation (Figure 5.5 A-C).

There was an overall impression of greatest effect at 5 mg/kg, but variability within each dose group for each of these features meant that it was not possible to accurately determine dose rate based on these radiographic changes. No obvious radiographic improvements were observed in the enzyme/dose modification group (except for 1 mg/kg SC described below) compared with 1 mg/kg rh4S, except epiphyses (shoulder and distal femoral) were possibly larger with generally smoother subchondral bone surfaces in the enzyme/dose modification group. Small group numbers and variability made it impossible to determine if this was significant.

Two of the untreated MPS VI cats had noticeably milder radiographic changes than the remaining untreated controls, and it was difficult to distinguish them from the 0.2 mg/kg treated cat. These animals could still be distinguished from the remaining IV ERT treated MPS VI cats by assessing lumbar vertebral bone density and cervical vertebral length. The skeletal changes in the 1 mg/kg SC treated cat was indistinguishable from more severely affected untreated MPS VI cats.

By 11 months of age, skeletal changes in response to ERT seen at 6 months were still evident. In addition, more uniform bone density was easily visible in the patellae of both of the treated cats, and less remodelling was evident in the femoral heads.

Figure 5.4: Lateral radiographs of the lumbar spine in untreated and ERT treated MPS VI, and normal control cats.

Radiographs of lateral view of the lumbar spine in ~6 month old cats, showing sclerotic, irregular epiphyses and radiopaque vertebral bodies in an untreated MPS VI cat (163m) (A); compared with more uniform bone density between lumbar vertebral epiphyses and vertebral bodies, and longer narrower dimensions, in an ERT treated MPS VI cat at 1 mg/kg rh4S (B); ERT treated MPS VI cat at 5 mg/kg rh4S (C); ERT treated MPS VI cat at 1 mg/kg PL4S (176f) (D); and a normal control cat (E). Milder degenerative changes in the vertebral articular facets were also seen in the ERT treated MPS VI cats except for the 0.2 mg/kg and 1 mg/kg SC dose rates, compared with untreated MPS VI controls. The changes seen in the untreated MPS VI cat shown is representative of the majority of untreated MPS VI cats.



Figure 5.5: Ventrodorsal radiographs of the pelvis in untreated and ERT treated MPS VI, and normal control cats.

Radiographs of ventrodorsal view of the pelvis and lumbar spine in ~6 month old cats. In addition to sclerotic lumbar vertebral endplates, the lumbar vertebrae and pedicles of the dorsal vertebral arches (arrow) were short and broad in untreated MPS VI cats (A), compared with longer, narrower vertebrae and pedicles (arrow) in MPS VI cats at 5 mg/kg ERT (B). A normal control is shown in (C). The femoral head epiphyses were also a more normal shape in the ERT treated MPS VI cats although some degree of bilateral coxofemoral subluxation was still present.

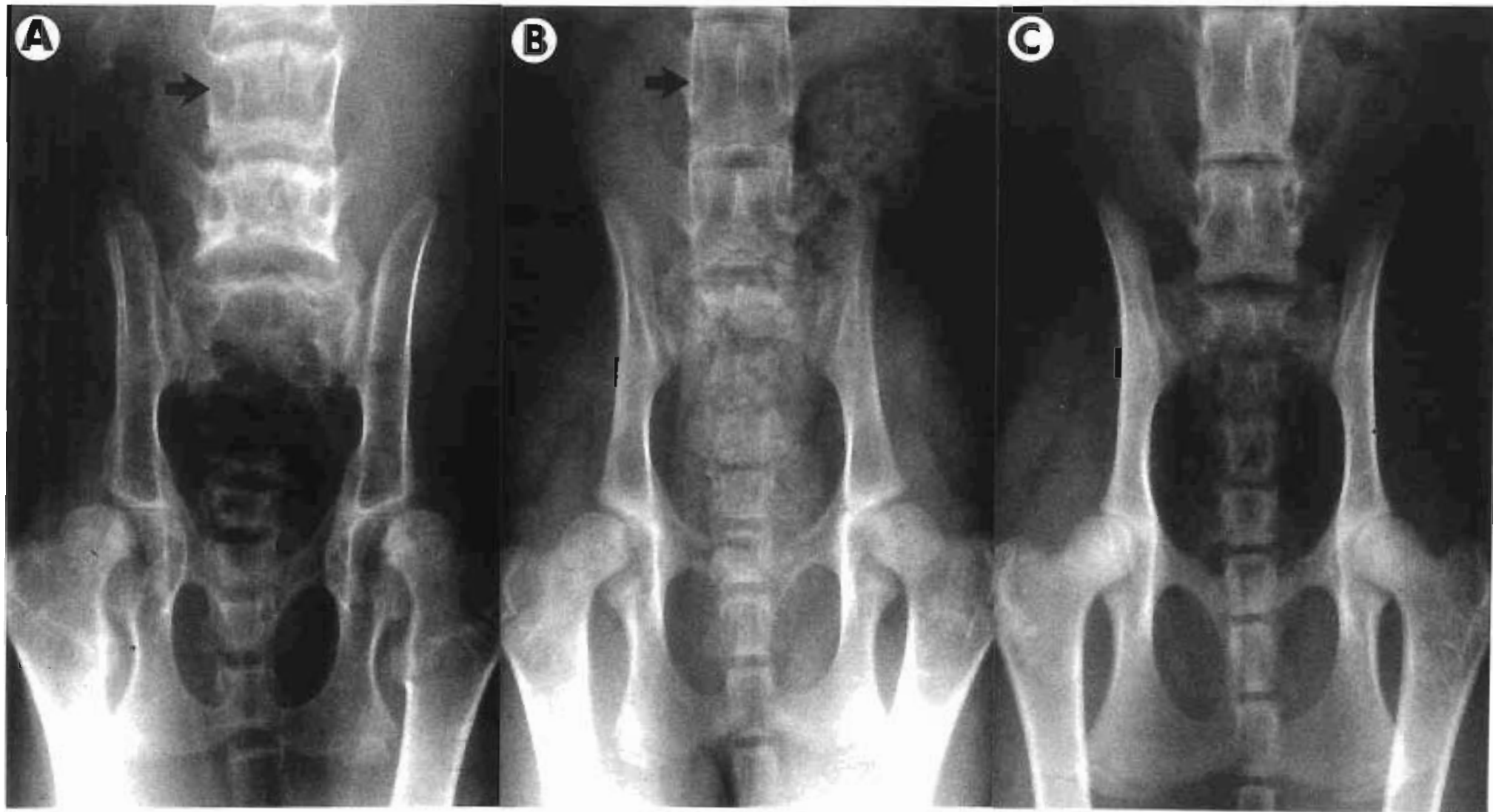


Figure 5.6: Lateral radiographs of the cervical spine in untreated and ERT treated MPS VI, and normal control cats.

Lateral radiographs of the cervical spine and shoulder joints in ~6 month old cats: untreated MPS VI (A), ERT treated MPS VI cat at 5 mg/kg rh4S (B), and a normal control cat (C). Cervical vertebrae were longer and vertebral epiphyses were generally more regular in ERT treated MPS VI cats. Proximal humeral epiphyses generally had a more uniform trabecular pattern, were larger, and in some ERT cats had smoother subchondral bone surfaces.



Figure 5.7: Lateral radiographs of the stifle joint in untreated and ERT treated MPS VI, and normal control cats.

Lateral view of the right femorotibial joint in ~6 month old cats: untreated MPS VI (A), ERT treated MPS VI cat at 1 mg/kg rh4S (B), ERT treated MPS VI cat at 5 mg/kg rh4S (C), and normal control (D). Trabecular pattern in the distal femur was more uniform and subchondral bone surfaces were overall smoother in MPS VI cats undergoing ERT at all dose rates, except at 0.2 mg/kg and 1 mg/kg SC.



From direct measurements of bone dimensions from standardised radiographs, the third cervical vertebra followed by the patella and the fifth lumbar vertebra were the bones most severely reduced in length in MPS VI cats at 6 months of age (see Section 3.3.4.8). To normalise the differences due to sex (Section 3.3.4.7), all measurements were expressed as a percentage of the mean values for the same measurements in either normal males ($n=5$) or normal females ($n=8$). Numbers of untreated MPS VI controls at 6 months old were: males, $n = 9$, and females, $n = 10$.

In the primary dose group at 1 mg/kg (6 months old) and 5 mg/kg (5 months old), there was a shift in values from each individual for all measurements (C3 length, C3 length/width ratio, L5 length, L5 length/width ratio, patella length, tibial length, tibial corticomedullary ratio) to the upper range, or higher than, the values observed in untreated MPS VI cats, resulting in increased mean dimensions for each parameter (Figure 5.8 A-C; C3, L5 and patella length shown only). Measurements from all 5 mg/kg cats were taken at 5 months of age, whereas all measurements from the remaining cats (Figure 5.8) were taken at 6 months of age. At 5 months of age, the mean value for most parameters from the 5 mg/kg group were higher than those from the 1 mg/kg rh4S dose group at 5 months (data not shown), except for tibial length and tibial corticomedullary ratio, which were the same as, and lower than (respectively) the mean values from the 1 mg/kg rh4S dose group. Hence at comparable ages, bone dimensions at 5 mg/kg were overall higher than at 1 mg/kg rh4S. Most values from the 0.2 mg/kg cat were within the upper range observed in untreated MPS VI cats. Greatest improvement in dimensions were observed in the L5 vertebral length. Conversely, increases in patella length and tibial length were small, which probably reflected both the wide range in values observed in the untreated MPS VI group for patella length, and the small differences in tibial length between untreated MPS VI and normal controls.

In the enzyme/dose modification group, most cats at 1 mg/kg PL4S, 1 mg/kg ED4S, 0.5 mg/kg rh4S twice weekly and 0.5 mg/kg ED4S twice weekly had increased values for all measurements, similar to those observed for 1 mg/kg rh4S (Figure 5.9 A-C; C3, L5 and patella length shown only). Some variability in bone dimensions was observed between individuals within the same dose group. Bone dimensions from the 1 mg/kg SC cat were

approximately equal to, or lower than the mean values for the same bones in the untreated MPS VI controls.

In the two MPS VI cats treated to 11 months of age at 1 and 5 mg/kg rh4S (cat 112f and 111m respectively), greatest improvement was observed in the tibial corticomedullary ratio. The dimensions for all other parameters except C3 width at both doses, and L5 length for the 5 mg/kg dose were similar to 11 month old untreated MPS VI controls (n=4). When compared with other animals in the same dose group, both of these treated cats at 5- 6 months of age had the lowest values for all measurements,. This helps to explain the apparently poorer response to therapy at 11 months compared with 6 months, and also reiterates the importance of larger group sizes before definitive conclusions can be made.

Figure 5.8: Bone dimensions in untreated and ERT treated MPS VI (primary dose group), and normal control cats.

Bone dimensions (% normal mean) from 6 month old MPS VI cats undergoing ERT in the primary dose group, compared with untreated MPS VI and normal control cats; (A) C3 vertebral length; (B) L5 vertebral length; (C) Patella length. Results for both males and females are combined. Dimensions for cats at the 5 mg/kg dose were taken at 5 months of age. The horizontal bar within each dose group (where $n \geq 2$) indicates the mean for each dose group, and individual points represent values obtained from individual cats. Numbers of animals in each group is indicated in the legend.

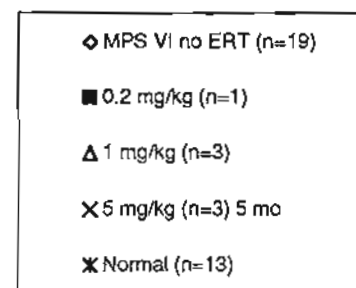
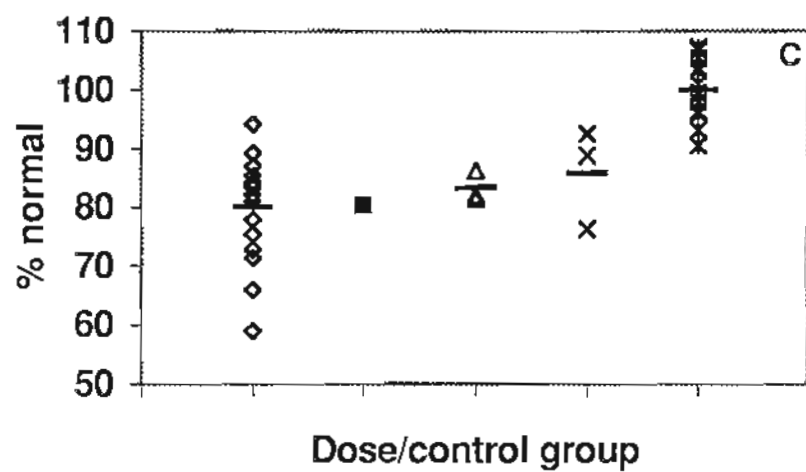
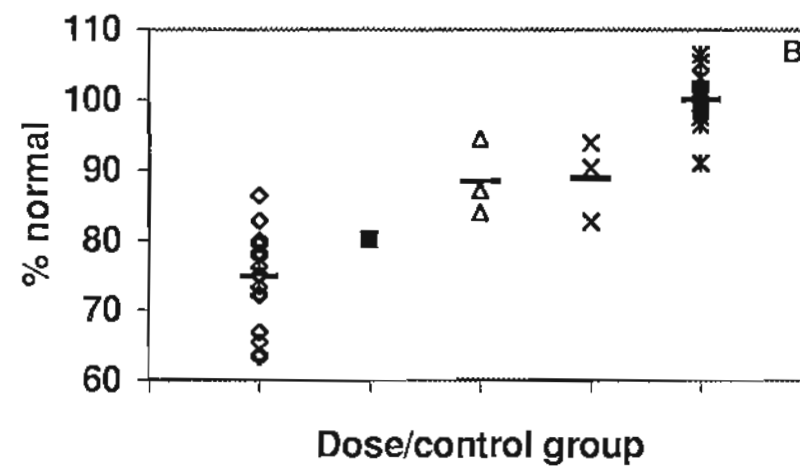
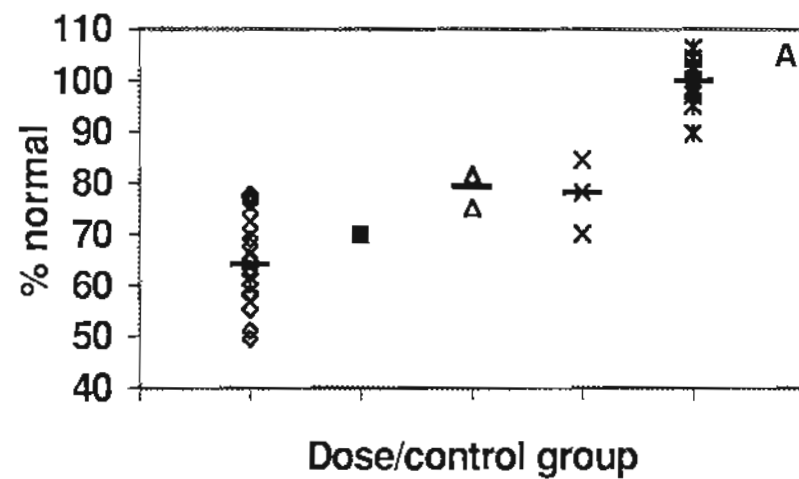
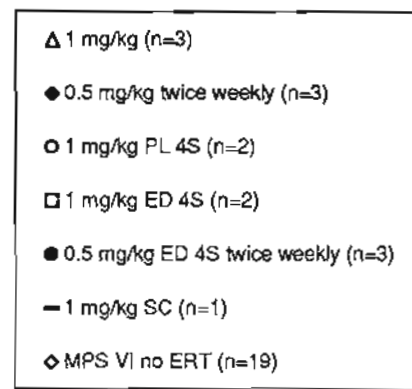
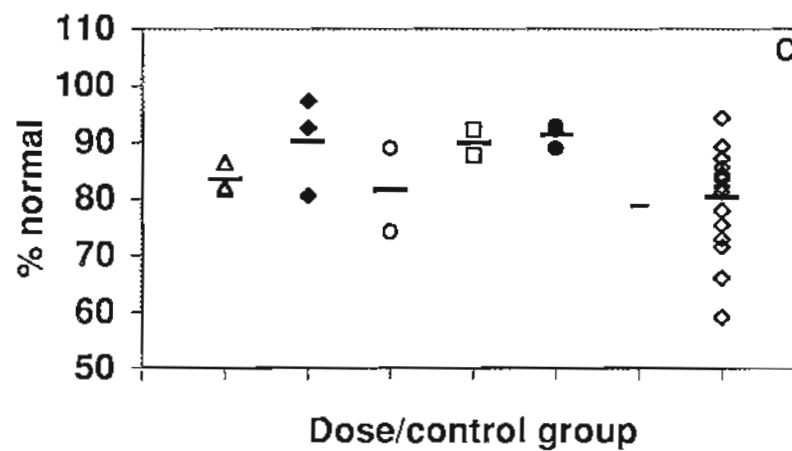
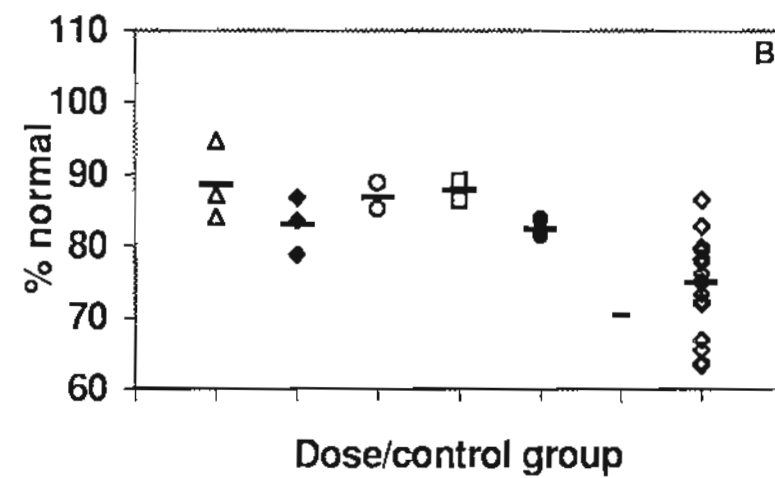
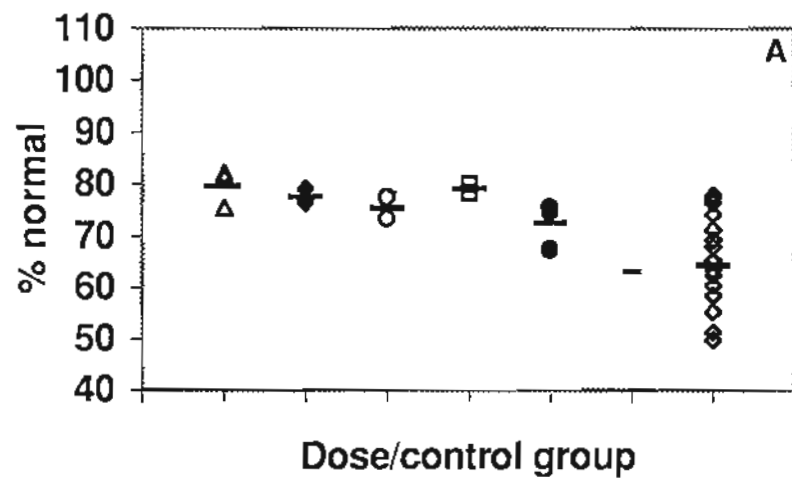


Figure 5.9: Bone dimensions in untreated and ERT treated MPS VI (enzyme/dose modification group), and normal control cats.

Bone dimensions (% normal mean) from 6 month old MPS VI cats undergoing ERT in the enzyme/dose modification group, compared with ERT treated MPS VI cats given 1 mg/kg unmodified rh4S weekly, and untreated MPS VI cats. (A) C3 vertebral length; (B) L5 vertebral length; (C) Patella length. Results for both males and females are combined. The horizontal bar within each dose group (where $n \geq 2$) indicates the mean for each dose group, and individual points represent values obtained from individual cats. Numbers of animals in each group is indicated in the legend.



5.3.5 Bone histomorphometry

The L5 vertebral sections clearly show improvements in overall vertebral dimensions (length and width), and in number and thickness of bone trabeculae present in the vertebral body (Figure 5.10). Proportional improvements were seen with increased enzyme dose.

As described in Section 3.3.5.3, no sex differences were observed in static bone histomorphometric parameters within normal control and untreated MPS VI groups at 6 months of age, therefore data from males and females was combined.

Histomorphometric quantitation of bone changes are presented in Figure 5.11 and Figure 5.12. In the primary dose group, a clear dose related improvement in bone mineral volume (BV/TV) at both 1 mg/kg and 5 mg/kg (Figure 5.11 A) was observed. This measurement showed a large difference between untreated MPS VI and normal controls and showed the clearest differences between different dose rates. The BV/TV in the 0.2 mg/kg cat was within the range observed for untreated MPS VI controls. The increased BV/TV was reflected in increased trabecular thickness and trabecular number, with decreased distance between trabeculae (trabecular separation)(Figure 5.11 B-D).

In the enzyme/dose modification group, no obvious differences in all four parameters were apparent due to the different dose regimes compared with the 1 mg/kg rh4S weekly dose from the primary dose group. The exception to this was all values obtained from the 1 mg/kg SC treated cat which were no different from the untreated MPS VI controls (Figure 5.12 A-D). The major limitation with the results were the very variable values obtained from different animals within the same group, notably for BV/TV within both the 1 mg/kg PL 4S and 1 mg/kg ED 4S groups (n=2 in both groups)(Figure 5.12 A). One animal in both of these groups had consistently higher values for BV/TV, Tb.Th. and Tb.N. compared with the other animal.

At 11 months of age in the ERT treated cats at 1 mg/kg and 5 mg/kg rh4S, a similar range of values were observed as those for the same dose rates at 6 months of age, although the values for the 1 mg/kg rh4S cat were closer to normal than for the 5 mg/kg cat (data not shown). As described at the end of Section 5.3.4, the two MPS VI cats given ERT to 11

months of age (111m and 112f) both showed the lowest improvement in bone dimensions compared with other animals in the same dose group at 5 or 6 months of age. Cat 111m (5 mg/kg) showed the greatest difference compared with other animals in the same dose group, and this may explain the lower bone histomorphometric parameters than those seen in the 11 month old 1 mg/kg cat (112f). Again this probably reflects the variability observed in disease severity between different MPS VI cats.

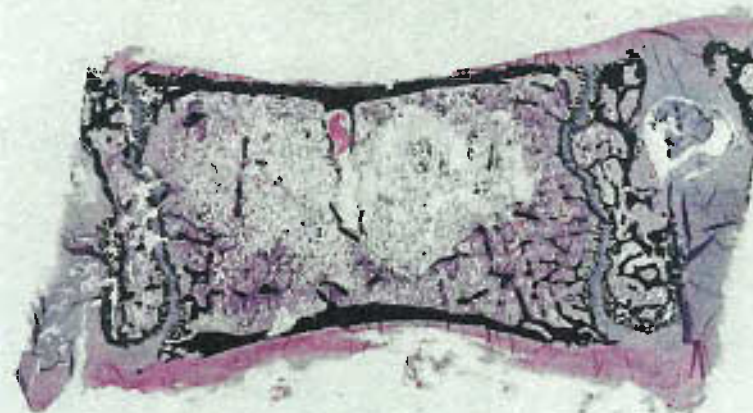
Figure 5.10: Fifth lumbar vertebral sections in 6 month old untreated and ERT treated MPS VI, and normal control cats.

Fifth lumbar vertebral sections from 6 month old cats: (A) an untreated MPS VI cat; (B) an ERT treated MPS VI cat at 1 mg/kg rh4S; (C) an ERT treated MPS VI cat at 5 mg/kg rh4S; (D) an untreated normal control cat. Sections stained with von Kossa/H&E. Magnification constant for all sections.

A



B



C



D



Figure 5.11: Bone histomorphometric analysis of the fifth lumbar vertebrae in 6 month old MPS VI cats undergoing ERT (primary dose group) and untreated MPS VI and normal control cats.

Bone histomorphometric analysis of the fifth lumbar vertebrae in 6 month old cats MPS VI cats undergoing ERT in the primary dose group compared with untreated MPS VI and normal control cats. (A) Bone mineral volume (BV/TV); (B) Trabecular thickness (Tb. Th.); (C) Trabecular number (Tb. N.); (D) Trabecular separation (Tb. Sp.). The horizontal bar within each dose group (where $n \geq 2$) indicates the mean for each dose group, and individual points represent values obtained from individual cats.

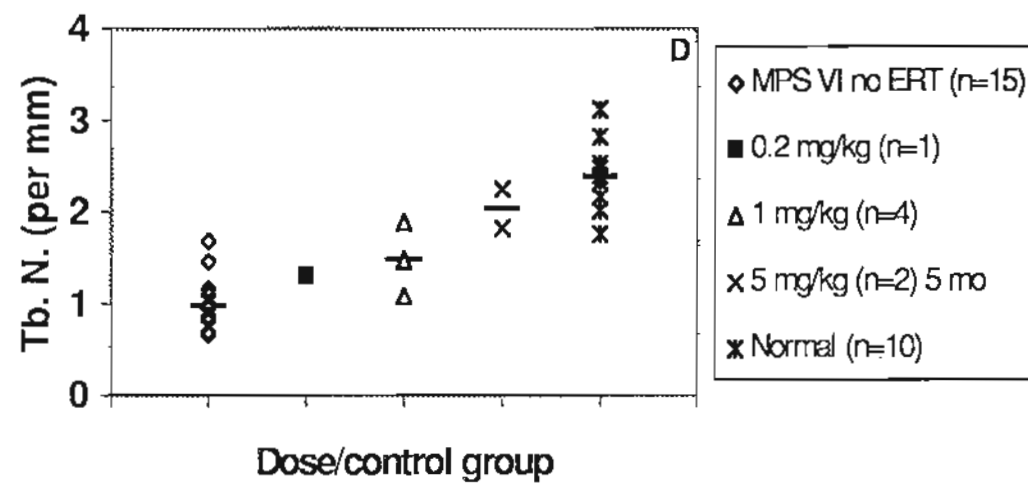
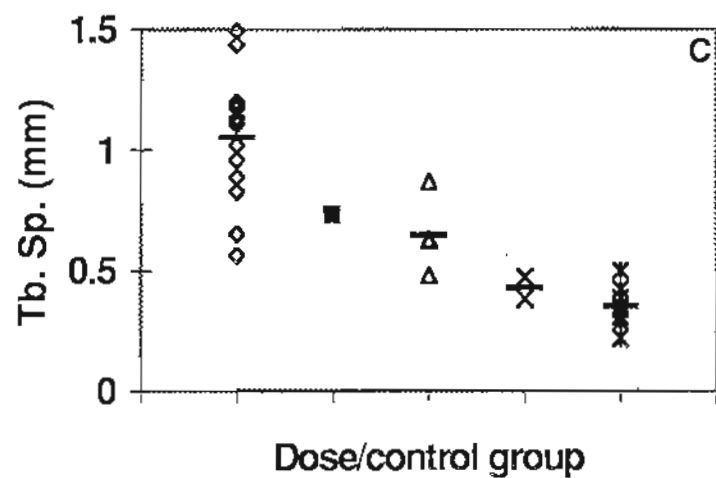
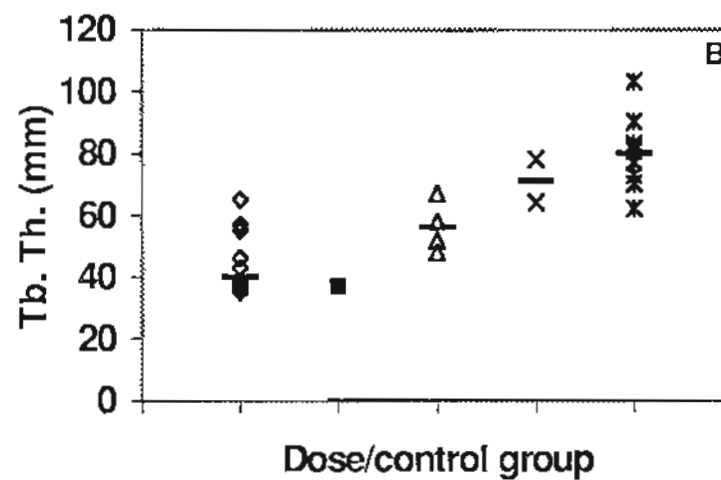
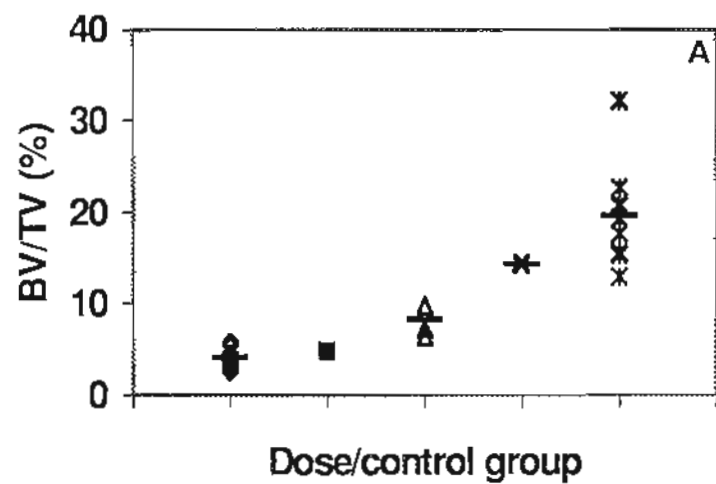
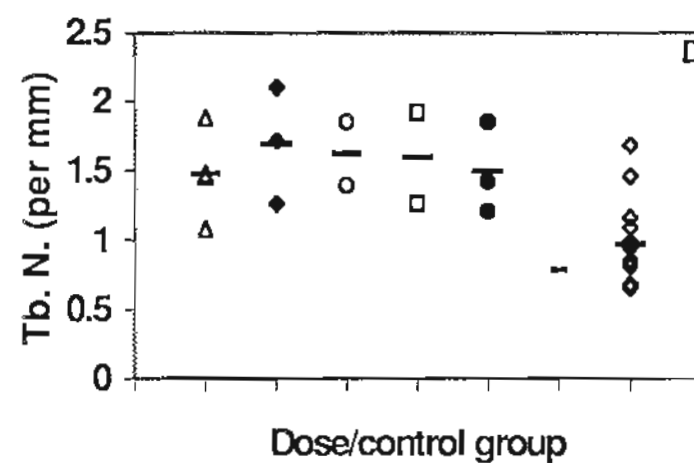
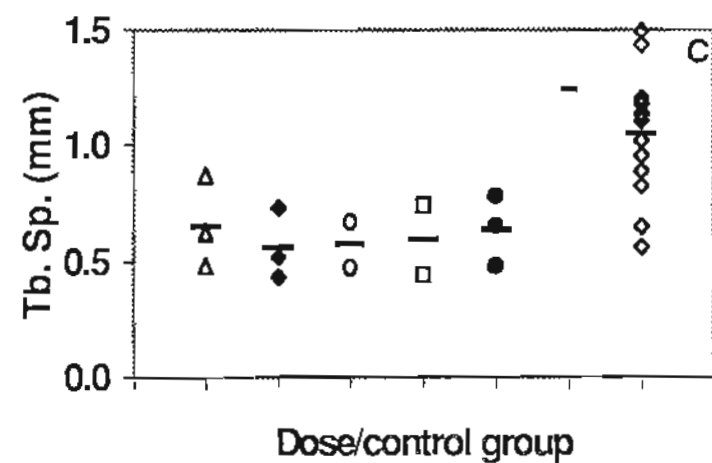
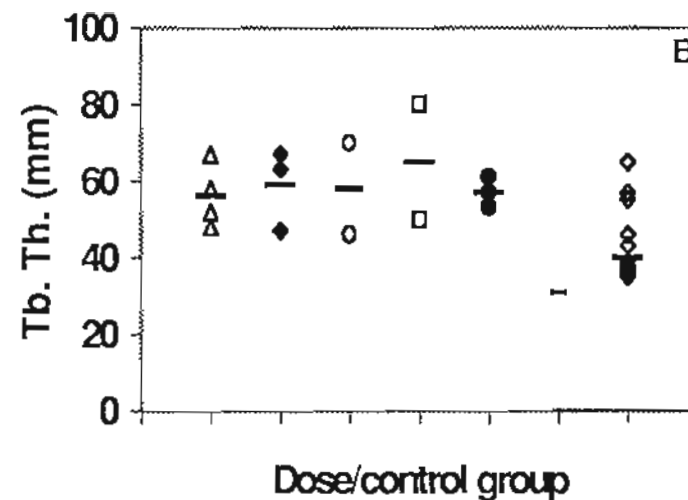
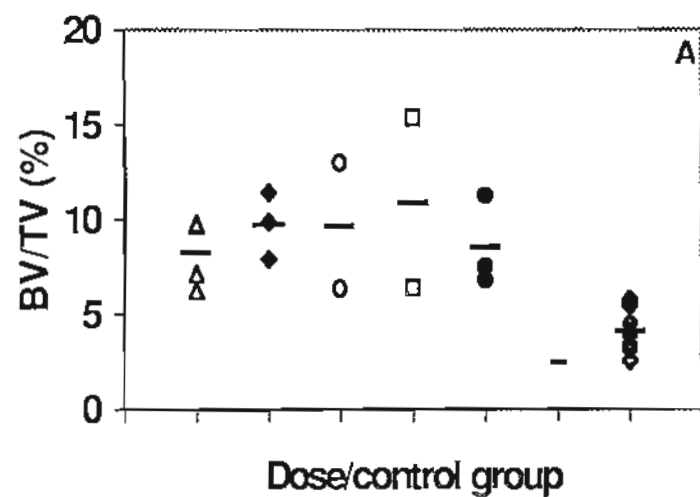


Figure 5.12: Bone histomorphometric analysis of the fifth lumbar vertebrae in 6 month old MPS VI cats undergoing ERT (enzyme/dose modification group) and untreated MPS VI and normal control cats.

Bone histomorphometric analysis of the fifth lumbar vertebrae in 6 month old MPS VI cats undergoing ERT in the enzyme/dose modification group compared with MPS VI cats undergoing ERT at 1 mg/kg unmodified rh4S and untreated MPS VI controls. (A) Bone mineral volume (BV/TV); (B) Trabecular thickness (Tb. Th.); (C) Trabecular number (Tb. N.); (D) Trabecular separation (Tb. Sp.). The horizontal bar within each dose group (where $n \geq 2$) indicates the mean for each dose group, and individual points represent values obtained from individual cats. Note y-axis scales are different from those in Figure 5.11.



- △ 1 mg/kg (n=4)
- ◆ 0.5 mg/kg twice weekly (n=3)
- 1 mg/kg PL 4S (n=2)
- 1 mg/kg ED 4S (n=2)
- 0.5 mg/kg ED 4S twice weekly (n=3)
- 1 mg/kg SC (n=1)
- ◇ MPS VI no ERT (n=15)

5.3.6 Urinary glycosaminoglycans (GAGs)

5.3.6.1 ABC method

GAG values in age matched urine samples from untreated MPS VI male and female cats, and normal control male and female cats were compared at different ages (Student t-Test) to determine if there were any sex differences in GAG concentration. No significant differences were observed, so values from males and females were combined as shown in Figure 5.13.

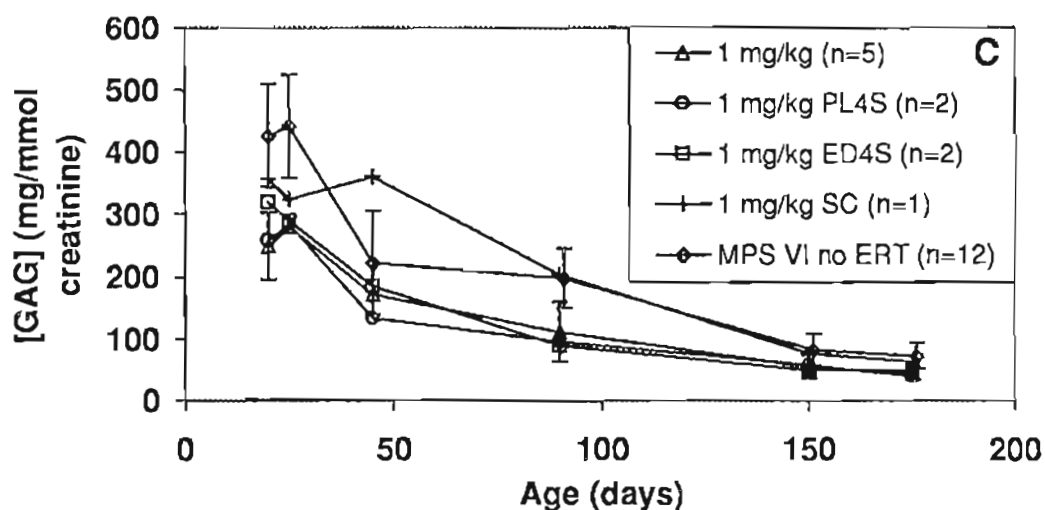
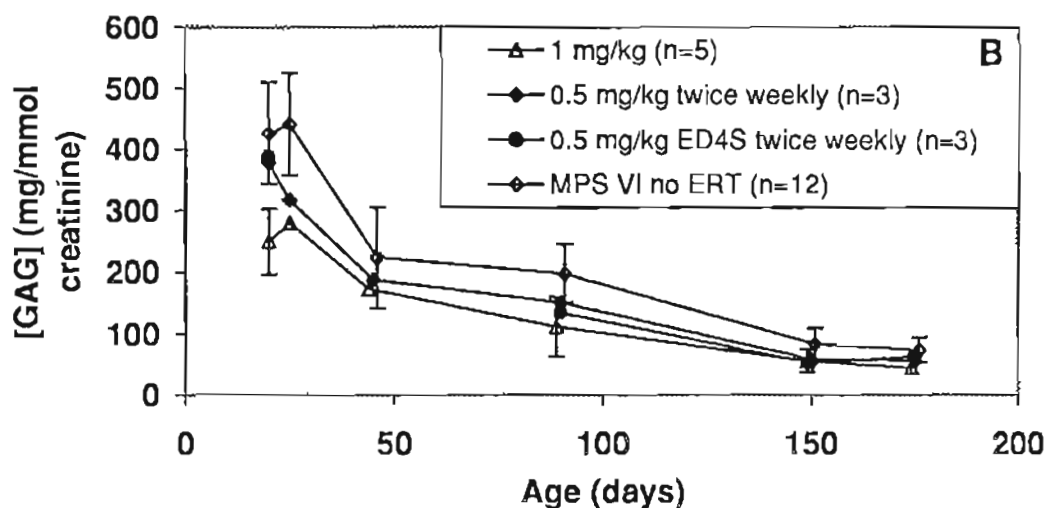
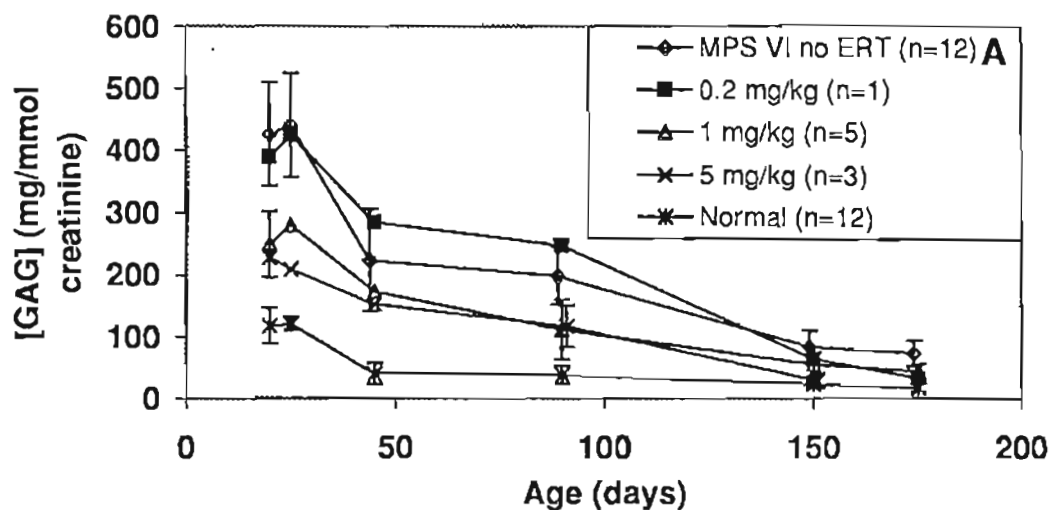
All cats including MPS VI and normal controls showed a rapid reduction in the amount of total urine GAG with increasing age to 6 months old (Figure 5.13). In the primary dose group (Figure 5.13 A), GAG levels in untreated MPS VI controls remained elevated above 1 and 5 mg/kg rh4S treated MPS VI cats and normal controls. GAG levels in the 0.2 mg/kg treated cat were within the range observed for untreated MPS VI controls. GAG levels at the 1 and 5 mg/kg rh4S dose rates appeared similar, although mean GAG values at the 5 mg/kg dose rate were slightly lower than those at 1 mg/kg at most ages.

In the enzyme/dose modification group, urine GAG levels in the 0.5 mg/kg rh4S twice weekly and the 0.5 mg/kg ED4S twice weekly groups were between those observed in the untreated MPS VI controls and the 1 mg/kg rh4S treated MPS VI cats (Figure 5.13 B). In the remaining dose groups (Figure 5.13 C), GAG levels in the 1 mg/kg PL4S and 1 mg/kg ED4S were very similar to those observed for 1 mg/kg rh4S, however the GAG levels from the 1 mg/kg SC treated cat were higher than, or within the range observed in untreated MPS VI controls.

From 7 to 11 months of age, urine GAG levels in ERT treated cats at 1 and 5 mg/kg were midway between an untreated MPS VI control and a normal control cat. At 11 months of age, GAG levels in the 5 mg/kg treated cat were the same as those in the normal controls, and in the 1 mg/kg treated cat, GAG levels were 1.5 times normal. Urine GAG in the untreated MPS VI control at 11 months of age was 4 times normal (data not shown).

Figure 5.13: Total urine GAG in MPS VI cats undergoing ERT, using the ABC method.

Total urine GAG in MPS VI cats undergoing ERT compared with untreated MPS VI and normal controls, from 0-6 months of age, using the ABC method. (A) ERT treated MPS VI cats in the primary dose group; (B) ERT treated MPS VI cats with twice weekly dose regimes from the enzyme/dose modification group compared with 1 mg/kg rh4S and untreated MPS VI controls; (C) ERT treated MPS VI cats with weekly dose regimes in the enzyme/dose modification group compared with 1 mg/kg rh4S and untreated MPS VI controls. Results for males and females combined. Error bars = 1SD. Numbers of animals at different ages within a dose/control group varied, and an approximate n value is given in the legend for each dose/control group. Numbers of animals at ~25 and ~45 days old were usually less than this value.



5.3.6.2 High resolution electrophoresis (HRE)

CPC purified urine GAG from urine samples at specific ages was separated by HRE, then scanned with a densitometer scanner to determine the proportion of each GAG species expressed as a percentage of the total GAG. In untreated MPS VI cats, DS2 was sometimes two to three times the level of DS1, and total DS (DS1 + DS2) as a percentage of the total GAG, increased with increasing age, with a concurrent decrease in the proportion of chondroitin sulphate (CS) in the urine with increasing age (Table 5.4).

Concomitant with a decrease in total GAG in cats at higher dose rates of ERT, was a decrease in the proportion of total DS in the urine in all cats at 5 mg/kg, and in most of the cats at 1 mg/kg and 1 mg/kg PL4S at most ages. At 0.2 mg/kg (data not shown) and 1 mg/kg ED4S, no difference in the proportion of DS was observed compared with untreated MPS VI controls (Table 5.4). In the initial samples from the 5 mg/kg treated cats (at ~20 and sometimes also 90 days of age), with moderate amounts of GAG being loaded on the plate, the DS1 band was not visible on the Alcian Blue stained cellulose acetate strips. DS2 was either not present in normal control cats or low amounts of DS2 was obscured by poor separation from the CS band/peak (Table 5.4).

Table 5.4: Average densitometric proportions of dermatan sulphate (%DS1+DS2) from high resolution electrophoresis of urine GAG in untreated and ERT treated MPS VI cats and normal controls.

Age (days)	MPS VI no ERT [§] Mean %DS ± SD	MPS VI no ERT [§] Range	5 mg/kg rh4S	1 mg/kg rh4S	1 mg/kg PL4S*	1 mg/kg ED4S*	Normal controls [§]
23	40.8 ± 4.8	33.0 - 47.6	19.4 [†]	37.8 ± 14.1 [‡]	44.5	42.1	1.6 ± 1.0
46	52.4 ± 5.0	44.8 - 53.4	-	-	30.3	46.1	3.9
91	61.9 ± 8.2	49.5 - 73.2	25.8 ± 7.1 [‡]	37.8 ± 5.3 [‡]	41.8	46.8	4.4 ± 3.5
151	58.9 ± 3.7	54.1 - 65.0	37.2 ± 12.9 [‡]	41.9 [†]	46.8	61.5	3.6 ± 1.2
175	62.8 ± 3.8	58.5 - 68.5	-	59.3 [†]	62.2	66.3	4.4 ± 1.5

* n=1; † n=2; ‡ n=3; § n=6 to 8;

5.3.6.3 DEAE method

The DEAE method was developed to try and improve sensitivity of total urine GAG quantitation compared with the results obtained from the ABC method. One major disadvantage of the DEAE method was that it was very time consuming compared with the ABC method. Consequently, only total urine GAG from animals in the primary dose group was quantitated using this second method.

The correlation between the two methods was very high, with a correlation coefficient (r) of 0.975 ($n=71$ samples analysed by both methods). When the values for the same sample obtained by both methods were expressed as a ratio, with increasing age in untreated MPS VI cats the ratio was approximately constant (Figure 5.14 A), however in normal cats this ratio decreased with increasing age (Figure 5.14 B). However, only a few samples were analysed between 7-11 months, which may have skewed the line of best fit in normal cats. The reasons for these differences between untreated MPS VI and normal cats is unknown. One hypothesis is that it may reflect preferential dye binding to the changing proportion of different GAG species with increasing age in normal cats which is masked by high DS levels at all ages in untreated MPS VI cats.

Using both GAG quantitation methods, urine collected at the beginning and then again at the end of anaesthesia had an apparent increase in the amount of urine GAG in MPS VI cats (with or without ERT) concomitant with a decrease in urine creatinine, even after normalising to creatinine concentration. On some occasions this led to an apparent increase in urine GAG in the final urine sample prior to euthanasia, due to longer duration of anaesthesia. The reason for this increase is unknown, and may be related to the anaesthetic used, however response to other anaesthetic agents was not examined.

The pattern of total urine GAG excretion in the primary dose group using the DEAE method was similar compared with the ABC method. A greater spread of values was observed between each dose/control group with the DEAE method, making comparison at different dose rates easier (Figure 5.15 A and B). GAG levels in untreated MPS VI controls remained elevated above ERT MPS VI cats and normal controls at approximately ten times normal. In all MPS VI cats undergoing ERT there appeared to be a dose responsive

reduction in the amount of urine GAG, with greatest reduction seen at 5 mg/kg to a level maintained at twice normal from 5 to 11 months of age. At 1 mg/kg rh4S, this level was maintained at approximately three times normal. There also appeared to be a marginal reduction in the amount of urine GAG excreted in the 0.2 mg/kg cat when compared with untreated MPS VI controls. Using the DEAE method, GAG levels in the 0.2 mg/kg treated cat were lower than the untreated MPS VI controls, which was different from that observed using the ABC method (Figure 5.13). This conflicting result is possibly due to the lower numbers of untreated MPS VI control urines analysed by the DEAE method (n=3) compared with the number analysed by the ABC method (n=12).

CPC is used to precipitate urine GAG in preparation for HRE, however CPC only precipitates GAG larger than approximately 3000 Da (Hopwood and Harrison, 1982). The DEAE method of urine GAG purification theoretically separates all charged GAG residues including those of molecular weights less than 3000 Da. Visualisation of GAG oligosaccharide lengths present is possible by gradient gel electrophoresis. Urine GAG from an untreated MPS VI cat and an MPS VI human, prepared by both methods, clearly illustrates the presence of lower molecular weight saccharides (approximately penta- and heptasaccharides) using the DEAE method, compared with urine purified using CPC precipitation which did not purify GAG below approximately a nonasaccharide (Figure 5.16). Human and feline MPS VI urine GAG patterns appeared to be almost identical.

To provide additional information regarding low molecular weight GAG less than 3000 Da, the average amount of GAG precipitated by CPC was compared with the yield from the DEAE method from the same urine sample. CPC precipitated an average of only: 30% of urine GAG in untreated MPS VI cats (range 28.6-31.7%), 28% at 1 mg/kg rh4S ERT (range 19.7-31.4%), 31% at 5 mg/kg ERT (range 18.5-37.6%) and 48% of urine GAG in normal control cats (range 44.1-55.9%), compared with the amount precipitated by the DEAE method. The amount precipitated by CPC was relatively constant at all ages examined (3-11 months). This indicates that approximately 70% of urinary GAG in an untreated MPS VI cat consists of low molecular weight GAG, and that there is no obvious shift in the proportion of low molecular weight GAG in the urine of ERT treated cats. HRE of DEAE prepared urine GAG may have provided further information, however time constraints limited any further analysis.

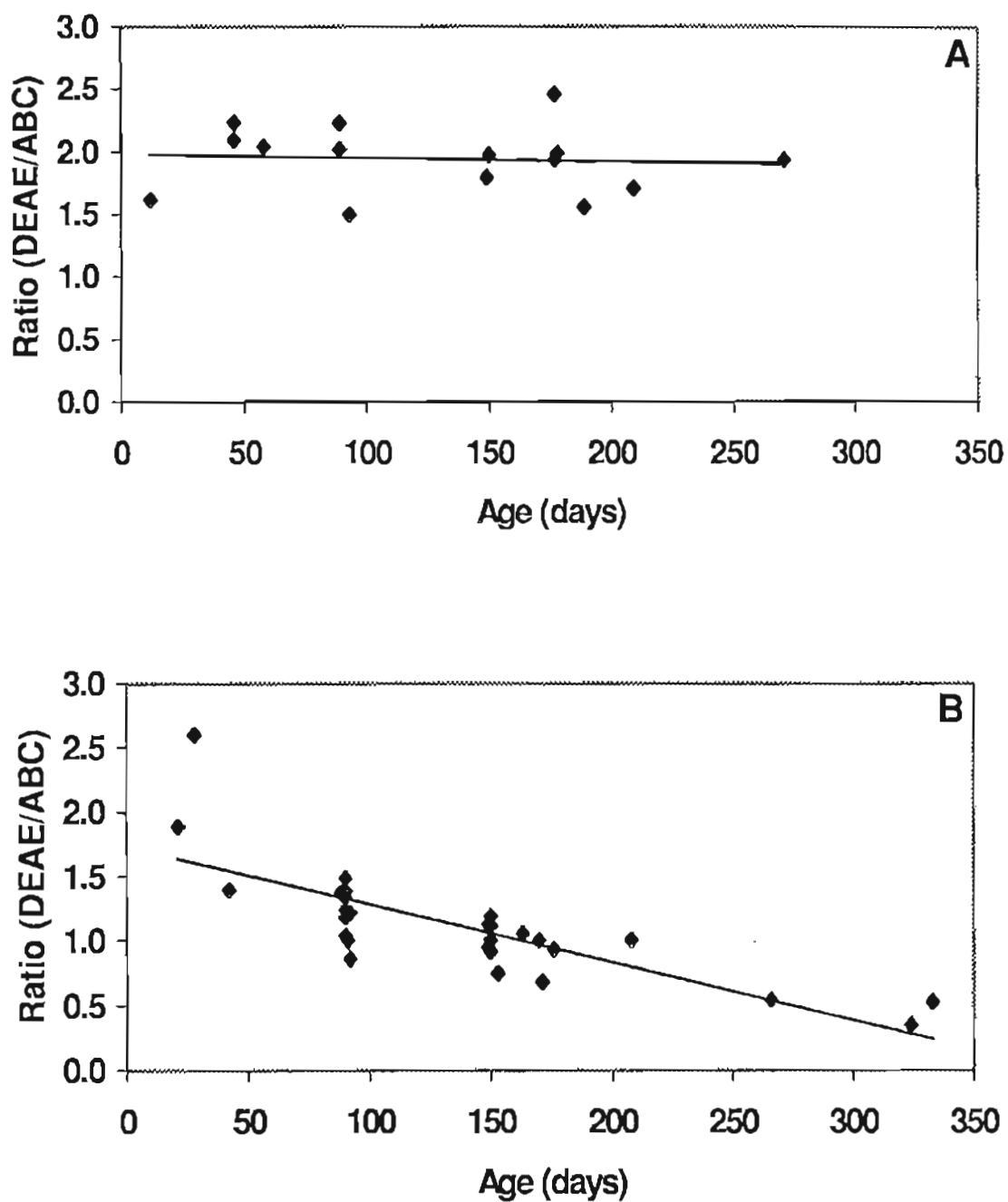


Figure 5.14: Ratio of total urine GAG using two quantitative methods (DEAE/ABC).

Ratio of total urine GAG (DEAE/ABC) using the DEAE and ABC methods in (A) untreated MPS VI cats and (B) normal control cats.

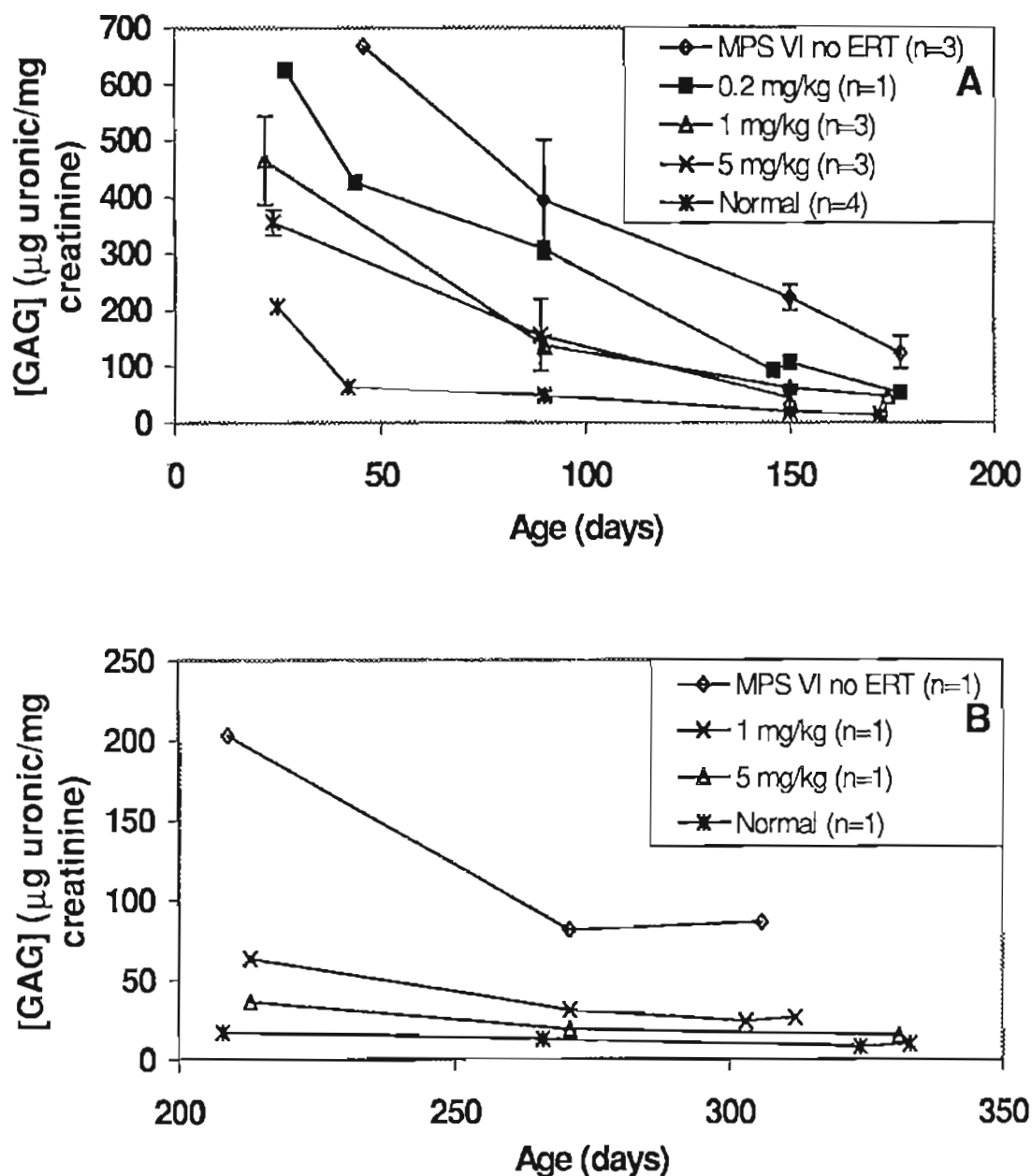
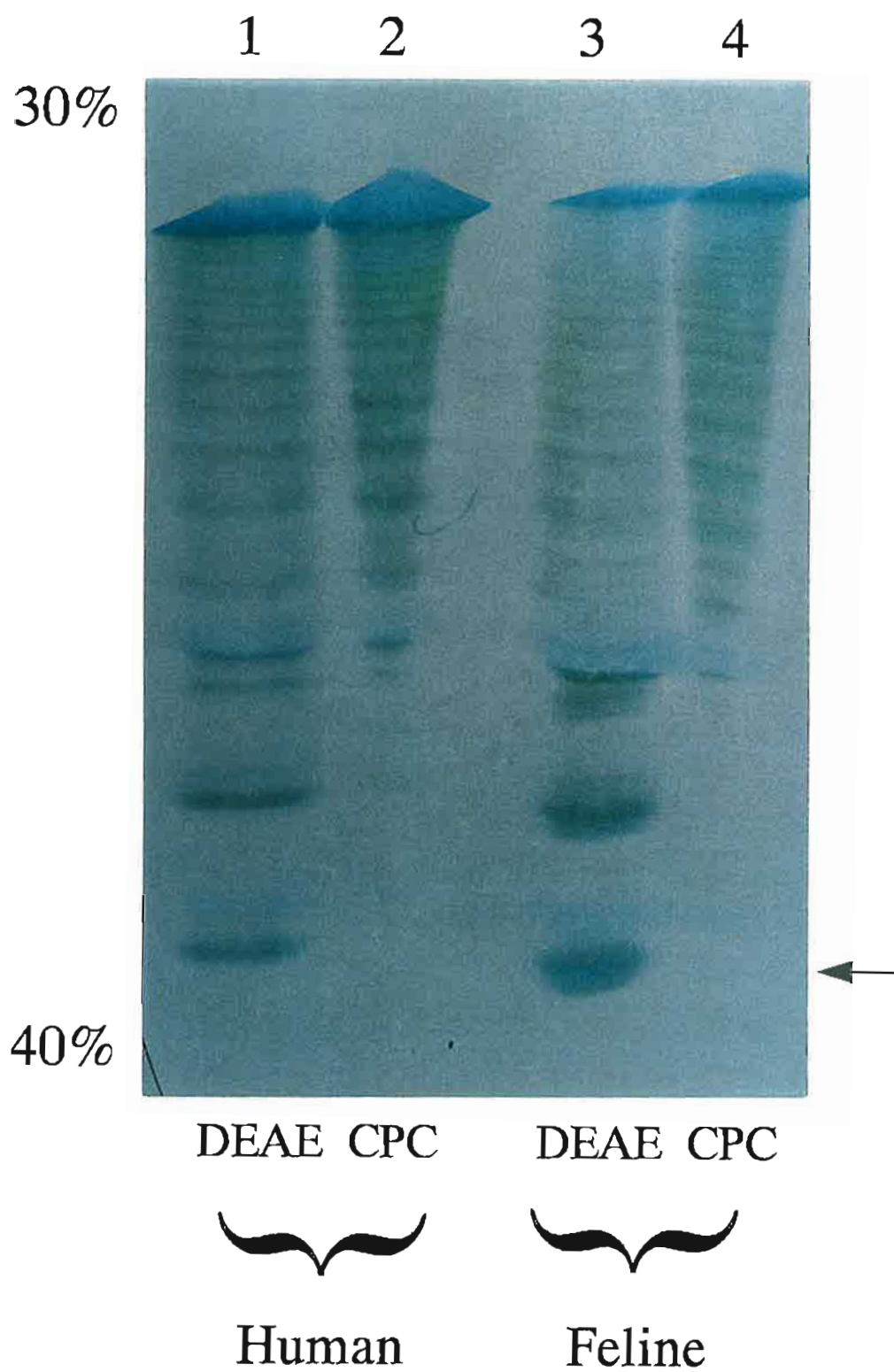


Figure 5.15: Total urine GAG in MPS VI cats undergoing ERT using the DEAE method.

Urine GAG excretion in untreated MPS VI cats, MPS VI 0.2 mg/kg ERT, MPS VI 1 mg/kg rh4S ERT, MPS VI 5 mg/kg ERT, and normal control cats: (A) from birth to 6 months. Error bars = 1SD. Points with no error bars indicates number of animals (n) < 3; (B) from 7 to 11 months, 1 and 5 mg/kg only (n=1 for all points).

Figure 5.16: Gradient gel electrophoresis of urine GAG from a human MPS VI patient and an untreated MPS VI cat prepared using two methods.

Urine GAG (~3.85 µg uronic acid/lane) was purified from an MPS VI human (lane 1 and 2) and an untreated MPS VI cat (lanes 3 and 4) urine using the DEAE method (lane 1 and 3) and CPC precipitation (lane 2 and 4) and run on a 30-40% gradient gel (gel concentration indicated on figure). Height of resolving gel was 10cm. The gel was stained with Alcian Blue. Mobility of oligosaccharides is dependent on both size and charge. The arrow approximately represents the mobility of a pentasaccharide by comparison with heparan sulphate standards (not shown)(Turnbull *et al.*, 1997). Separation between major bands are in disaccharide units.



5.3.7 Renal function

Urine protein/creatinine ratios (Section 2.2.15) were calculated from the final urine sample from all ERT treated MPS VI cats to evaluate renal function. Values less than 0.5 are considered normal. All cats had values less than 0.2, except one 11 month old 5 mg/kg ERT treated MPS VI cat (111m = 0.38) and one 11 month old normal control cat (121m = 0.5) which were still considered normal.

Plasma was available for determination of urea and creatinine levels in approximately half of the animals within each dose group at 6 months of age (n=11 ERT treated cats tested). Values from all of these cats were well within the reference range for both parameters, except for the creatinine level from the only 5 mg/kg cat tested (208m) was 12.6 mmol/l (reference range: 5.0 - 12.5) which was not considered significant. These results also indicated normal renal function.

5.3.8 Macroscopic pathology

At 6 months of age, there was an overall impression of decreased articular cartilage thickness in most of the ERT treated cats compared with the untreated MPS VI controls (Figure 5.17 A-I). This was observed most clearly in the shoulder, hip and knee joints, although cartilage thickness was not as uniform as seen in normal controls. There was noticeable variation between individuals in both the untreated MPS VI and ERT treated MPS VI groups, with one 5 mg/kg cat (208m) appearing to have thickened cartilage in all joints to a similar degree to the untreated MPS VI cats. Joints in the 0.2 mg/kg treated cat and the 1 mg/kg SC treated cat also appeared similar to untreated MPS VI controls. It was otherwise difficult to distinguish between different dose rates based on the general appearance of joints. In particular, there were no obvious improvements in joint appearance in cats treated with PL4S or ED4S at any dose rate (Figure 5.17 I)

The subchondral bone visible from the articular surface through thinner regions of cartilage, seen particularly in the shoulders, appeared to be more uniform in ERT treated cats, however the articular surface was sometimes slightly bumpy, as also seen in untreated MPS VI controls. The articular cartilage was thicker over the ball of the proximal humerus and

the ventral and caudal aspects of the distal femoral condyles, and underlying subchondral bone in these regions, seen only when the articular cartilage was removed, was irregular in all ERT treated cats. There was great variability in treated and untreated MPS VI cats in the presence and severity of erosive lesions on the cranial aspect of the shoulder joint where the biceps tendon passes over the joint into the intertubercular groove. There was no correlation with presence or absence, or severity of these lesions in animals with normal hindlimb use, or mild to moderate neurological deficits, or complete hindlimb paralysis.

However by 11 months, three out of four untreated MPS VI cats and both the 1 mg/kg (112f) and 5 mg/kg (111m) treated cats had erosive lesions in the shoulders, the most severe being found in the 5 mg/kg cat (Figure 5.17 F and H). This cat also had more severe fibrillation and flap formation in the elbows compared with the 11 month old untreated MPS VI cats (the 1 mg/kg 11 month cat was not examined).

Figure 5.17: Joint appearance at postmortem in ERT treated cats compared with untreated MPS VI and normal control cats at 6 and 11 months of age.

Shoulder joints (glenoid surface of the scapula on the left, proximal humerus on the right; cranial aspect at the top) from normal control cats (A, B); untreated MPS VI cats (C, D); MPS VI cats at 1 mg/kg rh4S ERT (E, F); MPS VI cats at 5 mg/kg rh4S ERT (G, H); and an MPS VI cat at 1 mg/kg PL4S ERT (176f)(I). Views A, C, E, G and I are from 5 to 6 month old cats, and views B, D, F and H are from 11 month old cats.

(A, B) In normal control cats the articular cartilage was thin and smooth, becoming thinner with increasing age. The cartilage was very thin at all ages on the cranial aspect of the proximal humerus joint surface (orientated at the top of the slide), where the biceps tendon passes over the articular surface into the intertubercular groove. The subchondral bone was uniform and pink and clearly visible through the cartilage surface. (C) In 6 month old untreated MPS VI cats, increased cartilage thickness was obvious, particularly extending caudally over the ball of the proximal humerus. Focal erosions in the cartilage surface were evident in the cranial aspect of the joint in over half of the 6 month old cats. Subchondral bone just caudal this region was irregular and spotty. (D) By 11 months of age, increased cartilage thickness was still obvious and erosions in the cranial aspect of the proximal humeral joint surface were generally increased in size. (E) At 1 mg/kg rh4S, erosions were still present in the cranial aspect of the humeral head, although cartilage directly caudal this region appeared thinner in most cats, and subchondral bone was more uniform. Cartilage was still very thickened over the caudal aspect of the joint. (F) In the one 11 month old ERT treated MPS VI cat at 1 mg/kg rh4S, cartilage generally appeared thinner than in untreated MPS VI controls, although a large defect in the articular surface was present. (G) At 5 mg/kg rh4S at 5 months of age, no erosions were present in either cat and cartilage was generally thinner in one cat, although was very thickened over the whole joint in the other cat. (H) By 11 months of age, the 5 mg/kg ERT treated cat had severe erosions in the articular surface. (I) Joint appearance in the 1 mg/kg PL4S cat was similar to those at 1 and 5 mg/kg rh4S at the same age.



5.3.9 Light and electron microscopy

In the primary dose group, dramatic reduction of storage to almost negligible levels was observed in the mitral heart valve and aorta at 5 mg/kg at 5 and 11 months (Table 5.5 and Figure 5.18). Similarly, at 1 mg/kg rh4S the degree of vacuolation due to storage was greatly reduced in these tissues, generally to a lesser degree, however there was also negligible storage in the heart valve at 11 months in the 1 mg/kg rh4S cat. There appeared to be variability in the extent of reduction in storage in heart valve among the different animals in the 1 mg/kg rh4S dose group, and also between different blocks from the same sample in all the dose groups. Normalisation of morphology in the aortic tunica media at 1 mg/kg rh4S was not uniform, and appeared to be corrected from the innermost layers, with gradual increase in the degree of vacuolation of cells towards the tunica adventitia (Figure 5.19). A thin band of vacuolated cells were present in the outer tunica media at 5 mg/kg (not shown).

Storage vacuoles were also reduced in a dose responsive manner in the 1 mg/kg and 5 mg/kg rh4S cats in fibroblasts in most connective tissues including skin, dura, hip joint capsule (Table 5.5) and kidney interstitium (data not shown), and also in perivascular cells in the cerebrum (Table 5.5). In the 0.2 mg/kg cat, there was no change in the degree of storage in these tissues and in heart valve and aorta, compared with untreated controls, except for marginal reduction in the degree of storage in the perivascular cells in the CNS and in skin fibroblasts, and dura was not examined.

Overall the reduction in lysosomal vacuolation at all doses except for 1 mg/kg SC in the enzyme/dose modification group followed the same trends in each tissue compared with 1 mg/kg unmodified rh4S. However some differences were apparent in certain tissues between rh4S, PL4S and ED4S. PL4S at 1 mg/kg appeared to lead to greater reductions in lysosomal storage in heart valve and aorta compared with 1 mg/kg rh4S. Conversely, increased lysosomal storage was present in brain, skin, dura, heart valve and aorta in one or both cats at 1 mg/kg ED4S compared with 1 mg/kg rh4S (Table 5.6).

Dividing the dose to 0.5 mg/kg twice weekly possibly lead to slight increases in the degree of lysosomal storage in aorta and heart valve compared with 1 mg/kg rh4S. Similarly there

was a suggestion of reduced ability to clear lysosomal storage in the same tissues with 0.5 mg/kg ED4S twice weekly, compared with 1 mg/kg ED4S weekly, although these differences were quite subjective (Table 5.6).

No change was seen in degree of lysosomal vacuolation in corneal keratocytes and chondrocytes from articular cartilage for all doses, including PL4S and ED4S (Figure 5.20). However storage was cleared from Kupffer cells in the liver at all dose rates including 0.2 mg/kg IV and 1 mg/kg SC (Table 5.5 and Table 5.6). The clearance of lysosomal storage in Kupffer cells at 1 mg/kg SC confirms the observation of increased rh4S activity in liver following SC administered enzyme distribution studies (see Table 4.4). The degree of lysosomal storage in the remaining tissues in this cat were no different from an untreated MPS VI cat, except for some reduction in degree of lysosomal vacuolation in skin samples. The skin sample was taken approximately 10 cm from the injection site and may suggest some correction of lysosomal storage due to diffusion of enzyme through subcutaneous tissues.

Characteristic white cell morphology in untreated MPS VI cats (heavy neutrophil granulation, non-staining eosinophils, and some vacuolated lymphocytes) was still present in MPS VI cats at all doses of ERT.

Fluorescent antibody staining to detect glomerular immune complexes was also performed on frozen kidney sections from all 5 mg/kg cats, one 1 mg/kg rh4S cat after 11 months ERT, and all cats at 1 mg/kg PL4S and ED4S. There was no evidence of positive staining of the glomerular basement membranes except for very slight staining in 1 cat (213m, 1 mg/kg PL4S). No evidence of immune complex deposition was present in these and additional cats (see Section 5.2.4) examined by electron microscopy except for evidence of very slight immunoglobulin deposits detected in less than 5% of the glomerular basement membrane in cat 213m (1 mg/kg PL4S). This cat also had grossly visible renal pathology at postmortem (see Section 5.3.3).

In paraffin embedded kidney sections from normal, ERT treated and untreated MPS VI cats, in a small proportion of glomeruli there was evidence of marked thickening of the basement membrane of Bowman's capsule, sometimes with proliferation of capsular

epithelium. There was also occasional atrophy of glomerular tufts and occasional lymphocytic infiltrates. The cause of these changes were unknown and not considered to be associated with ERT due to the presence of pathology in untreated MPS VI and normal controls.

A focal pale area with well defined margins was found in one liver lobe incidentally at postmortem in 1 cat (232f, 0.5 mg/kg ED4S, twice weekly). Histologically this appeared to be an acute infarct, although no thrombus was detected. This cat had no obvious illness at the time of euthanasia.

Table 5.5: Lysosomal storage in tissues from ERT treated MPS VI cats in the primary dose group after 5-11 months of ERT, compared with untreated MPS VI controls.

Tissue/cell type	MPS VI no ERT ≥ 6 mo	0.2 mg/kg 6 mo (n = 1)	1 mg/kg rh4S 6 mo (n = 4)	1 mg/kg rh4S 11 mo (n = 1)	5 mg/kg 5 mo (n = 2)	5 mg/kg 11 mo (n = 1)
Liver - Kupffer cells	3+	0	0	0	0	0
Brain - perivascular cells	3+	2+	0-1+	0	0	0
Skin - fibroblasts	3+	2+	1+-2+ [‡]	2+	0	0
Dura mater - fibroblasts	3+	NA	±-2+ [†]	NA	0*	NA
Heart-valve fibroblasts	3+	3+	1+-2+	0	0±	±-1+
Aorta - smooth muscle	3+	3+	2+ [†]	1+-2+	±-1+	1+
Hip joint capsule - fibroblasts	3+	NA	2+-3+ [‡]	2+	±-1+	±-1+
Cornea - keratocytes	3+	3+	3+	3+	3+	3+
Cartilage - chondrocytes	3+	3+	3+	3+	3+	3+

* n=1; † n=2; ‡ n=3;

NA, not available; 0, no lysosomal storage; ±, very mild vacuolation; 1+, mild; 2+, moderate; 3+, severe lysosomal vacuolation.

Note: no storage (0) was present in normal cats in all tissues.

Table 5.6: Lysosomal storage in tissues from ERT treated MPS VI cats in the enzyme/dose modification group after 6 months ERT, compared with ERT treated MPS VI cats at 1 mg/kg unmodified rh4S.

Tissue/cell type	1 mg/kg rh4S [§] (n = 4)	1 mg/kg PL4S [§] (n = 2)	1 mg/kg ED4S [§] (n = 2)	0.5 mg/kg rh4S twice weekly (n = 3)	0.5 mg/kg ED4S twice weekly (n = 3)	1 mg/kg rh4S [§] SC (n = 1)
Liver - Kupffer cells	0	0	0	0	0	0
Brain - perivascular cells	0-1+	1+*	1+-2+*	0-2+	±-1+	3+
Skin - fibroblasts	1+-2+ [‡]	±-2+	1+-3+	1+-2+	2+-3+	2+
Dura mater - fibroblasts	±-2+ [†]	NA	3+*	±-1+ [†]	2+-3+	NA
Heart-valve fibroblasts	1+-2+	±-1+	1+-3+	0-3+	2+-3+	3+
Aorta - smooth muscle	2+ [†]	0-1+	2+-3+	2+-3+	3+	3+
Hip joint capsule - fibroblasts	2+-3+ [‡]	2+	3+	2+-3+	3+	NA
Cornea - keratocytes	3+	3+*	3+	3+ [†]	3+	3+
Cartilage - chondrocytes	3+	3+	3+	3+ [†]	3+	3+

* n=1; † n=2; ‡ n=3; § dose administered weekly;

NA, not available; 0, no lysosomal storage; ±, very mild vacuolation; 1+, mild; 2+, moderate; 3+, severe lysosomal vacuolation.

Figure 5.18: Electron microscopy of heart valve following ERT.

Connective tissue cells in mitral valve: (A) in an untreated MPS VI cat, cells are enlarged and distended with storage vacuoles, (B) no storage vacuoles are observed in an MPS VI cat at 5 mg/kg ERT, (C) normal control cat. Bar, 10 μ m.

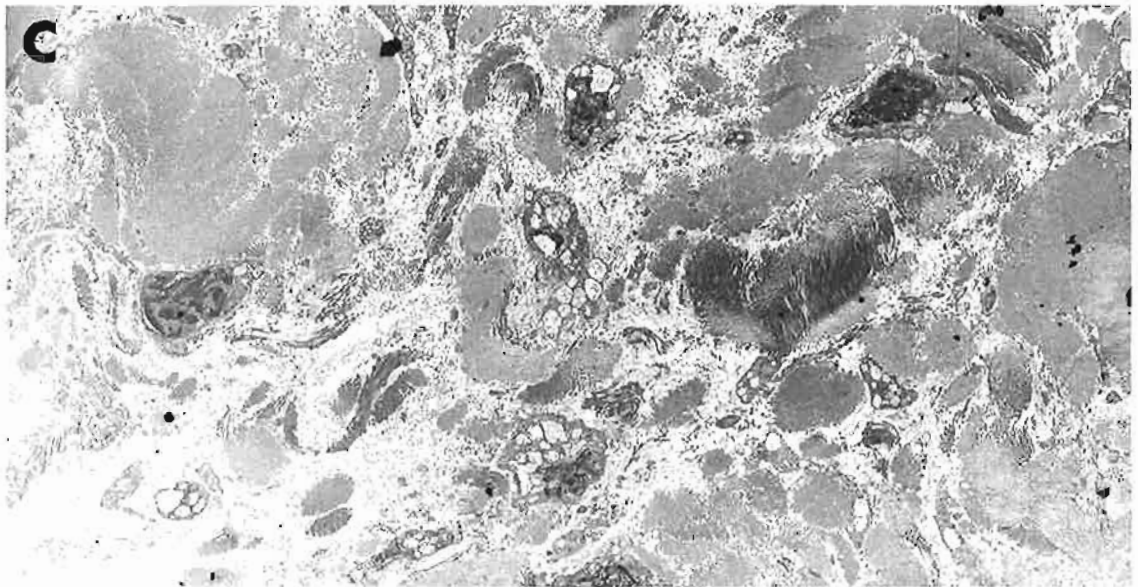
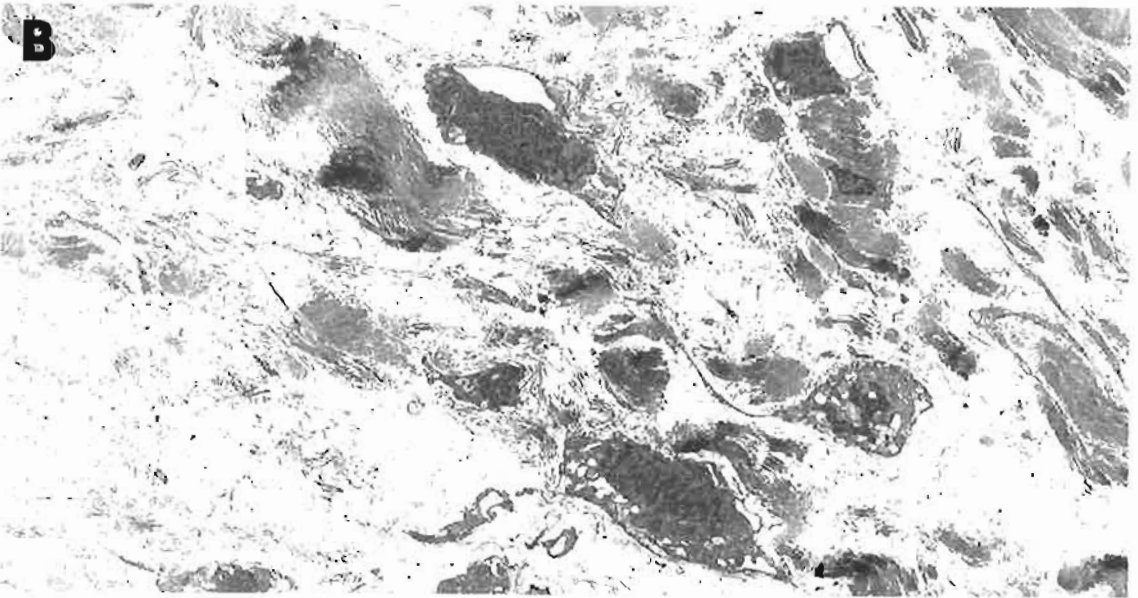
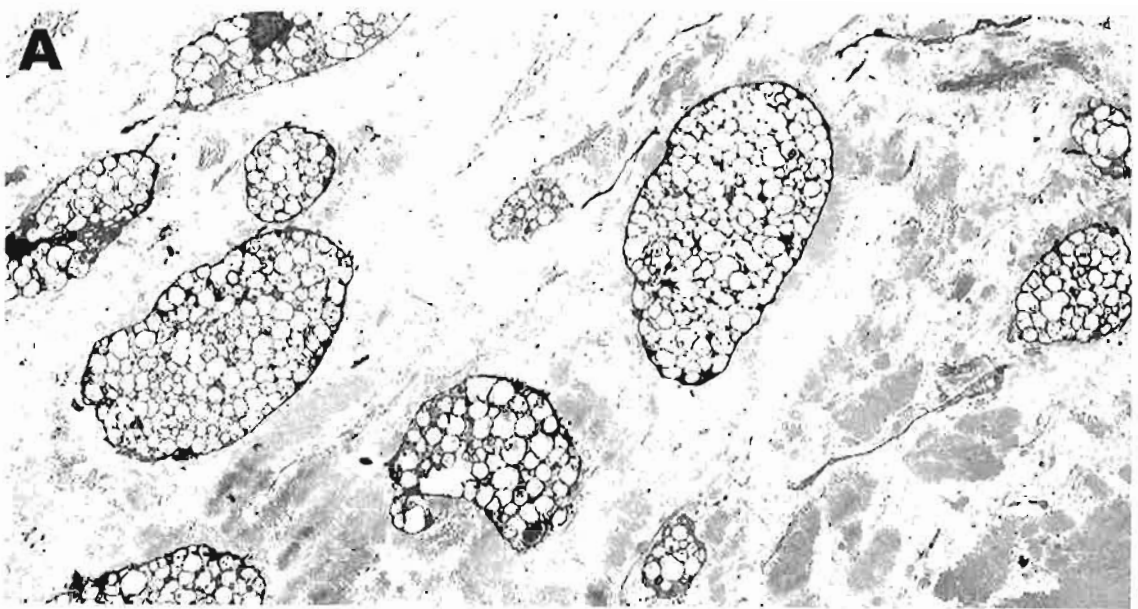


Figure 5.19: Electron microscopy of aorta following ERT.

Transverse section of aorta, with photographs from inner luminal (1), middle (2) and outer (3) regions of the Tunica media, in: (A1-A3) an untreated MPS VI cat, (B1-B3) an ERT treated MPS VI cat at 1 mg/kg rh4S cat showing absence of storage in inner third (B1), mild storage in majority of cells extending slightly more than the central third (B2), and almost maximal storage of cells in outermost quarter (B3), (C1-C3) a normal control cat. Bar, 12.5 μm .

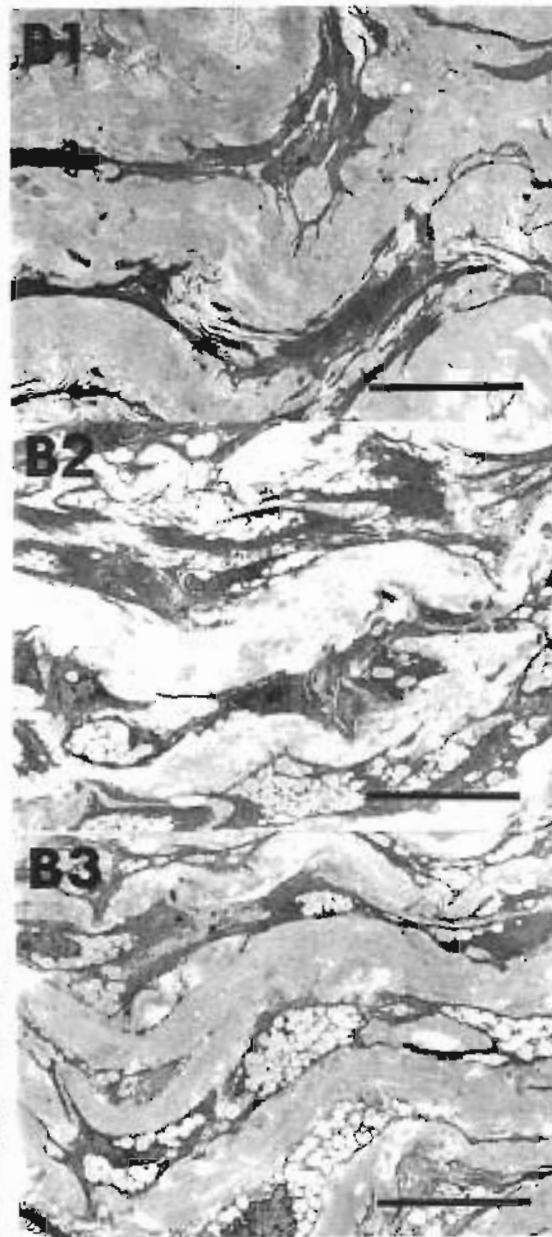
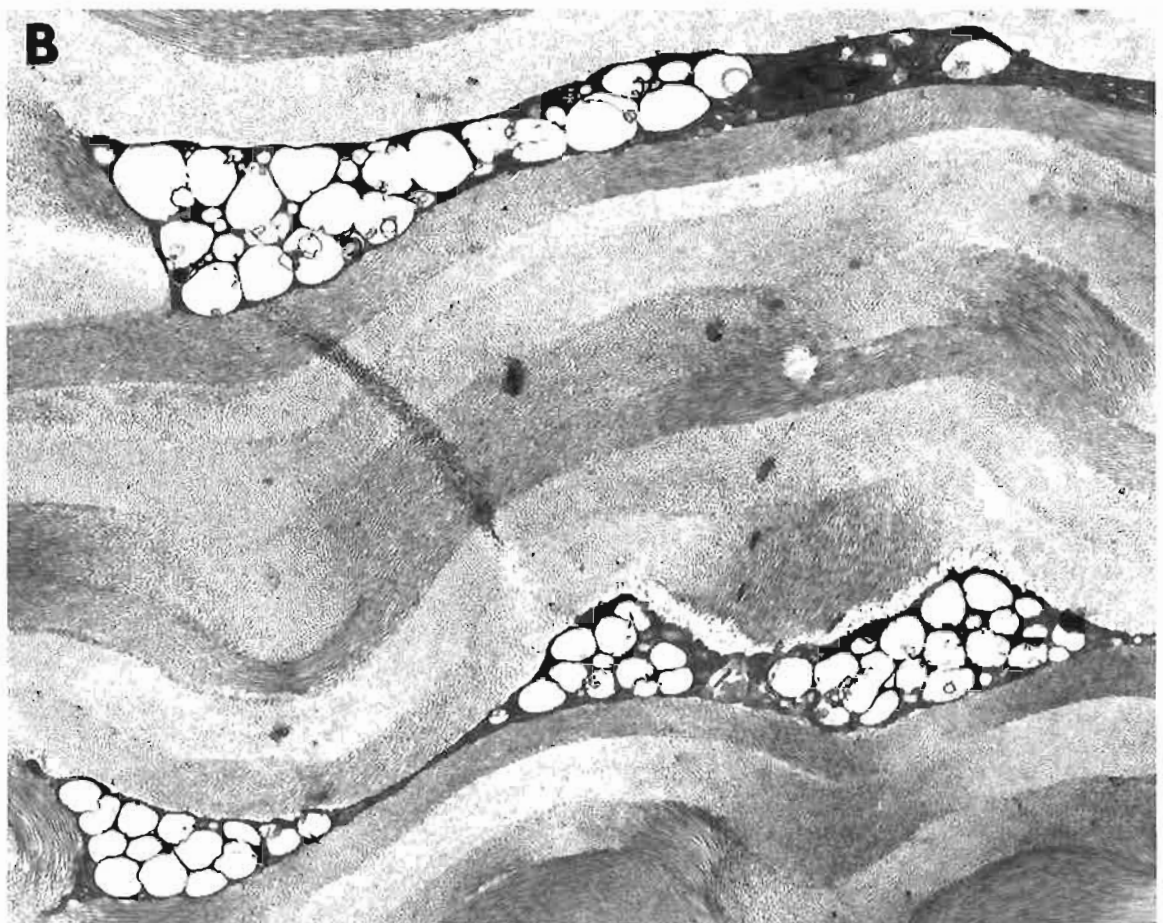
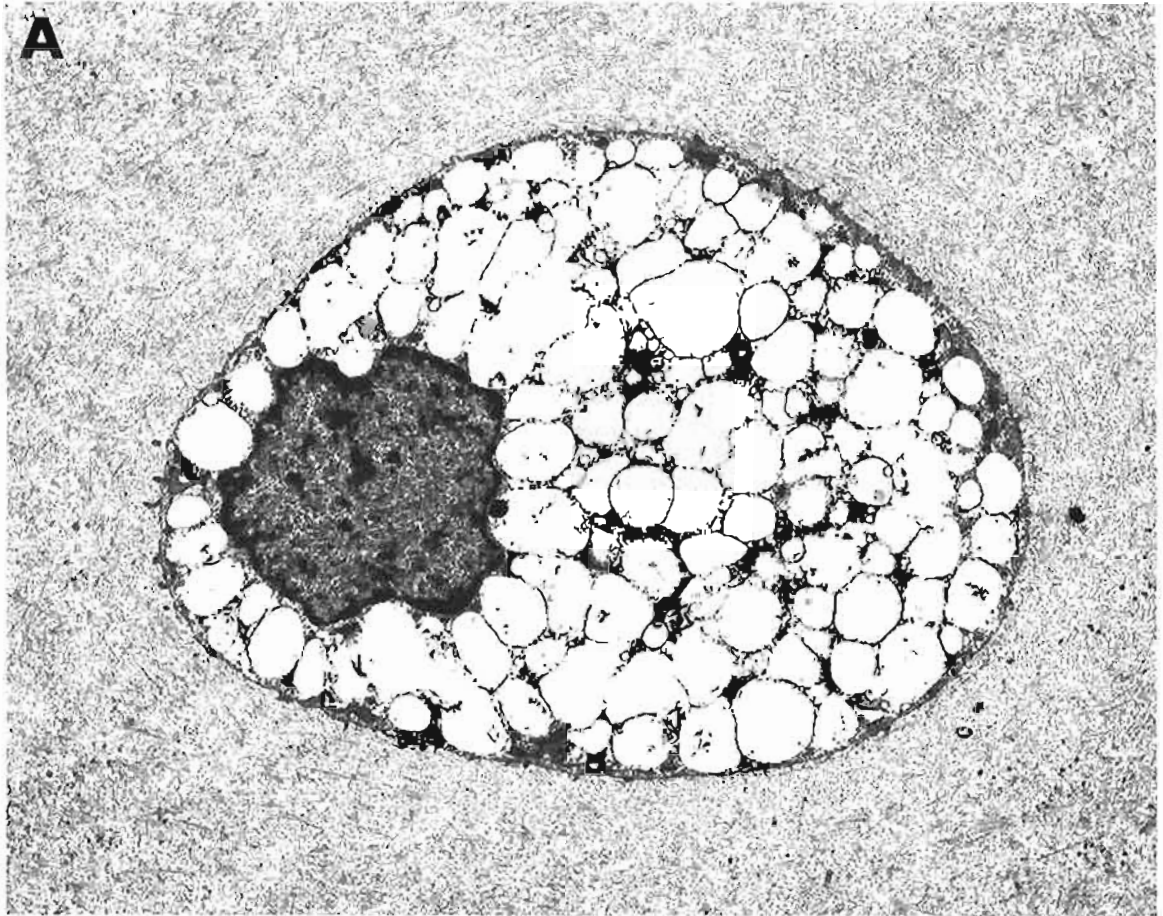


Figure 5.20: Electron microscopy of cornea and articular cartilage following ERT with ED4S and PL4S.

(A) Articular cartilage from an MPS VI cat treated with 1 mg/kg PL4S weekly showing no change in the degree of lysosomal vacuolation in chondrocytes compared with untreated MPS VI cats, and ERT treated MPS VI cats at other dose rates; (B) Cornea from an MPS VI cat treated with 1 mg/kg ED4S weekly showing no change in the degree of lysosomal vacuolation in keratocytes compared with untreated MPS VI cats and ERT treated MPS VI cats at other dose rates. Bar = 5 μ m (both figures).



5.4 Discussion

The major aims of experiments described in this chapter were to evaluate the efficacy of weekly ERT from birth in MPS VI cats for a duration of 5-11 months at three different dose rates (primary dose group), and to compare these findings with the efficacy of ERT using a twice weekly dose regime and also using modified enzyme to try and improve penetration of enzyme to cartilage (enzyme/dose modification group).

In the primary dose group, the results demonstrate a dose responsive effect of ERT, with clear improvements at 1 mg/kg rh4S given weekly, and greatest effect at 5 mg/kg weekly. ERT was effective in reducing development of soft tissue and skeletal pathology, correlating well with improvements observed clinically and with reductions in urinary GAG, including a specific decrease in DS. Comparison of response to therapy after 6 and 11 months of ERT indicated that the response to ERT was maintained for the 11 month duration of the study. The 0.2 mg/kg dose rate was insufficient to significantly alter progression of soft tissue and skeletal disease in the MPS VI cat.

In the enzyme/dose modification group, increasing dose frequency and coupling of rh4S to PL and ED did not appear to lead to significant improvements in efficacy compared with 1 mg/kg rh4S administered weekly. In particular, no changes in lysosomal storage were observed in cartilage. The 1 mg/kg SC dose rate was insufficient to alter progression of soft tissue and skeletal disease compared with untreated MPS VI cats. Comparing clinical appearance and soft tissue pathology, ED coupled enzyme (1 mg/kg ED4S weekly and 0.5 mg/kg ED4S twice weekly) overall appeared to be less effective than either rh4S (1 mg/kg weekly or 0.5 mg/kg twice weekly) or 1 mg/kg PL4S. PL4S was possibly slightly more effective at reducing lysosomal storage in heart valve and aorta than unmodified rh4S at the same dose rate.

Overall, histological response to ERT in the MPS VI cat was similar to observations following ERT in MPS VII mice and MPS I dogs. Following six weekly injections from birth, studies in MPS VII mice demonstrated that accumulation of lysosomal storage was prevented or delayed in hepatocytes, kupffer cells, spleen, heart and kidney interstitial cells

and retinal pigment epithelium, but not in corneal stroma, aortic media and chondrocytes. Lysosomal storage was also reduced in neurons in the brain, and skeletal growth was more normalised (Sands *et al.*, 1994). Following ERT confined to the first six weeks of life, adult MPS VII mice had increased longevity and had milder skeletal deformities than untreated mice (Vogler *et al.*, 1996). ERT in the canine model for MPS I also showed reduced lysosomal storage in hepatocytes, kupffer cells, spleen and some cells in the renal glomeruli, but no improvement was observed in brain, heart valves, cornea and chondrocytes (Shull *et al.*, 1994). Dogs were injected intravenously weekly for three months at very low doses compared with the ERT trials in the MPS VII mice and the studies described in this chapter. In further ERT studies in MPS I dogs with higher enzyme doses, some improvement in clinical appearance of the treated dog was also noted (Kakkis *et al.*, 1996).

Following ERT in MPS VI cats at the 0.5 mg/kg twice weekly to 5 mg/kg weekly dose rates, prevention or reduction of development of lysosomal storage in the majority of tissues examined, suggests that ERT is likely to lead to significant improvements in quality of life in human patients. Respiratory difficulty and cardiac valve insufficiency due to soft tissue thickening leads to death in many human MPS patients, and restriction of joint movement also causes major incapacitation (Neufeld and Muenzer, 1995). Therefore improvements seen in mitral heart valve and hip joint capsule provides great impetus to progress to therapy trials in human MPS VI patients. It is also interesting that decreases in lysosomal storage were not uniform in some tissues, seen clearly from the gradual increase in degree of lysosomal vacuolation towards the outer layers of the aortic tunica media with greatest improvement seen at 5 mg/kg and 1 mg/kg PL4S. Although it is unclear whether reduction of lysosomal storage in various cell types is due to direct uptake of rh4S or from cell to cell transfer of enzyme, the distribution of storage in the aorta suggests a dose related gradient of diffusion of rh4S in dense connective tissues, and improved diffusion and cellular uptake with PL4S compared with the same dose of rh4S.

ERT significantly altered skeletal disease in ERT treated cats at the 0.5 mg/kg twice weekly to 5 mg/kg weekly dose rates, with increased bone dimensions, resulting in improved flexibility and greatly reduced incidence of bony spinal cord compression. These changes were also visible radiographically, although improvements could not be clearly separated

according to dose rate. A more sensitive method of evaluating skeletal response to therapy was found to be bone histomorphometric analysis, which showed a definitive dose responsive effect of ERT on skeletal development (see also Byers *et al.*, 1997a). The more uniform bone density and more uniform trabecular pattern observed radiographically in ERT treated cats corresponded with increased trabecular number and thickness, and reduced trabecular spacing, as determined histomorphometrically. Good correlation ($R^2 = 0.50$ to 0.77) was observed in other studies between values from dual-energy X-ray absorptiometry of the lumbar vertebrae in anaesthetised MPS VI and normal cats, and bone histomorphometry of the L5 vertebra from the same animals after euthanasia (Turner *et al.*, 1995). This suggests that dual-energy X-ray absorptiometry may be better than radiography alone to monitor skeletal response to therapy in live animals, and ultimately in human patients.

A better understanding of the mechanisms behind the osteopaenia and development of skeletal pathology in untreated MPS VI cats is needed before it is possible to hypothesise why skeletal pathology was not completely reversed in ERT treated cats. There is a normal resorption of approximately 75% of bone trabeculae as they exit the growth plate (Howell and Dean, 1992) and new bone trabeculae cannot be created *de novo* outside of the growth plate. The greatly reduced trabecular number in untreated MPS VI cats maybe due to increased loss of poorly mineralised trabeculae as they exit the growth plate, but this process is not yet well understood. Conversely, the increased trabecular number in ERT treated cats can only indicate decreased loss of trabeculae as they exit the growth plate. This may be due to improved osteoblast function resulting in improved mineralisation and thicker trabeculae. The smoother subchondral bone surfaces and generally larger epiphyses in ERT treated cats also suggested improved mineralisation and transition of cartilage into bone. A dose responsive increase in bone formation rate was observed in ERT treated cats which also suggests improved osteoblast function (Byers *et al.*, 1997a). As observed in the previous chapter, high levels of enzyme are present in the bone marrow, and enzyme is therefore available to cells associated with trabeculae emerging from the growth plate. Decreased lysosomal storage following ERT in the MPS VI cats was evident in osteoblasts and osteocytes ultrastructurally (Byers *et al.*, 1997a). These changes suggestive of better osteoblast function may be due to improved function of individual osteoblasts or due to

increases in osteoblast number. However this was difficult to assess due to inaccurate identification of this cell type, and results were inconclusive (Byers *et al.*, 1997a). Further studies of these areas are needed before precise conclusions can be drawn.

It has also been suggested that improved radiographic bone density in ERT treated MPS I dogs (Kakkis *et al.*, 1996) and increased bone volume in BMT MPS VI cats (Norrdin *et al.*, 1993) could have been due to increased physical activity rather than a direct effect of therapy. It is not possible to determine if increased activity contributed to the increased bone volume in ERT treated cats. However despite comparable levels of physical activity at 3 months of age, significantly lower bone mineral volume is present in untreated MPS VI cats compared with normal cats at this age (Sharon Byers, unpublished observations), suggesting that physical activity is a minor contributor to bone pathology compared with the underlying biochemical abnormality.

Weekly ERT administered to MPS VII mice only in the first six weeks of life resulted in larger, longer lived animals (Vogler *et al.*, 1996), clearly indicating that early onset of therapy of limited duration had a lasting impact on subsequent skeletal development. Further studies in MPS VII mice comparing response to ERT started either at birth or at 6 weeks of age, demonstrated less severe skeletal dysplasia when ERT was started at birth (Sands *et al.*, 1997). Similarly histomorphometric results in the ERT treated cats in the previous chapter, although limited by the low dose rates and different dose regimes used, were suggestive of improved skeletal response the earlier therapy was instituted. These results, together with the significant improvements in skeletal disease observed in ERT in MPS VI cats from birth, indicates that early onset of ERT will be critical for optimal skeletal response to therapy in human patients.

Overall, few directly comparable results of responses to BMT and ERT in the MPS VI cat are available, so it is ultimately not possible to determine if one form of therapy had greater efficacy than the other. BMT studies in the MPS VI cat have shown some clinical improvement in mobility and general appearance (Wenger *et al.*, 1986), however no histological changes have been published. A reduction in CPC precipitable total urine GAG was observed in BMT cats to levels within or just above those observed in normal cats (Dial

et al., 1997). Using some common methods to those used in this chapter, urine DS in adult BMT cats was dramatically reduced to lower levels ($DS = 19 \pm 6\%$) than those observed in the 5 month old 5 mg/kg ERT cats ($DS = 37 \pm 13\%$). This may suggest greater efficacy of BMT compared with ERT in reducing urinary GAG. Extrapolating from our observations in ERT cats, this may therefore indicate greater reversal of lysosomal GAG accumulation in tissues. Alternatively some differences in these results may be due to different methods used to determine proportions of the GAG species separated by HRE, combined with different amounts of GAG loaded onto the HRE plates, or differences may be attributable to use of 24 hour urine samples in BMT cats.

BMT in human MPS VI patients is generally regarded to be very effective in alleviating widespread soft tissue related problems such as airway obstruction and joint stiffness (Hoogerbrugge *et al.*, 1995). However skeletal pathology is not significantly altered even in patients transplanted at a young age (Field *et al.*, 1994; Vellodi *et al.*, 1997). Our results suggest ERT may be more effective in reducing severity of skeletal disease compared with BMT in the MPS VI cat. However irradiation in preparation for BMT may also lead to growth retardation (Wagner *et al.*, 1990), complicating interpretation of efficacy of BMT on reversing skeletal pathology in the MPS VI cats. Slight changes in skeletal disease were seen radiographically following BMT in MPS VI cats (Wenger *et al.*, 1986; Haskins *et al.*, 1991b; Norrdin *et al.*, 1995), and bone histomorphometric analysis in BMT cats have shown some increases in iliac crest and L5 vertebral trabecular bone volume (BV/TV%) (Norrdin *et al.*, 1993; Turner *et al.*, 1995). The bone volume in the L5 vertebra increased to a level comparable with ERT treated cats at 1 mg/kg rh4S administered weekly (Turner *et al.*, 1995). The improved bone volume in BMT treated MPS VI cats may be due to low dose continuous enzyme replacement from donor marrow, compared with improved bone volume in ERT cats due to high bolus doses of enzyme. Some increases in bone volume may also be due to remodelling changes with increasing age in the presence of low enzyme levels. In support of this, bone volume in Alf and Duncan at 27 and 15 months of age respectively (low dose ERT started after birth; see Tables 4.1 and 4.7), reached values in between those from 6 month old 1 and 5 mg/kg ERT MPS VI cats treated from birth.

BMT has also been performed in neonatal MPS VI rats, however poor levels of engraftment were achieved, with clinical and radiographic improvements observed in only one out of twenty four rats which engrafted (Simonaro *et al.*, 1997). Both ERT and BMT from birth in MPS VII mice lead to increased long bone length, apparently to a similar degree in BMT animals at lower doses of irradiation (Sands *et al.*, 1993, 1997). Of further interest, were the even greater reductions in skeletal and soft tissue pathology in MPS VII mice when ERT started at birth to six weeks of age was directly followed by BMT, compared with ERT alone (Sands *et al.*, 1997). This suggests that ERT and BMT may reduce pathology in certain tissues to different degrees. These differences between ERT and BMT are presumably due to differences in enzyme distribution and enzyme dose, with enzyme delivered as a bolus in ERT compared with a lower level of continuously delivered enzyme in BMT. Migration of donor derived cells with subsequent enzyme transfer to surrounding cells in BMT may more effectively target the CNS, and avascular or dense connective tissues than by enzyme diffusion in ERT, however CNS uptake was not examined in our studies. If neonatal screening was able to identify MPS affected patients soon after birth, ERT could then be instituted during rapid skeletal growth, with therapy later changed to BMT to alleviate ongoing soft tissue pathology.

In further support of these differences, ERT and BMT had differing outcomes with corneal pathology. At all doses of ERT in the MPS VI cats, lysosomal vacuolation in corneal keratocytes was not altered, correlating with persistence of corneal clouding by slit lamp examination. This lack of correction was also observed in ERT studies performed in the MPS VII mouse (Sands *et al.*, 1994, 1997) and MPS I dog (Shull *et al.*, 1994). However BMT studies in MPS VII mice (Birkenmeier *et al.*, 1991) and MPS I dogs (Breider *et al.*, 1989), and combined ERT and BMT therapy in MPS VII mice (Sands *et al.*, 1997), demonstrated complete reversal or reduction of lysosomal inclusions in corneal keratocytes. These differences in outcome between ERT and BMT are probably due to migration of donor derived cells to the cornea in BMT treated animals. The lack of correction of corneal clouding in BMT MPS VI cats (Haskins *et al.*, 1991b) may indicate differences in corneal pathology due to MPS type, and hence different responses to therapy. In support of this, lack of correction of lysosomal inclusions in reciprocal corneal graft experiments in MPS VI

and normal cats suggests that pathology in the MPS VI cornea is not reversible (Aguirre *et al.*, 1992), however comparable experiments in other MPS types have not been performed.

Lysosomal storage in chondrocytes appears refractory to BMT and ERT in MPS animal models (Birkenmeier *et al.*, 1991; Sands *et al.*, 1994; Shull *et al.*, 1994) except for slight improvements seen in articular cartilage in BMT MPS I dogs (Breider *et al.*, 1989) and in trachea in several BMT MPS VI rats (Simonaro *et al.*, 1997). A maintained dose of 5 mg/kg rh4S for up to 11 months is the highest dose used in ERT studies in MPS animal models to date. Despite these high doses and charge modifications of enzyme to improve enzyme penetration into cartilage, ERT was unable to reduce the degree of lysosomal distension in articular cartilage or tracheal chondrocytes. This confirms the difficulty of enzyme transfer to chondrocytes in sufficient amounts, probably due to a combination of a high GAG load in these cell types and also the highly negatively charged and relatively avascular matrix surrounding the chondrocytes. However, although both were not objectively measured, the subjective reduction in articular cartilage thickness and hence improved transformation of cartilage into bone, and increased ear size in ERT treated cats, suggests some enzyme transfer has taken place, although perhaps only to peripheral chondrocytes. It also indicates that chondrocytes can function to some degree despite the presence of severe lysosomal distension that obscures the nucleus and other organelles when visualised ultrastructurally.

Great variability was observed in severity of joint disease in untreated MPS VI cats at 6 and 11 months (see Section 3.3.5.2). Although cartilage thickness appeared to be generally reduced in 6 month old ERT treated cats, at 11 months of age, the 5 mg/kg ERT treated cat had the most severe degenerative changes compared with untreated MPS VI controls. In BMT MPS I dogs (Breider *et al.*, 1989) and BMT MPS VI cats (Norrdin *et al.*, 1994) there was possibly a reduction in severity of joint lesions, however numbers of animals in both studies were small. Greater numbers of older ERT treated MPS VI cats would help to clarify if ERT altered the severity of joint disease compared with untreated MPS VI controls. Degenerative joint disease is probably due to a combination of reduced cartilage integrity and the ability of the subchondral bone to support the articular cartilage, as erosive lesions first appear in high load bearing regions such as in the shoulder.

Antibody production and its effects on efficacy of therapeutic proteins, and patient welfare due to immune mediated complications with the use of proteins is an important issue. Approximately 15% of Gaucher patients undergoing ERT develop antibody titres, with a small proportion of these experiencing various symptoms suggestive of immediate hypersensitivity reactions, although the presence of antibodies did not appear to alter efficacy of therapy (Pastores *et al.*, 1993; Richards *et al.*, 1993). However neutralizing antibody was recently described in two Gaucher patients undergoing ERT with Ceredase ®. These patients exhibited a lack of improvement with therapy or a worsening of disease symptoms (Ponce *et al.*, 1997). This may be an emerging problem in ERT treated Gaucher patients, although in only a very small proportion of patients. Withdrawal of ERT in the patient exhibiting lack of improvement lead to further disease progression, suggesting that ERT even in the presence of neutralizing antibody had some effect in stabilising disease symptoms (Ponce *et al.*, 1997).

Kittens undergoing ERT exhibited signs of reactions to enzyme infusions from an early age. This was partially controlled by antihistamine premedication, however in one 5 mg/kg cat (125m), moderate anaphylactic type reactions became resistant to premedication. Clinically and histologically this cat did not show any reduction in efficacy of therapy, and compared with the other 5 mg/kg treated cats, he was considered to generally show the greatest improvements as a result of therapy compared with two other cats at the same dose rate. The clinical reactions in ERT cats may have resulted from complement activation by immune complexes leading to anaphylotoxin release and mast cell degranulation, as seen in Gaucher patients undergoing ERT (Richards *et al.*, 1993), or from direct binding of immunoglobulins (typically IgE) to mast cells, although detection of IgE antibody class is currently unavailable in cats. There was no evidence of renal glomerular immune complex deposition or impaired renal function in the ERT cats compared with the controls for the duration of therapy studied. A total of 27 cats have undergone ERT with rh4S, with only 4 deaths due to infectious diseases or congenital abnormalities. Reactions to enzyme infusions were also observed in MPS VII mice, and these were readily controlled by premedication with antihistamines and corticosteroids (Sands *et al.*, 1997). Antibodies were detected in these mice against human serum albumin present in the enzyme preparation, but not against

β -glucuronidase (Sands *et al.*, 1997). Minimal complications following ERT in Gaucher patients and MPS VI cats and MPS VII mice suggests that ERT is a relatively safe therapy.

However patients or animal models with null mutations resulting in a complete lack of production of enzyme protein, are anticipated to produce antibodies against any administered enzyme, whether by ERT or gene therapy, as the replaced enzyme will be considered foreign by the immune system. This may result in long-term complications in these patients. A null mutation is present in the MPS I dog (Menon *et al.*, 1992), and IgG antibodies against human and canine α -L-iduronidase have been detected in ERT treated and myoblast-mediated gene therapy treated MPS I dogs, as well as clinical reactions following enzyme infusion (Shull *et al.*, 1994, 1996; Kakkis *et al.*, 1996). Despite this, reactions to infusions were controlled and efficacy of ERT was still observed.

Titres against rh4S in the ERT cats treated from birth by IV infusion were not significantly different from untreated MPS VI and normal controls (Brooks *et al.*, 1997). The MPS VI cat model has a small amount of feline 4S present (Yogalingam *et al.*, 1996), therefore potentially reducing the likelihood of the production of high titre reactive antibodies following ERT. In addition, human and feline 4S share greater than 90% homology (Jackson *et al.*, 1992), perhaps also reducing the possibility of generating a vigorous immune response against human rh4S in ERT treated cats. The high anti-rh4S titres observed in untreated MPS VI and normal control cats were unexpected. High serum immunoglobulin levels have previously been observed in cats reared in conventional catteries, compared with cats living in households or in specific pathogen free catteries. This was considered to be due to greater infectious disease problems within catteries (Pedersen, 1982), and persistent disease problems were certainly present within our MPS VI cat colony. The high background reactivity in all cats in general described in this chapter suggests the presence of high levels of low-affinity anti-rh4S antibodies, possibly due to cross-reacting epitopes from infectious disease antigens.

Following ELISA assay of sera to detect antibodies against the administered protein in ERT in human Gaucher patients (Richards *et al.*, 1993; Ponce *et al.*, 1997), MPS I dogs (Kakkis *et al.*, 1996), and in MPS VII mice (Sands *et al.*, 1997), antibodies were

immunoprecipitated to confirm antibody specificity. In an additional study evaluating antibody response in Gaucher patients, several serum samples had elevated immunoglobulin levels. However these results were not reproducible and the sera was unable to neutralise enzyme or precipitate radiolabelled enzyme, confirming the lack of specificity (Murray *et al.*, 1991). Therefore it would have been useful if either immunoprecipitation or enzyme neutralisation tests had been performed on ERT and control cat plasma samples, to provide additional information regarding antibody specificity in our studies. To that end, plasma samples from five IV ERT treated MPS VI cats, three untreated MPS VI and one untreated normal control cats were immunoblotted against rh4S. Plasmas from four out of the five ERT treated cats and all three untreated MPS VI cats reacted positively, however there was no apparent correlation with titre results and intensity of blotting. Blots from only two of the ERT treated cats stained more intensely than the untreated MPS VI controls. The normal control cat did not react (Doug Brooks, unpublished observations). This supports the hypothesis of cross-reacting antibodies in plasma samples causing high background reactivity in untreated controls and ERT treated MPS VI cats. The high antibody titre observed in the SC treated cat was possibly due to a different route of enzyme administration, although greater numbers of animals would need to be evaluated to assess this properly.

In the previous chapter, reduced efficacy of therapy due to antibody inactivation of rh4S was thought to have been a possibility in one cat (Alf). An *in vitro* dose dependent inhibition of rh4S activity by plasma from this cat was also observed (Crawley *et al.*, 1996). In high titre rats immunised against rh4S, following IV administration of rh4S, altered intracellular routing and more rapid turnover of rh4S was observed in percoll fractionated liver samples (Brooks *et al.*, 1997). In several ERT cats from the enzyme/dose modification group, intracellular routing of rh4S in fractionated liver samples was normal (Doug Brooks, unpublished observation). Extrapolating from these results, as the titres against rh4S in the vast majority of the remaining ERT treated cats were similar to these cats (and also to untreated MPS VI and normal controls), we conclude that the efficacy of therapy in cats undergoing ERT in the primary dose group and in the enzyme/dose modification group was not significantly compromised by production of antibodies against rh4S. However, the persistent high antibody titre in the SC treated cat suggests that reduced efficacy of rh4S

may have been possible in this cat, however no samples were available for liver fractionation. Evaluation of antibody production and efficacy of ERT studies with recombinant feline 4S (rf4S) may help to better understand results collected from ERT with rh4S, and whether reduced efficacy due to antibodies against rh4S has occurred.

Cationization of proteins to alter systemic distribution, particularly to try and increase protein targeting to the CNS across the blood brain barrier, has been evaluated previously, using poly-L-lysine coupled hexosaminidase in normal cats (Rattazzi, 1983; Rattazzi *et al.*, 1983; Rattazzi and Dobrenis, 1991). Enhanced uptake *in vitro* and some alterations in distribution in normal cats *in vivo* was observed, however an anaphylactoid reaction occurred in several cats infused with poly-L-lysine coupled Hexosaminidase A (Rattazzi and Dobrenis, 1991). A previous report of a dose-dependent histamine release after addition of poly-L-lysine to isolated rat peritoneal mast cells (Ennis *et al.*, 1980) suggested that this may be a significant problem if this form of enzyme modification was pursued, although the same response was not observed in guinea-pig peritoneal mast cells.

Persistent mild reactions from an early age were observed in the two cats treated with weekly PL4S, but this was readily controlled using antihistamine premedication and slow rates of infusion. These reactions were not obviously different in severity from the reactions in most of the 1 and 5 mg/kg rh4S treated cats. It is possible that the poly-L-lysine may have directly caused some mast cell degranulation, however the reactions may also have been due to the relatively higher protein levels in the enzyme solution compared with unmodified rh4S due to the reduced enzyme specific activity after enzyme coupling. Increased antigenicity of cationized bovine serum albumin (BSA) compared with native BSA administered to mice has also been observed following immunization (Muckerheide *et al.*, 1987). None of the ED4S or PL4S treated cats (n=7) demonstrated elevated titres against rh4S, and compared with the remaining IV ERT cats (n=14) treated with unmodified enzyme, no significant difference in antibody titres was observed (student *t*-test). In addition, cationized BSA administered IV to rabbits induced an immune complex glomerulonephritis due to IgG and C3 deposits in the glomerular capillary wall compared with a lack of pathology in rabbits injected with native BSA (Adler *et al.*, 1983). Neither of these sequelae were observed in any of the PL4S or ED4S treated MPS VI cats, except for

a very slight increase in glomerular immunofluorescence and suggestion of very slight glomerular basement membrane immunoglobulin deposits by electron microscopy in one 1 mg/kg PL4S treated cat (213m). It is unknown if this slight reactivity was due to a urinary tract infection in the neonatal period resulting in unilateral renal fibrosis, or whether it was a direct cause of ERT with PL4S.

Cationization of rh4S with PL possibly reduced the severity of lysosomal storage in the dense connective tissues of aorta and heart valve compared with 1 mg/kg rh4S, however ED4S was less effective histologically than rh4S. Due to cationization, PL4S and ED4S may be internalised by absorptive endocytosis as well as by mannose-6-phosphate receptor (M6PR) mediated endocytosis. As pointed out by Rattazzi and Dobrenis (1991), whether increased levels of cell uptake of cationized protein occurs, ultimately reflects the affinity of the cationized protein for the plasma membrane and hence concentrations of the protein at the plasma membrane, and also the rate of endocytosis of a particular cell type. GAGs impart a negative charge to connective tissues, and possibly the charge density in heart valve and aorta is greater than many of the other soft connective tissues examined. Therefore the much higher pI of PL4S compared with unmodified rh4S would have increased the ability of PL4S to diffuse through these tissues. The reduced efficacy of ED4S compared with PL4S (and rh4S) may be due to the lower pI resulting in decreased affinity for plasma membranes, and hence lower levels of internalisation, compared with the rate of endocytosis of unmodified rh4S via the M6PR. An alternative explanation for the slightly improved response with PL4S, is that enzyme inactivated due to the coupling process was able to restore activity following infusion, hence PL4S treated cats received a higher enzyme dose than calculated. However this explanation seems less likely as enzyme activity of both ED4S and PL4S were stable at 4°C over the course of the study. Evaluation of increased numbers of animals treated with PL4S would be useful to clarify the observations made.

An improved method for quantitating urine GAG was developed based on a previous method (Di Ferrante, 1967) to improve sensitivity of total urine GAG quantitation compared with the results obtained from the ABC method (Gold, 1979). The DEAE method binds all sulphated GAG, and allows almost complete separation from urine

pigments and proteins which interfere with uronic acid quantitation. Some uronic acid content was evident in the column flow through, although was not quantifiable due to pigment, and probably contains non-sulphated chondroitin and hyaluronic acid (Varadi *et al.*, 1967). Compared with CPC precipitation which fails to precipitate GAG below 3000 Da (Hopwood and Harrison, 1982), the DEAE method although more labour intensive, allows greater sensitivity in detecting changes in GAG excretion associated with therapies. Perhaps even greater sensitivity in urine GAG quantitation would have been observed if 24 hour urine samples had been collected (Di Ferrante and Lipscomb, 1970), however this was not considered practical due to the large numbers of animals being examined.

Due to these differences observed between the CPC and DEAE methods of GAG purification, it was also questionable whether HRE of CPC prepared GAG was representative of the total GAG present in the urine. By comparing urine GAG isolated by CPC precipitation with the DEAE method, the lack of any change in proportion of low molecular weight GAG excreted in the urine of ERT treated MPS VI cats suggests that HRE of CPC prepared GAG is adequate to monitor response to therapy. Further work examining HRE of DEAE prepared GAG would be useful to confirm this. However, HRE of CPC prepared GAG did reveal a dose related decrease in urine DS which also correlated with reductions in lysosomal storage in connective tissues.

Our observations of no improvement or normalisation in leukocyte morphology at all doses of ERT indicate that this may not be useful for monitoring efficacy of ERT in human MPS VI patients. It also suggests that leukocytes may not have the appropriate cell surface receptors for uptake of rh4S, or that these cell types have high 4S requirements. Leukocyte morphology is discussed in more detail in the next chapter.

As described extensively in Chapter 3, clinical heterogeneity seen in both untreated and treated MPS VI cats, including between siblings, made analysis of results more difficult in this chapter. This suggests that greater difficulties in evaluation of efficacy will be met in human MPS VI patients undergoing ERT, because of the many different genotypes and therefore phenotypes expected in human patients. Factors contributing to the variability throughout this study included infectious disease outbreaks at different times, differences in

numbers of animals housed together and food availability was not always completely *ad libitum*. There has been a general trend over the last 5 years of a general increase in size of all colony cats, probably due to a gradual decrease in numbers of animals in the colony.

5.5 Summary and conclusions

ERT was effective in reducing development of soft tissue and skeletal pathology in the MPS VI cat, correlating well with dose related improvements in bone histomorphometric parameters and reductions in urine GAG excretion. Greatest response was observed at 5 mg/kg rh4S, with almost no storage seen in heart valve, aorta and skin, however cartilage chondrocytes and corneal keratocytes remained unchanged at all doses. Cationization of rh4S with PL lead to slight reductions in lysosomal storage in some soft tissues compared with the same dose of rh4S, however there was no evidence of reduced storage in cartilage chondrocytes. There was suggestion of improved transformation of cartilage into bone in higher dose cats, however significant joint pathology was present at postmortem in MPS VI cats, with or without ERT, at 11 months of age. Increasing dose frequency did not appear to lead to significant improvements in efficacy compared with 1 mg/kg rh4S administered weekly, and ED4S was slightly less effective than rh4S at the same dose rates. The 0.2 mg/kg and 1 mg/kg SC dose rates appeared insufficient to significantly alter progression of soft tissue and skeletal disease compared with untreated MPS VI cats. The majority of treated cats only experienced mild clinical reactions to enzyme infusions and there was no suggestion of immune mediated complications or reduction in enzyme efficacy.

These observations suggests that ERT is likely to lead to significant improvements in quality of life in human patients. In particular, the improvements observed in bone growth and bone structure, and minimal or no lysosomal storage in heart valve, indicates that ERT can target important sites of pathology and alter disease course. Our studies also suggest that the greatest effect of ERT on skeletal disease may occur the earlier the age of onset of therapy, although further studies are needed to fully clarify this aspect. The lack of correction of lysosomal storage in cornea and cartilage, despite attempts to improve cellular uptake by enzyme modifications, indicates that diffusion of enzyme into avascular tissues is minimal. Corneal pathology in human MPS VI patients can be alleviated by corneal transplants,

however our results suggest that alternative therapies directed to cartilage, in particular articular cartilage, need to be developed.

Several anomalies within the breeding colony were identified throughout the course of this study, including difficulty in identifying true heterozygotes, making production of MPS VI kittens for ERT studies difficult. Mutation analysis identified a number of different genotypes within the colony, and in the next chapter, a detailed description of the resulting phenotypes is made, which clarifies the anomalies observed previously.

6. Colony genotype to phenotype relationships

6.1 Introduction

During the course of this study, molecular analysis of the cat colony demonstrated that MPS VI affected cats used to evaluate the efficacy of ERT, were homoallelic for an amino acid substitution at codon 476 (L476P)(Yogalingam *et al.*, 1996). Despite this, correlation between leukocyte 4S/ β hex ratios and heterozygote and normal genotypes was still poor, with some phenotypically normal cats with one or no L476P alleles demonstrating leukocyte 4S activities similar to L476P homozygotes (Figure 3.1). This suggested an additional mutation modifying 4S activity may be present in these cats. If additional genetic factors were present within the colony, it was also unknown whether this had contributed to the clinical variability observed in untreated MPS VI cats. A second feline MPS VI mutation was subsequently discovered within our MPS VI cat colony, which involved a base change leading to an amino acid substitution at codon 520 (D520N)(Yogalingam *et al.*, 1998).

The aim of this chapter was to determine the origin and inheritance of both mutations within the colony, and to describe and compare the clinical, biochemical and histological features of the different genotypes in the colony. In particular, L476P/D520N compound heterozygote and D520N homozygote cats were examined, and were compared with L476P homozygotes, which were previously described in detail in Chapter 3.

To evaluate phenotype due to different genotypes, leukocyte 4S/ β hex ratios and morphology, urinary GAG analysis, clinical and radiographic skeletal appearance, postmortem changes and tissue histopathology were assessed.

6.2 Specific methods

6.2.1 Animal origins and mutation analysis

As described in earlier chapters, MPS VI was originally identified in purebred Siamese cats, and the colony was established from five “Family 3” cats, believed to be heterozygotes, imported from the Philadelphia, USA. The original identification numbers of these animals were 1189m, 1310m, 1035f, 1141f, and 1143f. Unrelated normal cats were intermittently introduced to the colony for subsequent outbreeding.

Genotype of animals within the colony was established from PCR amplification of DNA from dried blood spots or DNA prepared from frozen white cell pellets (Section 2.2.6.1). Restriction analysis of PCR products using *Hae III* restriction enzyme was used to identify an L to P change at codon 476 (Yogalingam *et al.*, 1996), and *Bsl I* or *Ava II* to identify a D to N change at codon 520 (Yogalingam *et al.*, 1998), as outlined in Section 2.2.6.3. Due to the presence of these two mutations, there were six genotypes observed within the colony: normal for both alleles (annotated normal/normal), L476P homozygotes (L476P/L476P), L476P heterozygote (L476P/normal), D520N homozygotes (D520N/D520N), D520N heterozygote (D520N/normal), and L476P and D520N compound heterozygotes (L476P/D520N). The L476P/L476P homozygotes which exhibit a severe MPS VI phenotype, were described in detail in Chapter 3, and were evaluated for the efficacy of ERT in Chapters 4 and 5.

Limited numbers of some genotypes were only available due to previous culling of breeding animals to optimise breeding of L476P/L476P cats for ERT studies. Most of the material available for analysis were archived samples. This study is therefore largely retrospective, with only small numbers of young live animals from some genotypes available for evaluation.

6.2.2 Enzymology

Peripheral leukocytes were prepared and assayed for 4S activity as outlined in Section 2.2.7. Activity was expressed as a 4S/ β hex ratio. One-way analysis of variance and the

Tukey-HSD test for post-testing was used for statistical comparison of 4S/ β hex ratios between D520N/D520N, L476P/D520N and L476P/L476P genotypes, accepting a p -value of < 0.05 as statistically significant.

6.2.3 Urinalysis

Normal cat urines were obtained from cats outside the colony. Urine creatinine was measured using an autoanalyser method (Section 2.2.15). Total urine GAG was quantitated using the ABC method (Section 2.2.16), and values from males and females were combined. One-way analysis of variance and the Tukey-HSD test for post-testing was used for statistical comparison of total urine GAG at 90 days among D520N/D520N, L476P/D520N, L476P/normal and D520N/normal genotypes, accepting a p -value of < 0.05 as statistically significant. Some samples were also analysed for total GAG by the DEAE method (Section 2.2.18).

Urine samples were prepared by CPC precipitation to visualise GAG species by HRE. Total uronic acid concentrations of prepared samples were determined using the hydroxydiphenyl method (Section 2.2.19) and approximately the same amounts (μg uronic acid) were loaded for HRE. Increased run times and a larger cellulose acetate strip were used to improve separation of bands (Section 2.2.17). Densitometric estimations of the approximate relative proportions of total DS (DS1 + DS2) in urine from cats 1 year or older (except for L476P homozygotes), were expressed as a percent of total GAG (Section 2.2.17). The relative proportions of the different GAG species varies in young cats (see Table 5.4), hence urine from cats less than 1 year old was excluded to minimise variability.

6.2.4 Leukocyte morphology

Air dried blood films were stained with May-Grunwald Giemsa and examined at 400 - 1000 $\times$ magnification. Leukocytes were prepared from buffy coats for electron microscopy as described in Section 2.2.22.

6.2.5 Clinical and radiological examination

A “blinded” operator performed slit lamp examinations (see Section 2.2.11) of the corneas in the oldest available cats of each genotype without anaesthesia (L476P/D520N, 3.8 - 6.6 years; D520N/D520N, 2 months; L476P/normal, 1.2 - 1.7 years; D520N/normal, 2.5 months; normal/normal, 7 months; L476P/L476P, 5 months). Two additional D520N/D520N cats (4 years old) were examined for corneal clouding without the slit lamp. Corneal clouding in L476P/L476P cats is apparent without the aid of a slit lamp from as early as 6-7 weeks of age. Cervical spine flexibility was subjectively measured by determining the ability to move the head laterally.

Standardised radiological examination under general anaesthesia and standardised bone measurements were taken directly from radiographs, as described in Sections 2.2.13 and 2.2.14. Length/width dimensions for L5 and C3 were expressed as a ratio. Measurements were taken from female cats at 3, 5, and 6 months, and female cats greater than 18 months of age to compare growth and final adult size among the different genotypes. Due to lack of data from older L476P homozygotes, 1 year old female L476P homozygotes were included as a comparison for the adult female group. Skeletal growth in L476P homozygotes appears to largely cease by 7 to 11 months with only marginal increases in tibial long bone growth until 12 to 18 months. Numbers of adult females in each group were: normal/normal, L476P/normal and D520N/normal (n=9 combined), L476P/D520N (n=12), D520N/D520N (n=8), and L476P/L476P (n=4). Statistical comparison of bone dimensions between these four groups was performed using one-way analysis of variance and the Tukey-HSD test for post-testing, accepting a *p*-value of < 0.05 as statistically significant. These radiographs, and additional radiographs of adult males and cats 11 to 18 months old were also examined for qualitative changes in skeletal appearance.

6.2.6 Pathology

Cats of various ages were euthanased using an overdose of intravenous barbiturate. Various tissues for electron microscopy were fixed and processed (Section 2.2.22) and 1 µm thick Toluidine Blue stained survey sections were evaluated at 100 - 400 × magnification to assess overall distribution of vacuolation due to lysosomal storage. Cross-sections of rib

cartilage samples surrounded by outer perichondrial connective tissue were taken from approximately midway along the cartilage portion of the rib. Articular cartilage sections were cut from the articular surface through to the subchondral bone. Tissues from both colony bred and external normal/normal cats were examined as controls. Samples from the majority of non-cartilagenous tissues were also evaluated further under electron microscopy for the presence of abnormal lysosomal vacuolation, particularly those from the oldest available animals for each genotype (L476P/D520N up to 6.5years; D520N/D520N up to 4.3 years). The shoulder and stifle joints were examined in six adult L476P/D520N compound heterozygotes, one 30 month old L476P homozygote, and the stifle joints only, in 2 adult D520N homozygotes.

6.3 Results

6.3.1 Genotype analysis - origin of the L476P and D520N mutation

Six genotypes were clearly distinguishable using both *Hae* III and *Bsl* I digestion of PCR products (Figure 6.1).

The pedigree of our colony shows the origin of both the L476P and D520N alleles in one of the original imported heterozygote cats (cat 1189m)(Figure 6.2). DNA was not available from cats 1035f and 1189m, however all remaining cats were genotyped by PCR based mutation analysis. The L476P/D520N genotype of cat 1189m was deduced from breeding outcomes. This cat had an abnormally low leukocyte 4S/ β hex ratio of 0.11 (see normal reference ranges, Table 6.1). The genotype of cat 1035f could not be completely deduced. This cat may have been a compound heterozygote (L476P/D520N) as she also had a moderately low leukocyte 4S/ β hex ratio of 0.19, however she may have been an L476P/normal heterozygote.

The D520N mutation appears to have an autosomal recessive mode of inheritance, and was not detected in all L476P/L476P cats tested (n=39), indicating that the two mutations are not linked. The D520N mutation was also not present in normal cats unrelated to the colony (n=23).

In collaboration with Dr Mark Haskins (University of Pennsylvania, USA) and Dr Paola Di Natale (University of Naples, Italy) dried blood spots and DNA prepared from fibroblasts were supplied for genotype analysis from Family 2 and Family 3 cats (from M.H.) (Haskins *et al.*, 1979b), and from an MPS VI cat identified in Italy (from P.D.) (Di Natale *et al.*, 1992). The L476P allele was identified in both Family 2 and Family 3 cats, and MPS VI affected cats from both families, as well as a Family 2/3 genetic compound MPS VI cat were L476P homozygotes. The D520N allele was not detected in any cats, however only a total 5 colony derived cats were tested. The Italian MPS VI cat was also identified to be an L476P homozygote.

Figure 6.1: Identification of genotypes using *Hae* III and *Bsl* I restriction analysis of PCR products.

The L476P mutation introduces a *Hae* III restriction site, while the D520N mutation removes an *Ava* II and *Bsl* I restriction site in the 296 base pair PCR product. (A) ***Hae* III digestion:** Three restriction fragments (51, 75 and 163 base pairs) were generated from *Hae* III digestion of PCR product from an L476P homozygote cat (lane 1). L476P/D520N compound heterozygote (lane 2) and L476P/normal heterozygote (lane 3) PCR products were partially digested with *Hae* III (51, 75, 163 and 245 base pair fragments), and a small fragment is produced in D520N/D520N (lane 4), D520N/normal (lane 5) and normal/normal (lane 6) genotypes (51 and 245 base pair fragments). (B) ***Bsl* I digestion:** In a D520N homozygote cat (lane 10), no digestion of the PCR product takes place with *Bsl* I (296 base pair product remains intact), while in L476P/D520N compound heterozygotes (lane 8) and D520N/normal heterozygotes (lane 11), three fragments (93, 203 and 296 base pairs) are generated from a partially digested PCR product. Complete digestion occurs in L476P/L476P, L476P/normal and normal/normal cats (lanes 7, 9 and 12 respectively), producing two fragments (93 and 203 base pairs). The sizes of the fragments detected is indicated in base pairs on the side of the gels, and the sizes of the molecular weight markers ("m" lanes) in base pairs are 501/489, 404, 331, 242, 190, 147, 110, 67 and 34.

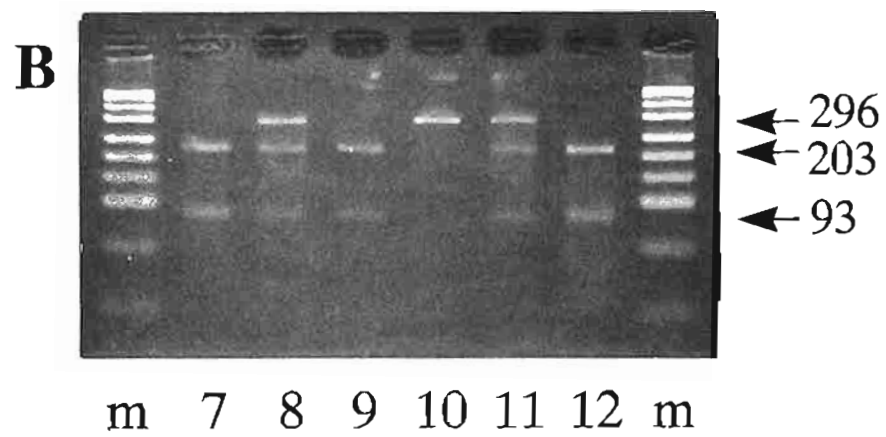
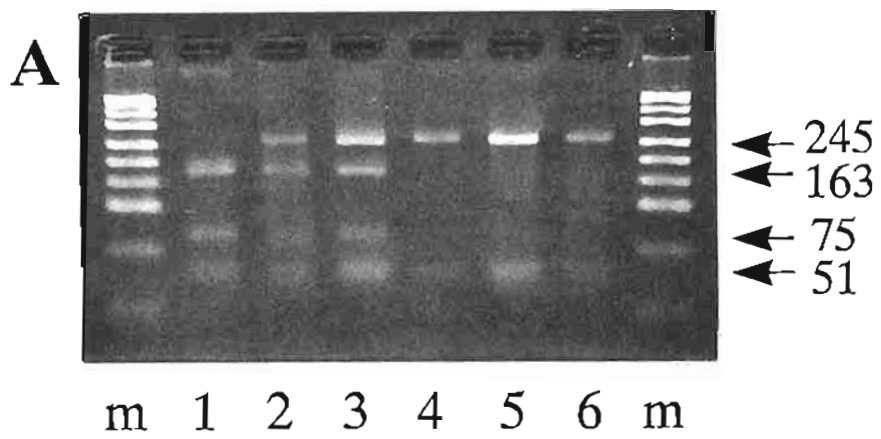
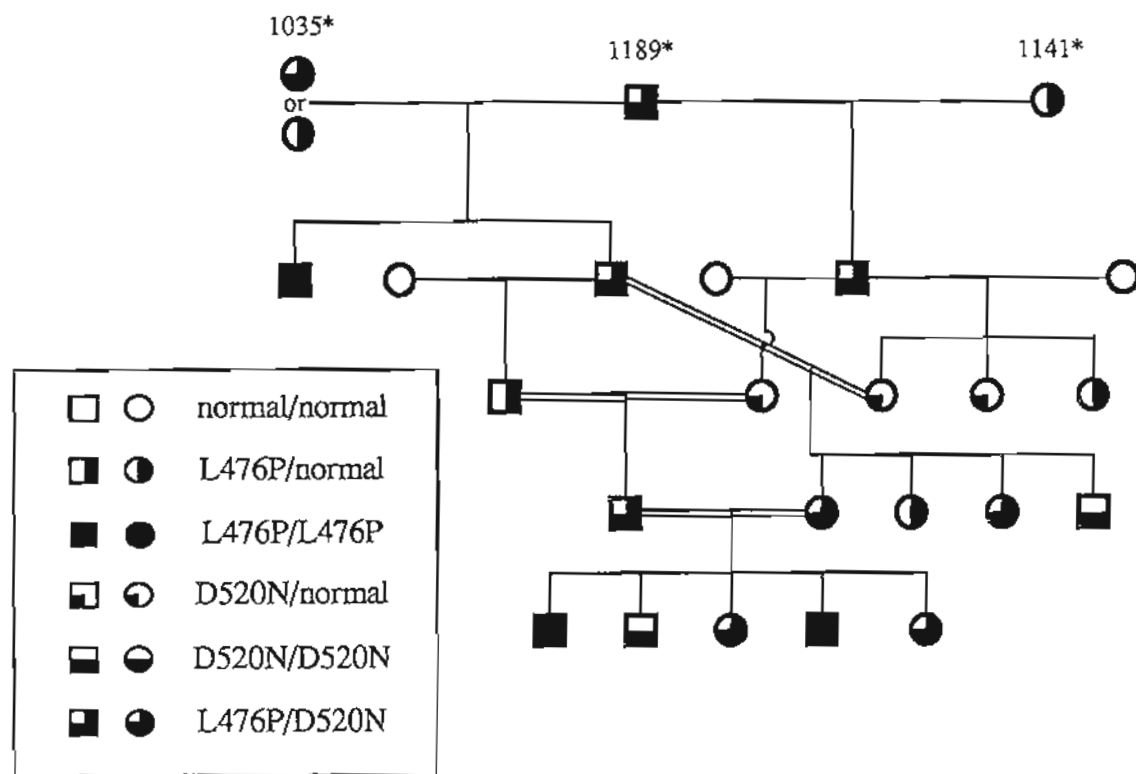


Figure 6.2: Pedigree of the MPS VI cat colony - origin of the L476P and D520N mutations.

Pedigree of the MPS VI cat colony showing origin of both the L476P and D520N alleles in founder heterozygote cats (asterisked) imported from the USA. Identification numbers of these individuals are also shown. Males and females are represented by square and round symbols respectively. See figure for description of symbols which represent the six possible genotypes within the colony. Severely affected MPS VI cats are represented by fully shaded symbols (L476P/L476P). Double parallel lines indicate consanguinity.



6.3.2 Enzymology

L476P/L476P, L476P/D520N and D520N/D520N genotyped cats all had significantly lower leukocyte 4S/ β hex ratios than normal/normal, L476P/normal and D520N/normal cats (Table 6.1). Leukocyte 4S/ β hex ratios in L476P/L476P and L476P/D520N cats were statistically different from D520N/D520N cats (ANOVA; $p < 0.001$ and Tukey-HSD at $p < 0.05$). L476P/normal and D520N/normal heterozygotes showed enzyme ratios approximately one third of normal/normal. The vast majority of cats were 2-3 months of age at the time of leukocyte collection for enzymology, except normal/normal cats, which were obtained from outside the colony and were usually adult at the time sampling. Additional normal/normal cats bred within the colony and sampled at 3 months of age had much lower leukocyte 4S/ β hex ratios (mean $\pm$ SD = 1.25 ± 0.41 , $n=5$). This adds support to previous observations that the 4S/ β hex ratio may alter with age, possibly due to changes in cell type within the white cell population (Viv Muller, unpublished observations). This may help to explain why L476P/normal and D520N/normal heterozygotes had mean ratios less than the expected half of normal/normal ratios.

Table 6.1: Peripheral leukocyte 4S/ β hex ratios in six different genotypes.

Genotype	4S/ β hex ratio	4S/ β hex ratio range	n
normal/normal	$2.32 \pm 0.99^*$	0.94 - 4.0	16
L476P/normal	0.874 ± 0.499	0.36 - 2.5	21
D520N/normal	0.831 ± 0.557	0.23 - 2.5	30
D520N/D520N	0.052 ± 0.047	0 - 0.19	29
L476P/D520N	0.028 ± 0.024^a	0 - 0.105	38
L476P/L476P	0.008 ± 0.008^b	0 - 0.018	15

* mean $\pm$ 1 SD; n number of cats in each group;

a $p < 0.05$, L476P/D520N vs D520N/D520N; b $p < 0.05$, L476P/L476P vs D520N/D520N

6.3.3 Urinalysis

Total urinary GAG values for each genotype decreased with increasing age. Values from L476P homozygotes were significantly higher than the remaining genotypes (Table 6.2). Mean total GAG in L476P/D520N and D520N/D520N cats was higher than D520N/normal cats at 20 days of age, however this was not statistically significant, probably due the moderate standard deviations and due to the low numbers of animals tested. At 90 days, mean total GAG from both L476P/D520N and D520N/D520N cats was significantly higher than in L476P/normal, and levels in D520N/D520N cats was also significantly higher than in D520N/normal cats (ANOVA; $p < 0.0000$ and Tukey-HSD at $p < 0.05$). In older cats there were no obvious differences in total urine GAG between different genotypes (except for L476P homozygotes), although smaller numbers of animals were examined (Table 6.2).

Table 6.2: Genotype differences in total urine GAG (mg/mmol creatinine) using the ABC method at different ages.

Genotype	20 days (mean $\pm$ sd)	n	90 days [†]	n	175 days	n	>330 days	n
normal/normal	- [†]	-	-	-	-	-	17 $\pm$ 6	6
L476P/normal	-	-	33 $\pm$ 6	11	15 $\pm$ 4	4	8	2
D520N/normal	115 $\pm$ 32	6	38 $\pm$ 14	20	17 $\pm$ 6	12	16 $\pm$ 5	5
D520N/D520N	137 $\pm$ 18	5	68 $\pm$ 22 ^a	11	24	2	16 $\pm$ 7	14
L476P/D520N	131 $\pm$ 31	7	52 $\pm$ 21 ^b	21	16 $\pm$ 3	4	16 $\pm$ 3	6
L476P/L476P	439 $\pm$ 97*	7	198 $\pm$ 47*	12	73 $\pm$ 21*	12	-	-

[†] samples unavailable

* L476P/L476P values at all ages statistically different from remaining genotypes

‡ due to insufficient numbers of animals at different ages in some genotype groups, statistical testing between different genotypes was only performed with the 90 day old groups.

a $p < 0.05$, D520N/D520N vs D520N/normal, and D520N/D520N vs L476P/normal

b $p < 0.05$, L476P/D520N vs L476P/normal

Total urine GAG was quantitated in a small number of animals using the more sensitive DEAE method. No differences in total urine GAG were apparent at two different ages between the genotypes examined (Table 6.3). However urine from greater numbers of animals would need to be analysed to confirm this, particularly due to the large range of values obtained from the D520N/D520N genotype at 90 days, resulting in a large standard deviation either side of the mean.

Table 6.3: Total urine GAG (μg uronic acid/mg creatinine) using the DEAE method in three different genotypes.

Age	90 days	150 days
	(mean $\pm$ sd)	
D520N/normal (n=5)	50 $\pm$ 8	21 $\pm$ 5
D520N/D520N (n=4)	47 $\pm$ 40	18 $\pm$ 3
L476P/D520N (n=4)	47 $\pm$ 15	17 $\pm$ 4

Following HRE of CPC precipitated GAG from L476P/L476P urine, a characteristic pattern was observed, as also seen in human MPS VI patients. Dermatan sulphate (DS) migrated in 2-3 bands: an intense, narrow band near the origin (DS1), and one or two less distinct bands (DS2, sometimes seen as a doublet) closer to the cathode end (Figure 6.3). The other major component was a broad chondroitin sulphate band (CS), and a heparan sulphate band (HS) became more obvious with increasing age.

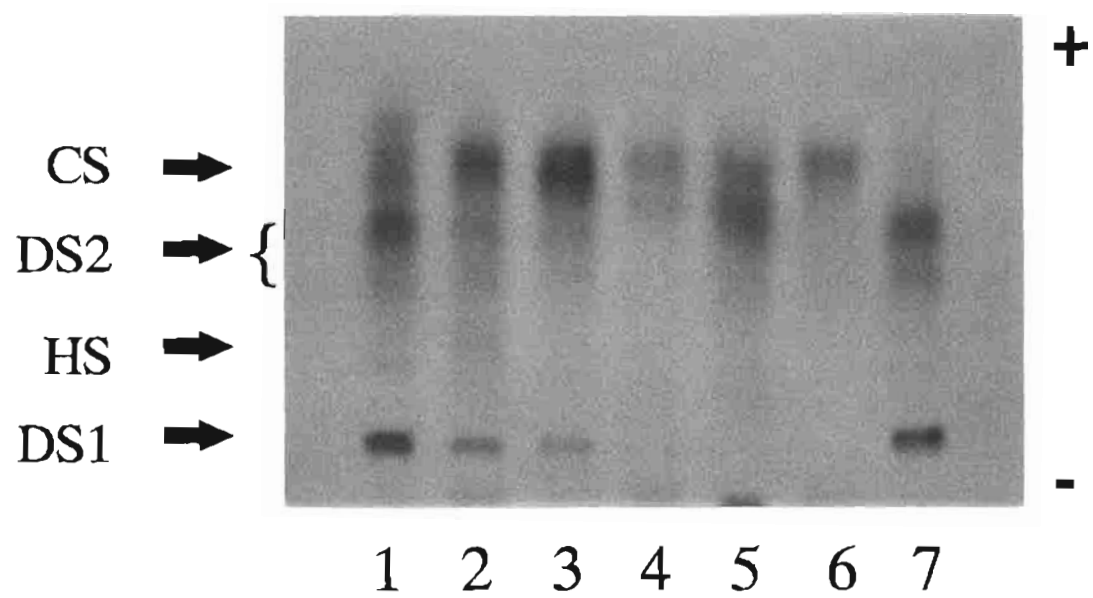
Clearly visible DS bands were seen in the majority of adult L476P/D520N and D520N/D520N cats (mean $\pm$ SD of %DS = 36 $\pm$ 6, and 32 $\pm$ 4, n=16 for both groups)(Figure 6.3), however the intensity of the bands were not as heavy as seen in L476P/L476P cats (%DS $\geq$ 60% in 6 month old cats and older). Very faint bands corresponding to DS were observed in over half of the normal/normal cats (mean $\pm$ SD of %DS = 17 $\pm$ 6, n=6), L476P/normal cats (mean $\pm$ SD of %DS = 23 $\pm$ 3, n=6) and

D520N/normal cats (mean $\pm$ SD of %DS = 18 ± 8 , n=4) cats. In several normal/normal, L476P/normal and D520N/normal cats, additional unidentified bands were present on HRE. Most of these bands migrated in a similar pattern to known bacterially contaminated human urines prepared in the same way (Wendy Norton, unpublished observation). These samples were excluded from further densitometric analysis.

Urine from large numbers of kittens (the majority at 2-5 weeks of age) was run on HRE, originally to screen for L476P homozygotes before other diagnostic methods had been developed. The genotype of most of these kittens was unknown, but most were either L476P/D520N or D520N/D520N, by deduction from parental genotype. Very faint DS1 bands were visible only in several genotyped L476P/D520N cats at 3 months of age. The lack of DS in most young L476P/D520N and D520N/D520N cats compared with adult cats with the same genotype is probably due to the higher proportion of CS excreted in young cats.

Figure 6.3: High resolution electrophoresis of urine GAG from six different genotypes.

HRE of urine GAG precipitated with CPC. Cellulose acetate plates were stained with Alcian Blue. Lane 1: Six month old L476P/L476P homozygote. All remaining urine samples were from adult cats (>18 months old). Lanes 2-6: L476P/D520N, D520N/D520N, L476P/normal, D520N/normal, and normal/normal genotypes respectively. Lane 7: 30 month old L476P/L476P homozygote with increased levels of DS1 and DS2 compared with the 6 month old L476P homozygote in lane 1. Dermatan sulphate (DS1 and DS2) bands were clearly visible at all ages in the L476P homozygotes, and were also present in the majority of adult L476P/D520N and D520N/D520N cats. Chondroitin sulphate (CS) was present in all genotypes, and heparan sulphate (HS) was faintly visible in some samples.



6.3.4 Leukocyte morphology

By light microscopy in L476P/L476P cats, the normal granules of eosinophils did not stain, with the granules appearing as empty vacuoles (Figure 6.4 A). Coarse basophilic cytoplasmic granulation was observed in neutrophils and basophils, and some lymphocytes contained vacuoles (Figure 6.4 A and B), as described previously (Alroy *et al.*, 1989). In both L476P/D520N and D520N/D520N cats, eosinophils and lymphocytes were normal (Figure 6.4 C and E). Abnormal neutrophil granulation was also present in these two genotypes, with intensity ranging from normal, or fine to very heavy basophilic granulation (Figure 6.4 C-F). Basophils also had variable numbers of abnormal basophilic cytoplasmic granules with variable staining intensity in both L476P/D520N and D520N/D520N cats (Figure 6.4 D and F), some with a similar appearance to those found in L476P/L476P cats and some almost normal. There was no obvious difference in leukocyte morphology between D520N/D520N and L476P/D520N genotypes. No abnormal leukocyte morphology was observed in normal/normal, L476P/normal (Figure 6.4 G and H), and D520N/normal cats (not shown).

Therefore, the presence of neutrophil granulation was unreliable for detection of L476P homozygotes at birth. However L476P/L476P cats were reliably and rapidly distinguished at birth by looking for the presence of non-staining eosinophils on blood films, as all cats were later confirmed to be L476P/L476P by genotype analysis. No discrepancies have occurred in a total of 65 L476P/L476P cats evaluated using both methods. Eosinophils stained normally in three severely affected human MPS VI patient blood films stained under the same conditions (PG, MF, KF). However, abnormal eosinophil staining was not observed in L476P/L476P blood films stained with Diff-Quik stain. This stain is not a true Romanovsky stain and also doesn't normally stain mast cells (Martin Copland, Veterinary Pathology Services, Adelaide, personal communication).

Corresponding to changes observed by light microscopy, and compared with ultrastructural detail from a D520N/normal cat (Figure 6.5 A) abnormal inclusions or altered specific granules were observed by electron microscopy in neutrophils, eosinophils and basophils in L476P/L476P cats (Figure 6.5 C), as described previously (Haskins *et al.*, 1979b; Alroy *et*

al., 1989). Similar, but fewer abnormal inclusions were observed in some neutrophils in L476P/D520N (Figure 6.5 B) and D520N/D520N cats (not shown), however eosinophils and basophils appeared normal. Some neutrophils in these two genotypes were normal or only contained 2 or 3 inclusions (Figure 6.5 B). No abnormalities were detected in basophils of L476P/D520N compound heterozygotes and D520N homozygotes, however only small numbers were examined.

Figure 6.4: Light microscopy of peripheral leukocytes in the different genotypes.

Light microscopy of peripheral leukocytes: e = eosinophil, n = neutrophil, l = lymphocyte, b = basophil. (A) L476P/L476P with an abnormal non-staining eosinophil, an abnormal neutrophil with heavy basophilic granulation in the cytoplasm, and an abnormal lymphocyte with several cytoplasmic vacuoles; (B) L476P/L476P with an abnormal basophil with intensely basophilic granules obscuring the nucleus, between an abnormal neutrophil showing heavy basophilic granulation, and an abnormal lymphocyte; (C) L476P/D520N, showing a normal staining eosinophil surrounded by five abnormal neutrophils with basophilic cytoplasmic granulation of variable intensity; (D) L476P/D520N, with an abnormal basophil showing intensely basophilic granules present in the cytoplasm, similar to those observed in L476P/L476P basophils, and an abnormal neutrophil containing basophilic granulation; (E) D520N/D520N, with a normal staining eosinophil between two abnormal neutrophils demonstrating variable basophilic granulation present in the cytoplasm (F) D520N/D520N with an abnormal neutrophil containing cytoplasmic basophilic granulation and an abnormal basophil with increased basophilic staining of some cytoplasmic granules; (G) normal/normal cat with a normal eosinophil, two normal neutrophils with clear cytoplasms, and a normal lymphocyte; (H) L476P/normal heterozygote, with a normal appearing basophil showing irregular but slight basophilic staining of the cytoplasm, a normal neutrophil with a clear cytoplasm, and a normal staining eosinophil; Original photos all taken at 400 × magnification (May-Grunwald Giemsa).

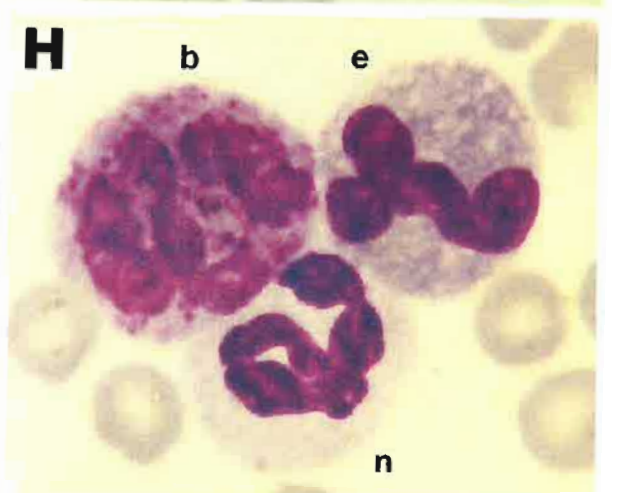
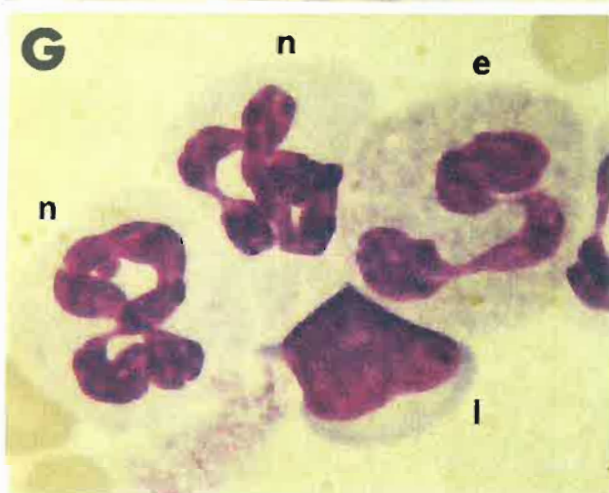
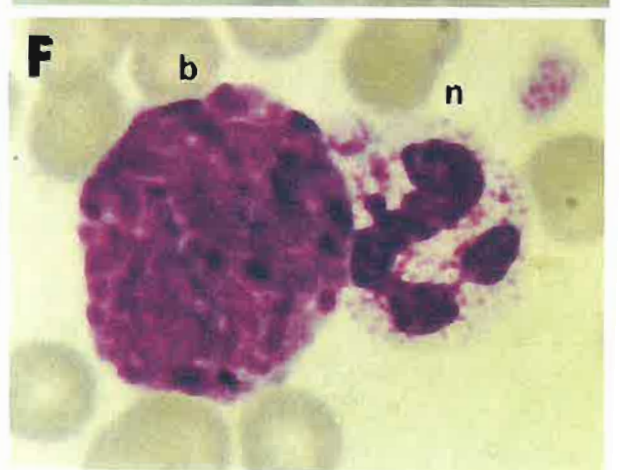
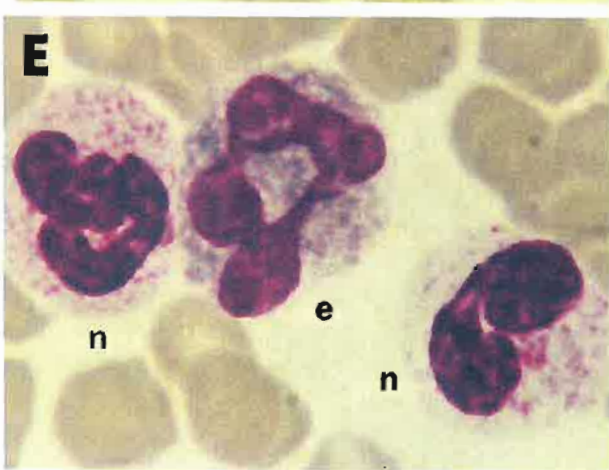
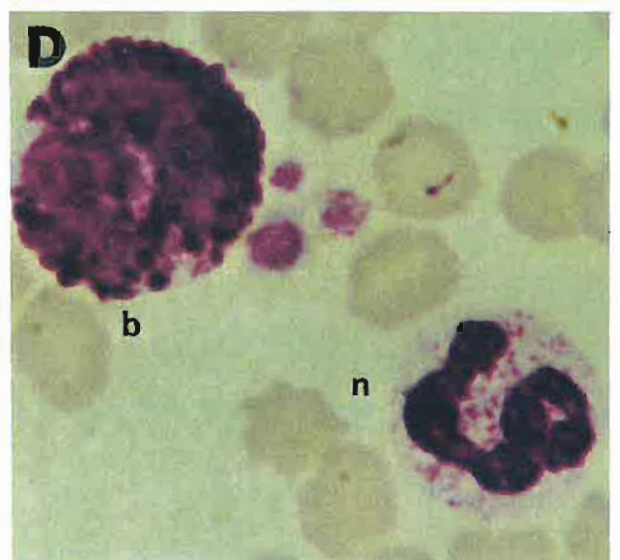
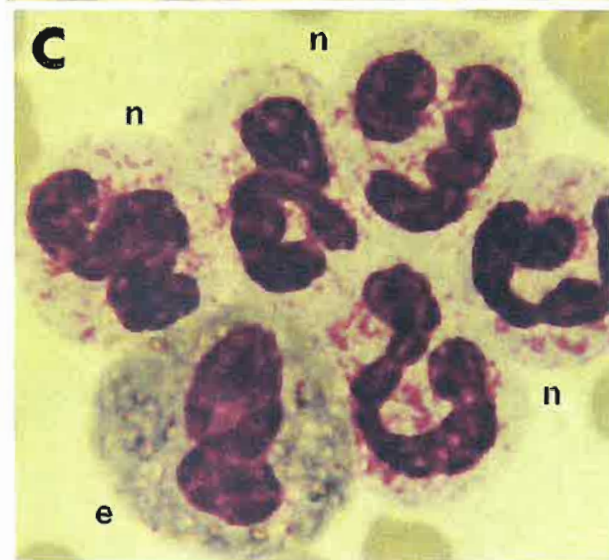
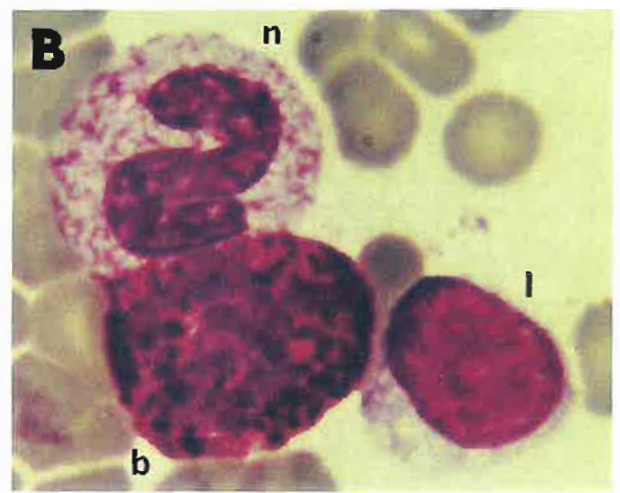
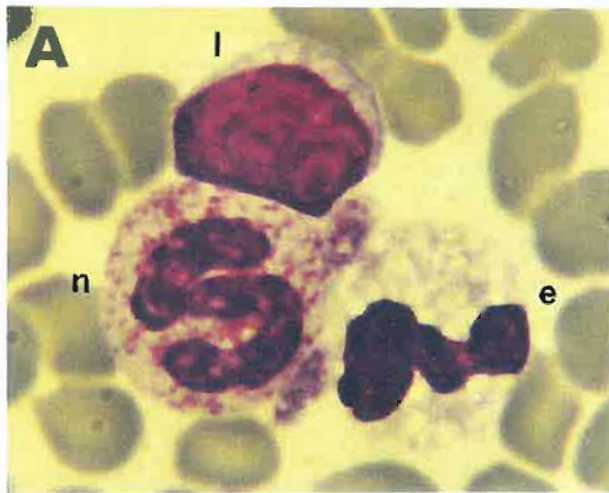
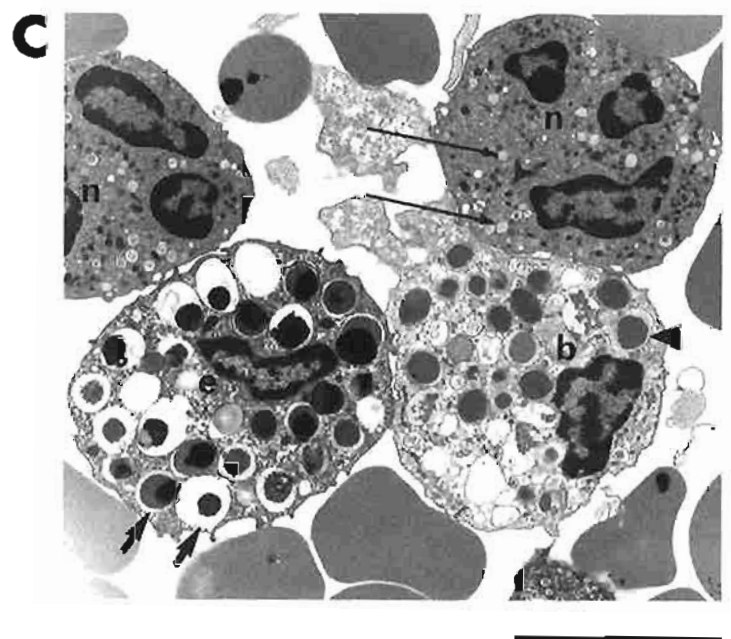
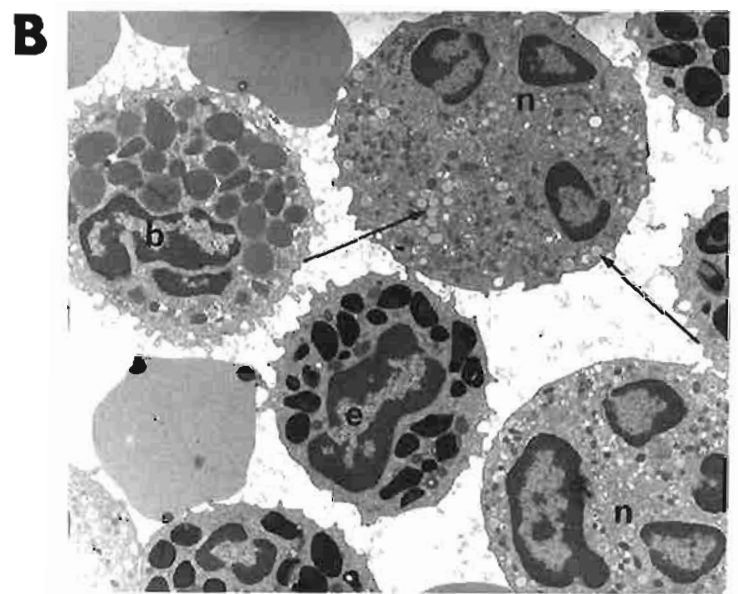
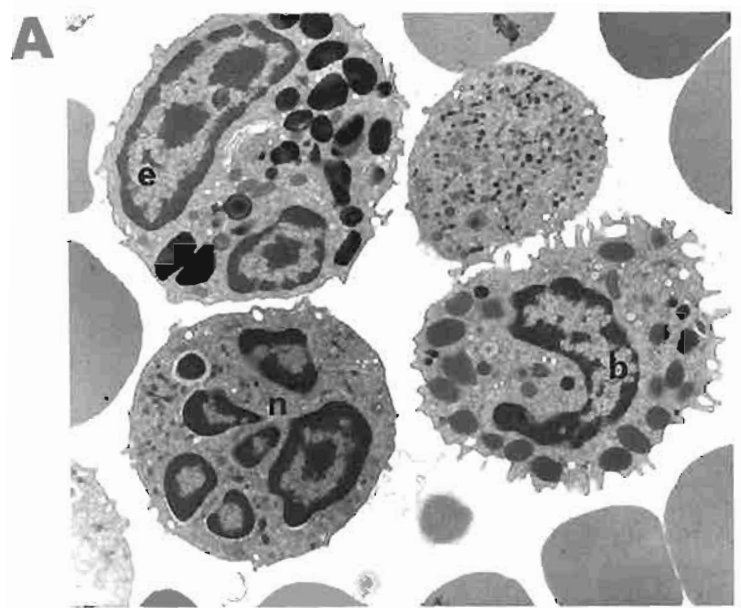


Figure 6.5: Electron microscopy of leukocytes in three different genotypes.

Ultrastructural detail of a neutrophil (n), eosinophil (e) and basophil (b) in: (A) a D520N/normal cat, (B) an L476P/D520N cat, and (C) an L476P/L476P cat. Small abnormal oval to round membrane bound inclusions, 0.2 - 0.5 μm in diameter, containing fine granular material are observed in neutrophils in the L476P/D520N and L476P/L476P genotypes (long thin arrows, B and C), which are not present in D520N/normal neutrophils in (A). These inclusions were not obvious in some L476P/D520N and D520N/D520N neutrophils. The eosinophil specific granules in L476P/L476P cats were severely altered, with either empty specific granules or containing variable amounts of granular or lamellar material (short thick arrows, C). Basophil specific granules in the L476P/L476P cats were also modified with fine fibrillar material at the periphery of the granules (arrowhead, C) or specific granules were empty or contained granular material. Ultrastructure of leukocytes in D520N/D520N cats was no different from L476P/D520N cats. Bar = 5 μm .



6.3.5 Clinical and radiographic examination

By slit lamp examination, from physical appearance and flexibility, and from bone dimensions from radiographs at all ages measured (3, 5, 6 months - data not shown; and greater than 18 months) L476P/D520N and D520N/D520N genotypes were indistinguishable from normal/normal, L476P/normal and D520N/normal cats, with no statistical significance between these groups for bone dimensions (Table 6.4). However L476P/L476P cats exhibited corneal clouding, progressive reduction in flexibility with increasing age (see Section 3.3.4), statistically significant reduction in dimensions of all bones measured at all ages compared with the five other genotypes (except tibial length at 90 days, data not shown)(Table 6.4), and characteristic skeletal changes observed radiographically as described previously (Section 3.3.4.6). These findings have been summarised in Table 6.7 together with additional observations outlined below.

Table 6.4: Mean bone dimensions from standardised radiographs in female cats greater than 18 months of age, with six different genotypes.

genotype	n	Ratio L5	Ratio C3	Patella	Tibial
		length/width	length/width	length (cm)	length (cm)
normal/normal	1	4.32	2.52	1.29	10.6
L476P/normal	5	4.46 ± 0.20*	2.61 ± 0.17	1.39 ± 0.08	10.88 ± 0.35
D520N/normal	3	4.23 ± 0.12	2.80 ± 0.27	1.20 ± 0.04	10.38 ± 0.10
D520N/D520N	8	4.44 ± 0.29	2.74 ± 0.20	1.28 ± 0.05 [‡]	10.68 ± 0.39 [‡]
L476P/D520N	12	4.09 ± 0.42	2.51 ± 0.19	1.30 ± 0.07	10.97 ± 0.36
L476P/L476P	4 [§]	2.57 ± 0.38 ^a	1.43 ± 0.17 ^a	1.08 ± 0.05 ^a	9.93 ± 0.22 ^a

n number of cats in each group; * mean ± 1 SD; ‡ n=7; § 3 of the 4 cats were only 1 year of age - see explanation Section 6.2.5;

a ANOVA; $p < 0.001$ and Tukey-HSD at $p < 0.05$, L476P/L476P vs L476P/D520N, D520N/D520N, and normal/normal, L476P/normal, D520N/normal combined.

On radiographs of L476P/D520N cats, bilateral degenerative changes were also observed in 64% of shoulder (scapulohumeral) joints (Table 6.5 and Figure 6.6 A-C). Changes observed included loss of smooth subchondral bone outline, mild to severe remodelling of the caudal aspect of the proximal humeral epiphysis, subchondral bone sclerosis, intraarticular calcification, and mild to severe osteophyte formation. The degree of severity of degenerative changes in the shoulder was quite variable and did not appear to be directly correlated with age. For example, moderate to severe remodelling was observed in 2 L476P/D520N cats that were only 1 and 1.2 years old, and only mild irregularities were observed in the subchondral bone of a 4.1 year old cat. The degenerative joint changes observed were not as severe as those seen in L476P homozygotes, which included almost complete loss of the proximal humeral epiphysis (Figure 6.6 D). Radiographs of the shoulder of five 5 to 6 month old L476P/D520N compound heterozygotes were also examined, and very mild subchondral bone irregularities were observed in 1 cat at 6 months. Mild remodelling changes of the proximal humeral epiphysis were present in only 1 (6%) 1.3 year old D520N/D520N of 18 examined with this genotype (Table 6.5). The genotype of this cat was confirmed from 2 separate blood samples. The remaining D520N/D520N cats appeared normal (Figure 6.6 E). No degenerative shoulder changes were observed on radiographs from 21 additional cats with normal/normal, L476P/normal and D520N/normal genotypes (Table 6.5).

A subset of L476P/D520N cats with degenerative shoulder joint changes also had generally less severe bilateral degenerative changes in the stifle (femorotibial) joints (20% of the total number of L476P/D520N cats examined)(Table 6.5). Remodelling was predominantly localised over the caudoventral aspect of the femoral condyles (Figure 6.7 A and B). Intraarticular calcification without evidence of other changes was observed in an additional three L476P/D520N cats, one D520N/normal and three L476P/normal heterozygotes, however this is a common feature of non-specific stifle joint disease in cats (Graeme Allan, Specialist Veterinary Radiologist, personal communication) so was not considered atypical. No stifle abnormalities were observed in D520N/D520N cats (Figure 6.7 C), normal/normal cats and the remaining L476P/normal and D520N/normal heterozygotes. Mild bilateral coxofemoral subluxation and remodelling of the femoral heads was also observed in one 1.2 year old L476P/D520N compound heterozygote. Radiographic changes of joints in L476P

homozygotes were severe and as described previously (Section 3.3.4.6)(Cowell *et al.*, 1976; Haskins *et al.*, 1979b).

Table 6.5: Evaluation of standardised radiographs for the incidence of degenerative joint disease in the shoulder and stifle of male and female cats, with six different genotypes.

Genotype	n	age range (years)	Shoulder pathology	Stifle pathology
normal/normal	1	3.5	none	none
L476P/normal	11	0.9 - 5.8	none	none*
D520N/normal	9	0.9 - 4.2	none	none [‡]
D520N/D520N	18	0.9 - 5.2	1/18 (6%)	none
L476P/D520N	25	0.9 - 6.7	16/25 (64%)	5/25 (20%) [§]
L476P/L476P	5	0.9 - 2.5	5/5 (100%)	5/5 (100%)

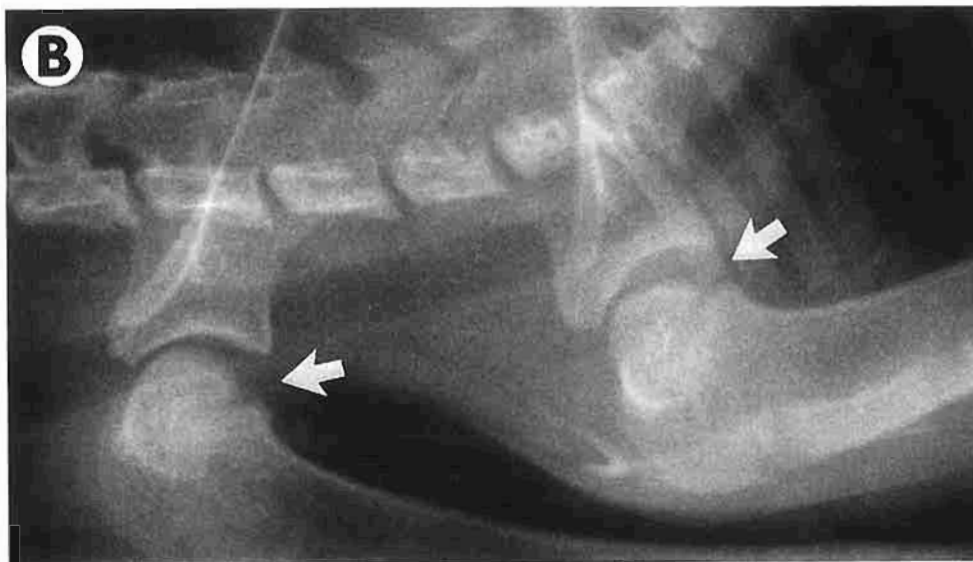
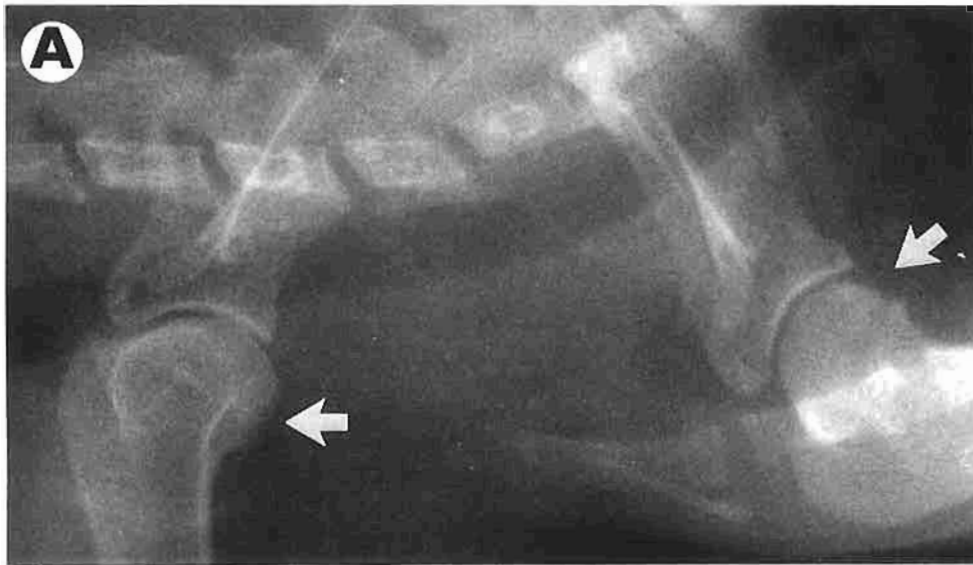
n number of cats in each group

* 3/11 had very mild intraarticular calcification; ‡ 1/9 had very mild intraarticular calcification

§ 3/25 additional cats had mild to moderate intraarticular calcification without obvious bone remodelling

Figure 6.6: Radiographs of the cervical spine and shoulder joints in adult cats with different genotypes.

Lateral radiographs of cervical spine and shoulder joints in adult cats (age in brackets): L476P/D520N cats with (A) mild (2.6 years), (B) moderate (1.2 years), and (C) severe (6.7 years) remodelling and loss of subchondral bone with mild to severe osteophyte formation in the caudal aspect of the proximal humeral epiphyses in the shoulder (arrows) and normal cervical spine; (D) an L476P/L476P cat (2.5 years) with severe degenerative shoulder changes (arrows), and short cervical vertebrae with irregular epiphyses; (E) a D520N/D520N cat (3.9 years) with normal shoulder joints and cervical spine.



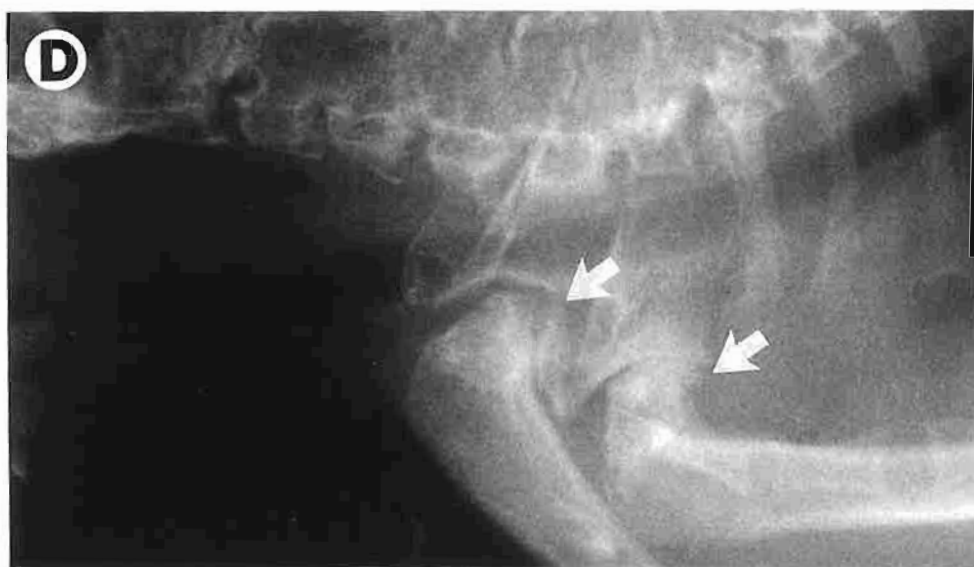
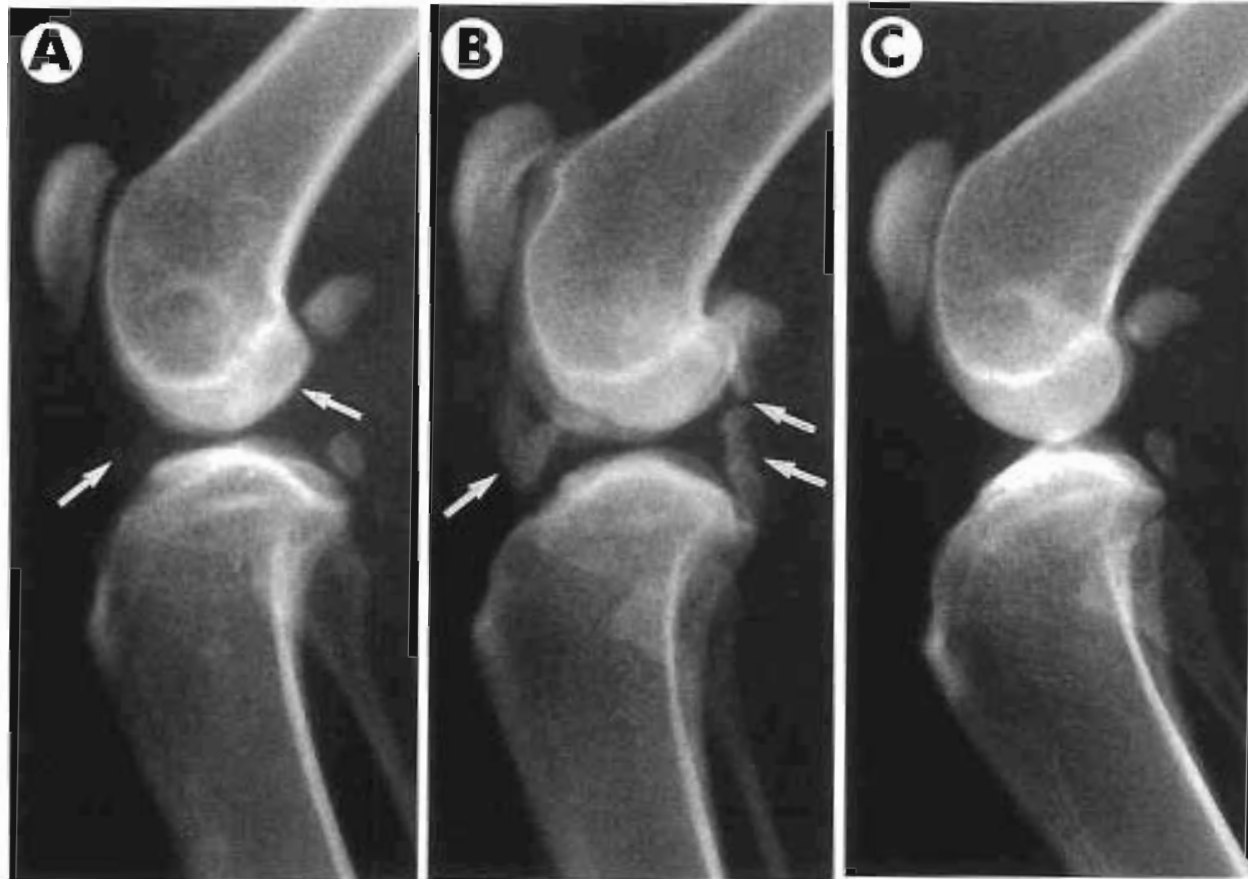


Figure 6.7: Radiographs of the stifle joint in adult cats with different genotypes.

Lateral views of the stifle joint in adult cats (age in brackets): (A) L476P/D520N (2.8 years) with moderate remodelling of the caudoventral aspect of the femoral condyles (arrow) and very mild intraarticular calcification (arrow); (B) L476P/D520N (5.5 years) with severe remodelling, osteophyte formation and intraarticular calcification (arrows); (C) No abnormalities were observed in the stifles of D520N/D520N cats (5.2 years).



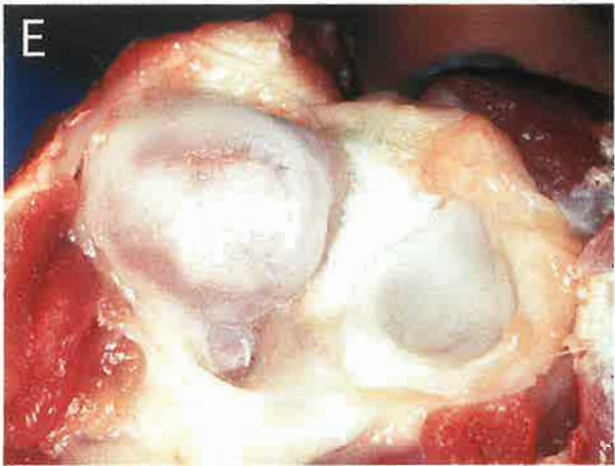
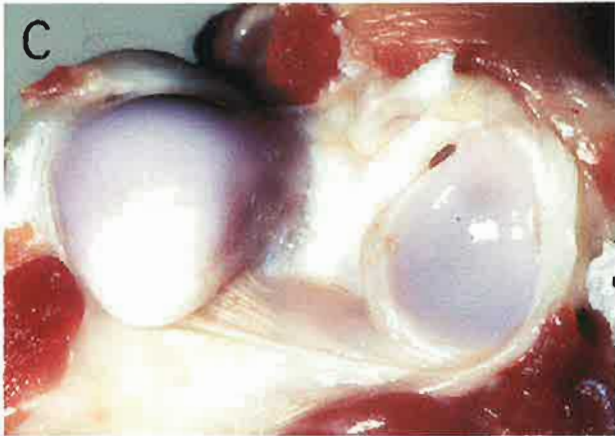
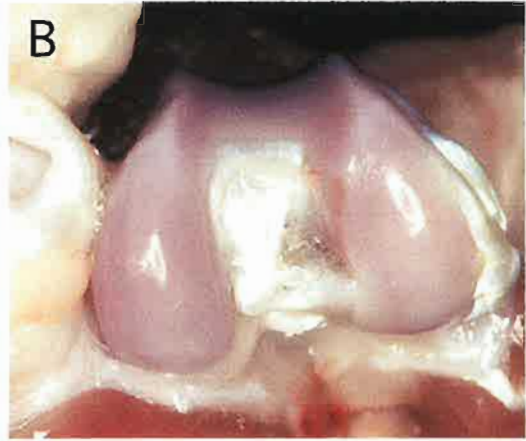
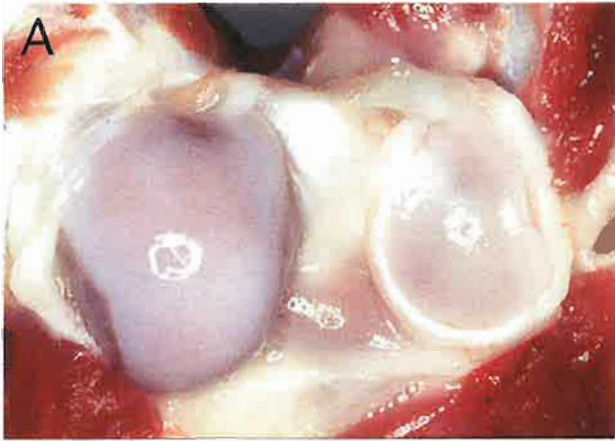
6.3.6 Postmortem joint examination

Degenerative joint changes observed on radiographs in L476P/D520N cats corresponded to severe abnormalities of the articular surface at postmortem in both the shoulder and stifle joints. There were variable extents of fibrillation, erosion and remodelling of the joint surfaces, particularly over the caudoventral aspect of the proximal humeral epiphysis and on the caudoventral aspect of the distal femoral condyles, with large osteophytes and/or loose bodies present in some joints (Figure 6.8 E and F). In one L476P/D520N, dissection into the stifle joint was made difficult by very extensive intraarticular calcification. Corresponding to normal joint appearance on radiographs, the articular surfaces in some L476P/D520N cats appeared normal (Figure 6.8 A and B). In several L476P/D520N cats with mild radiographic evidence of degenerative changes on the most ventrocaudal aspect of the humeral head (shoulders), at postmortem the articular cartilage appeared very white (i.e. thickened) and was mildly fibrillated in the same region (Figure 6.8 C). Very superficial cartilage fibrillation and erosion was observed in the stifles of several L476P/D520N cats which both had normal stifle radiographs (Figure 6.8 D) or mild to moderate intraarticular calcification without bone remodelling. The shoulder joints in a 30 month old L476P/L476P were more severely affected than any of the L476P/D520N cats, with more extensive remodelling, loss of epiphyseal bone and loss of articular cartilage (Figure 6.8 G). Stifle joint changes also appeared different in the 30 month old L476P/L476P cat, with very thick cartilage without any erosion on the caudoventral region of the distal femoral condyles, but with erosion around the intercondylar notch (not shown). The cartilage fibrillation and osteophyte formation was more severe in the remainder of the stifle joint of the L476P homozygote compared with the L476P/D520N cats.

No abnormalities were observed in the stifle joints of both D520N homozygotes, but unfortunately the shoulder joints were not examined.

Figure 6.8: Articular surfaces at postmortem.

Shoulder and stifle joint surfaces at postmortem: (A) normal articular surface in the shoulder joint of a 2.3 year old L476P/D520N (proximal humerus left, glenoid surface of the scapula on the right) and (B) normal appearance of the ventral aspect of the distal femoral condyles (stifle joint) in the same cat; (C) the shoulder joint of a 2.6 year old L476P/D520N showing increased thickness of cartilage on the most ventrocaudal aspect of the humeral head with mild fibrillation of some regions of the articular surface, and (D) the distal femoral condyles in the same L476P/D520N cat, showing fibrillation and superficial erosion of focal regions of the articular surface which was not visible radiographically; (E) the shoulder joint from a 6.7 year old L476P/D520N showing severe degenerative changes in articular cartilage with erosion, loose bodies and ventral osteophytes corresponding to the same regions on radiographs (see Figure 6.6 C); (F) caudoventral aspect of the distal femoral condyles (stifle joint) in a 5.5 year old L476P/D520N with severe focal erosion of articular cartilage, corresponding to the same regions on radiographs (see Figure 6.7 B); (G) shoulder joint from a 2.5 year old L476P/L476P with severe remodelling and loss of articular cartilage surface, and eburnation of subchondral bone, corresponding to the same regions on radiographs (see Figure 6.6 D).



6.3.7 Light and electron microscopy

Abnormal chondrocyte morphology was observed in rib, tracheal and articular cartilage samples from L476P/L476P, L476P/D520N and D520N/D520N cats, with changes seen most clearly in cross sections of rib cartilage. In rib sections from L476P/L476P cats at all ages, all chondrocytes showed severe lysosomal distension (Figure 6.9 A), with larger and more elongated superficial chondrocytes in the outer zone of the cartilage than chondrocytes in the inner layers (Figure 6.9 A and B). Fibroblasts extending into the perichondrium also had severe lysosomal storage. In 3 month old normal/normal (n=3), L476P/normal (n=2) and D520N/normal (n=2) cats, the superficial chondrocytes in approximately the first 2-4 cell layers were long and slender (Figure 6.9 C and D) with occasional chondrocytes showing single or multiple vacuoles, and chondrocytes in inner layers were broader and rounder, with increasing lipid and occasional vacuoles (Figure 6.9 C). In contrast, severe vacuolation and distension of superficial chondrocytes, similar to that seen in L476P/L476P cats was present in age matched L476P/D520N cats (n=3) (Figure 6.9 E and F). Abnormal but less severe vacuolation was also present in the same region in D520N/D520N cats (n=2) (Figure 6.9 G and H), but this was not uniform throughout the circumference of the rib, and chondrocyte morphology was almost normal in some areas of the superficial zone. In both L476P/D520N and D520N/D520N cats, the degree of vacuolation decreased to normal levels in the inner cartilage zones, and by about 5-6 cell layers, chondrocytes appeared normal. In addition, large extracted glycogen lakes were observed in some chondrocytes from all genotypes, predominantly in the inner cartilage zones.

The severity of vacuolation and distension of the superficial chondrocytes appeared to alter with age, and was most severe at approximately 3 months. In adult L476P/D520N compound heterozygotes, the most superficial rib chondrocytes mostly appeared normal, however distended vacuolated chondrocytes were still present in slightly deeper layers. These changes were present minimally in 6 month old D520N homozygotes. Rib from adult D520N homozygotes was not available. The location and severity of abnormal vacuolation in rib sections of the different genotypes is summarised in Table 6.6.

Table 6.6: Location and severity of lysosomal inclusions in rib cartilage sections of 3 month old cats with six different genotypes.

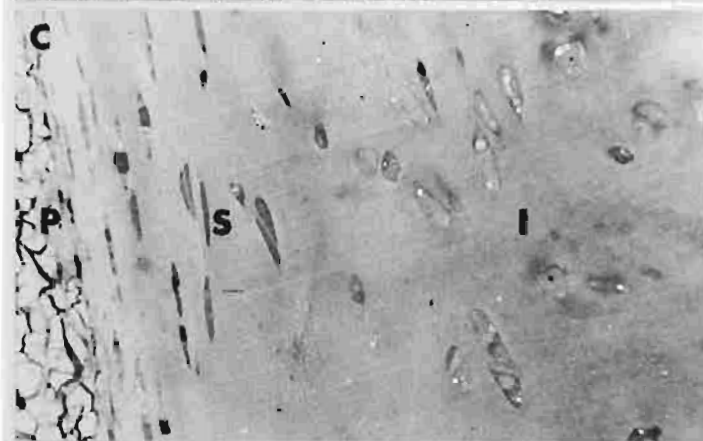
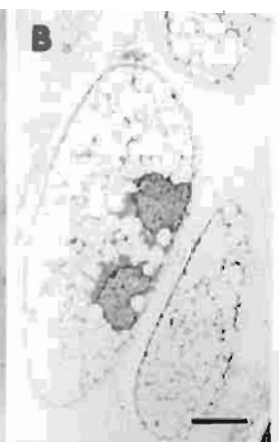
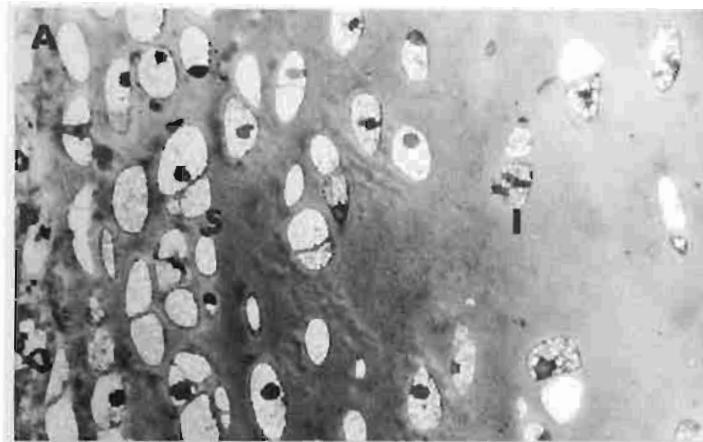
Genotype	n	Perichondrial Cells	Superficial Chondrocytes (superficial zone)	Mature Chondrocytes (inner zones)
normal/normal	3	normal	normal*	normal
L476P/normal	2	normal	normal	normal
D520N/normal	1	normal	normal	normal
D520N/D520N	2	normal	mild to moderate	normal
L476P/D520N	3	normal	moderate to severe	normal
L476P/L476P	2	severe	severe	severe

n number of cats in each group

* some inclusions in these cells are normal

Figure 6.9: Light and electron microscopy of rib cartilage in 3 month old cats from different genotypes.

Rib cartilage sections from 3 month old cats: (A, C, E, G) Toluidine Blue stained 1 μm thick sections orientated with the perichondrial connective tissue (P) on the left, superficial cartilage zone (S) middle, and inner cartilage zones on the right (I), original magnification 400 $\times$; (B, D, F, H) Electron micrographs show the corresponding ultrastructure of chondrocytes in the superficial zones of the rib cartilage (magnification constant, Bar = 5 μm). (A, B) severe lysosomal storage and distension of all chondrocytes in all zones and fibroblasts in the perichondrial connective tissue was observed in L476P/L476P cats; (C, D) minimal vacuolation was present in chondrocytes in L476P/normal heterozygotes (D520N/normal and normal/normal not shown) with occasional chondrocytes showing single or multiple vacuoles and increasing lipid towards the inner zone; (E, F) severe distension and lysosomal vacuolation was observed in chondrocytes in the superficial zone in L476P/D520N, and to a slightly lesser degree in D520N/D520N cats (G, H), however the degree of vacuolation decreased to normal levels in the inner cartilage zones (E, G).



The pattern of abnormal chondrocyte morphology in tracheal cartilage in L476P/L476P, L476P/D520N and D520N/D520N cats compared with normal/normal cats was similar to that seen in rib sections, however the pattern and severity of abnormal chondrocyte morphology was different in articular cartilage. In normal/normal, L476P/normal and D520N/normal articular cartilage at any age, only very occasional isolated vacuoles were observed in chondrocytes (Figure 6.10 A). In articular cartilage from L476P/L476P cats, all chondrocytes were increased in size and completely filled with lysosomal vacuoles (Figure 6.10 B). In 3 month old L476P/D520N cats, abnormal but overall much milder chondrocyte vacuolation was observed than in age matched L476P/L476P cats, with both normal and completely vacuolated chondrocytes scattered throughout all depths of the articular cartilage with no apparent pattern to the degree of vacuolation (Figure 6.10 C; Figure 6.11). Similar but milder changes in abnormal morphology were observed in age matched D520N/D520N cats (Figure 6.10 D).

Some adult L476P/D520N compound heterozygotes and D520N homozygotes had very occasional fully vacuolated chondrocytes, however in one adult L476P/D520N compound heterozygote, all chondrocytes appeared fully vacuolated. This cat also had severe degenerative joint changes, and the cartilage sample was taken from regions of articular cartilage that were grossly normal.

One 6 month old L476P/D520N out of four examined, appeared to have mild Kupffer cell storage in the liver (Figure 6.12). Apart from this, no obvious storage was observed in non-cartilage connective tissues from L476P/D520N, D520N/D520N, L476P/normal and D520N/normal cats of various ages compared with normal/normal cats. Tissues examined included liver, skin, heart valve, aorta, cornea and hip joint capsule. Spurious results due to mitochondrial changes were initially obtained from tissues that had undergone mild autolysis. So care was taken subsequently to quickly place tissues in fixative, and to distinguish between lysosomes (single membrane bound) and mitochondria (double membrane) ultrastructurally.

Figure 6.10: Light microscopy of articular cartilage sections from different genotypes.

Toluidine Blue stained 1 μm thick sections of articular cartilage sectioned from the articular surface (top) towards the subchondral bone (bottom) in 3 month old cats (A) normal chondrocytes from a D520N/normal heterozygote with almost no cytoplasmic vacuoles present; (B) L476P/L476P homozygote, with severe lysosomal vacuolation and distension of all chondrocytes throughout all regions of the articular cartilage; (C) L476P/D520N compound heterozygote with chondrocytes exhibiting no vacuoles (normal), mild vacuolation and complete vacuolation in various regions of the cartilage matrix; (D) D520N/D520N homozygote with very mild vacuolation in some chondrocytes. Original magnification 400 $\times$.

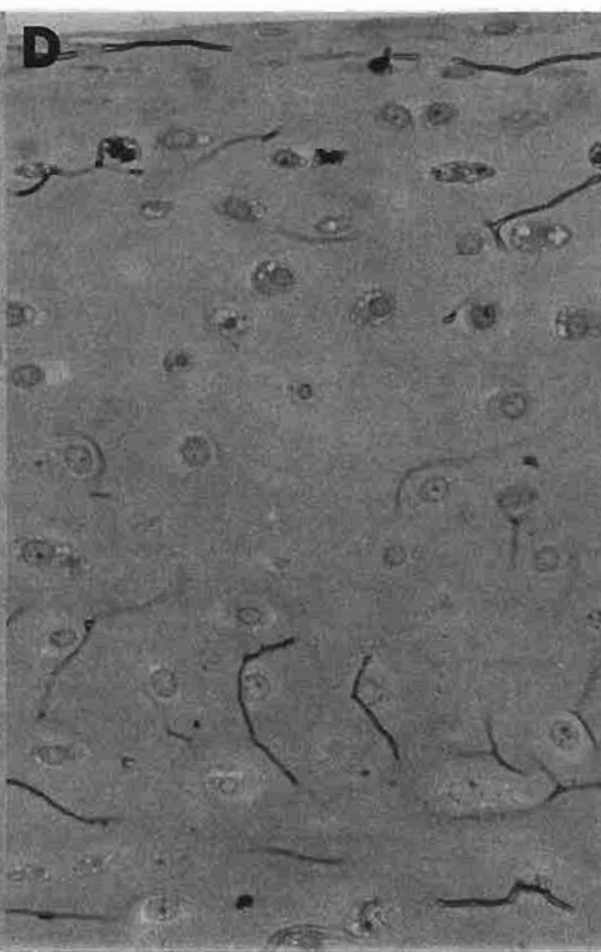
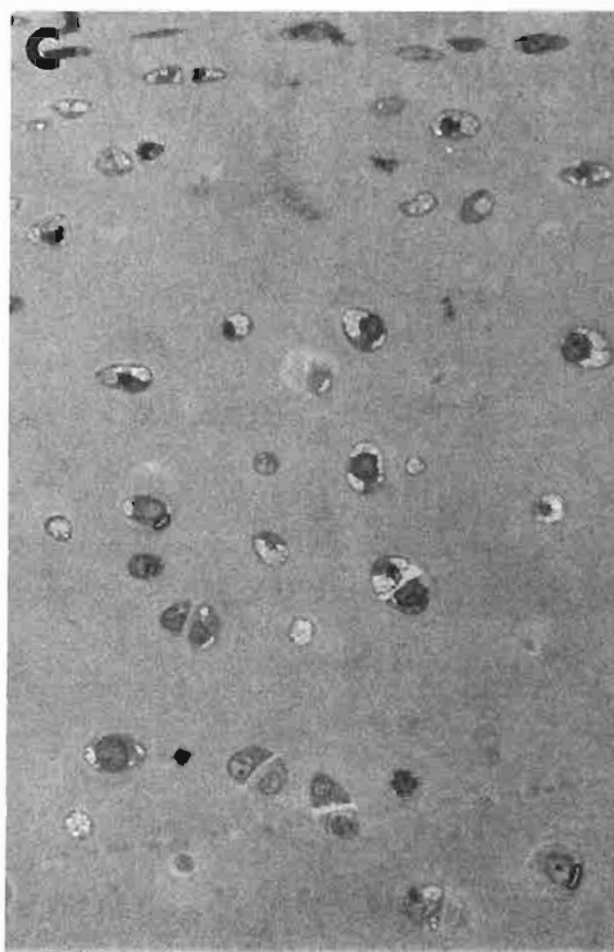
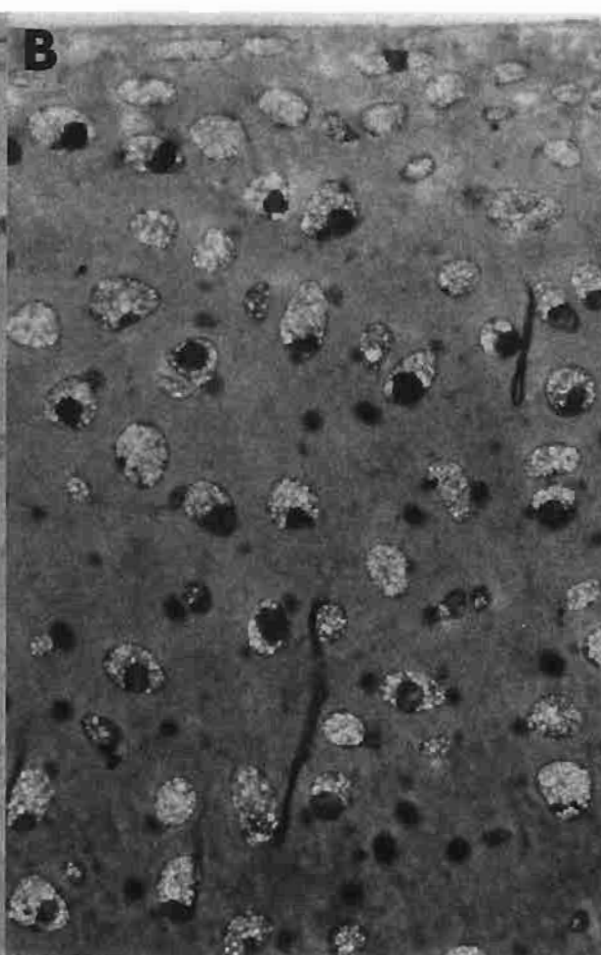
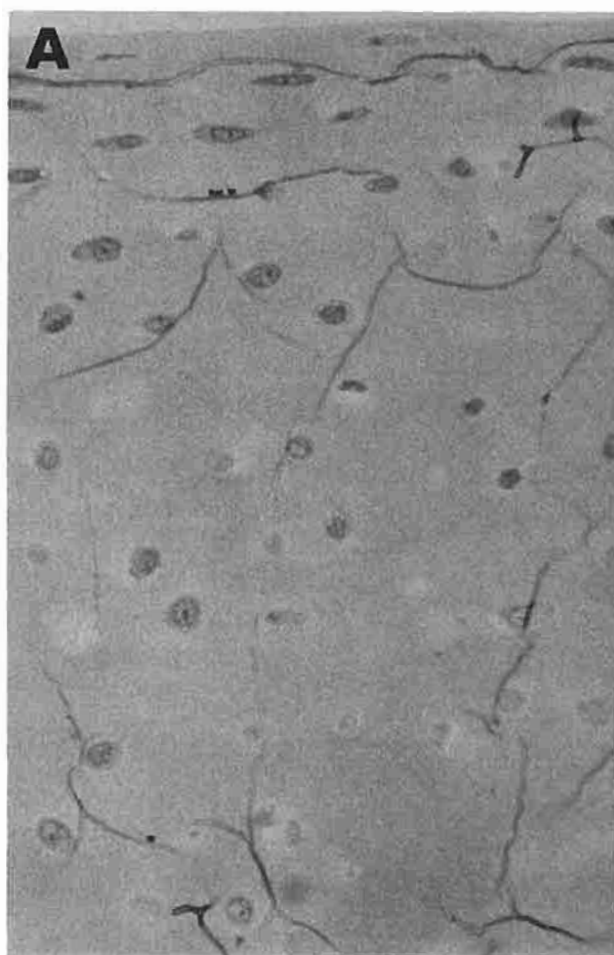


Figure 6.11: Electron microscopy of articular cartilage from an L476P/D520N compound heterozygote.

Ultrastructural detail of articular cartilage chondrocytes from a 3 month old L476P/D520N compound heterozygote illustrating chondrocytes with variable degrees of vacuolation. Normal chondrocytes contain only occasional vacuoles. Empty membrane bound vacuoles appear identical to those observed in L476P/L476P chondrocytes (see Figure 4.7). Bar = 5 μm .

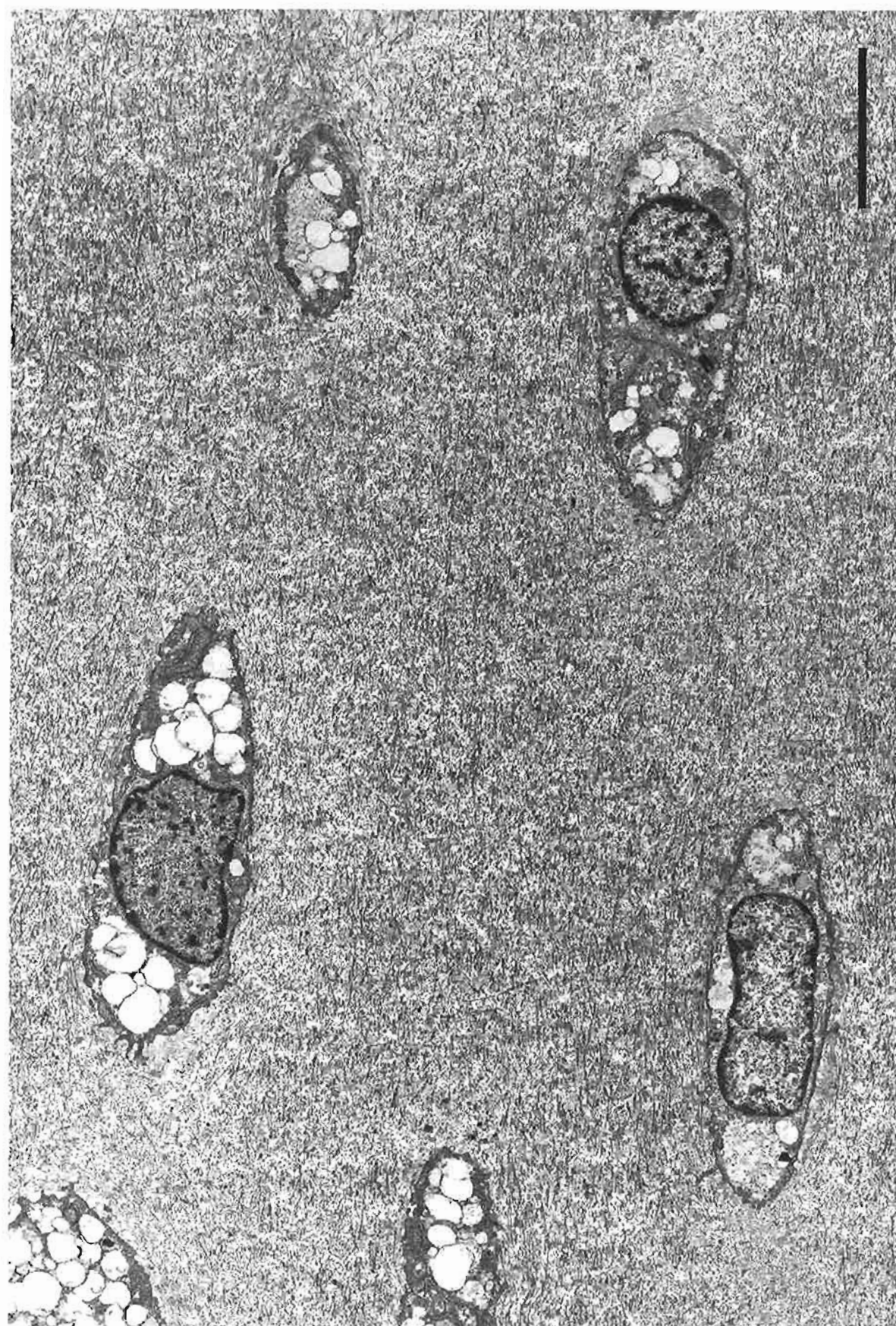


Figure 6.12: Electron microscopy of storage in Kupffer cells from an L476P/D520N compound heterozygote.

Empty membrane bound vacuoles (thick arrows) were present in some Kupffer cells in liver samples from a 6 month old L476P/D520N compound heterozygote. These vacuoles appeared similar to those observed in an untreated L476P/L476P homozygote (see Figure 4.4 A) and were therefore assumed to be distended lysosomes containing undegraded GAG. Normal lipid inclusions were also observed (thin arrows) and were clearly distinguishable from lysosomal inclusions. Some Kupffer cells without abnormal inclusions were also observed. Bar = 5 μ m.

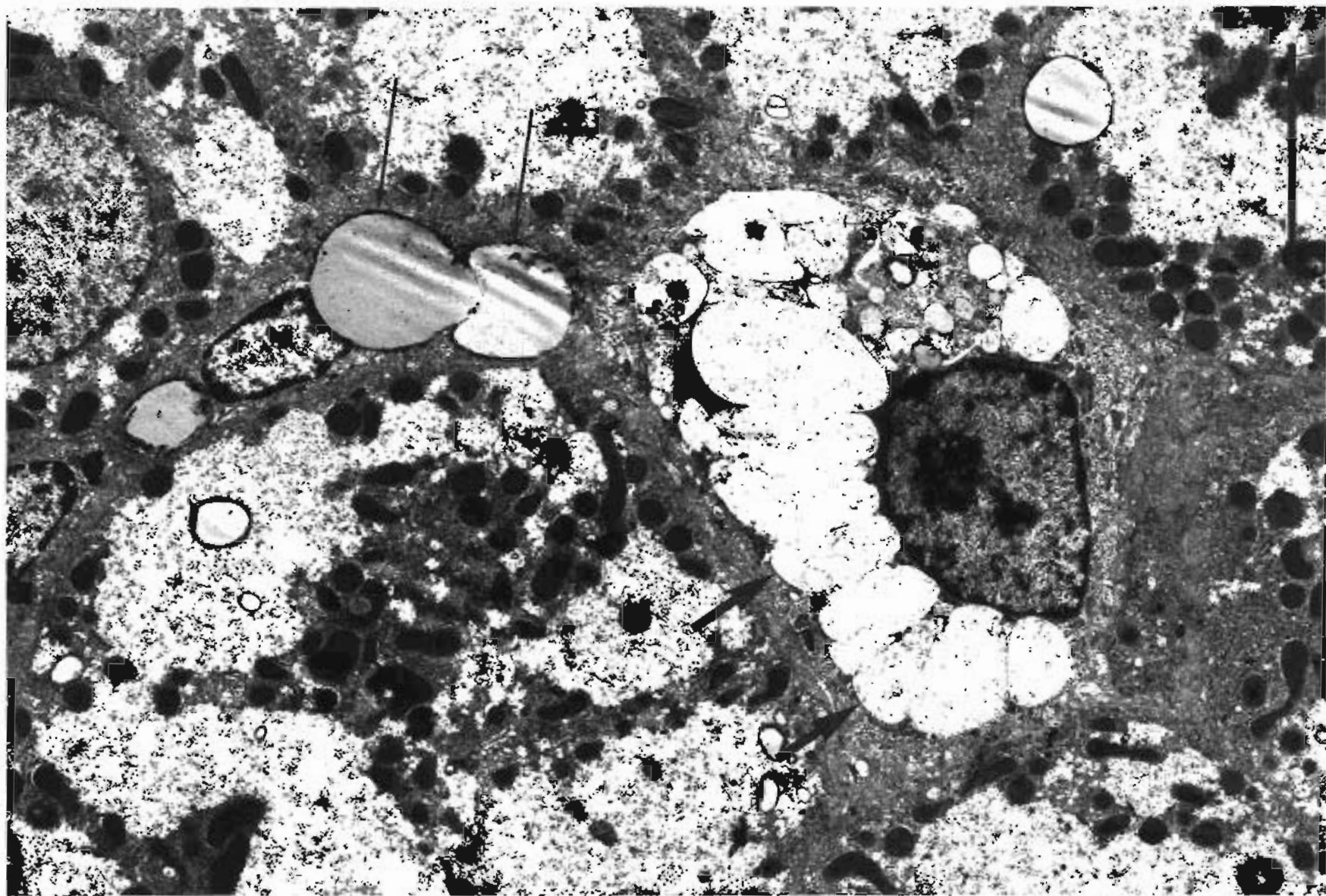


Table 6.7: Summary of feline MPS VI genotype and resulting phenotype.

Genotype	Mean white cell 4S/βhex ratio (% normal ± 1SD)	DSuria (× normal)	Skeletal growth	Joint disease	Corneal clouding	Chondrocyte inclusions	Connective tissue inclusions	White cell inclusions
L476P/L476P	0.008 (0.3 ± 0.3 %)	>3×	reduced	severe	mild	severe	widespread	neutrophils basophils eosinophils lymphocytes
L476P/D520N	0.028 (1.2 ± 1.0 %)	~1.8×	normal	mild to moderate	clear	mild	± normal*	neutrophils ?basophils
D520N/D520N	0.052 (2.2 ± 2.0 %)	~1.6×	normal	± normal [‡]	clear	very mild	normal	neutrophils ?basophils

* 1/4 cats had lysosomal storage in Kupffer cells (liver); ‡ 1/18 cats had degenerative joint disease

6.4 Discussion

Soon after establishing the feline colony, it was evident that abnormally low leukocyte 4S/ β hex ratios were found in a number of apparently clinically normal animals, and only one clinically affected MPS VI cat was produced from early breeding attempts. Selection of heterozygote offspring from colony cats outbred with outside normal cats became difficult due to the wide variation in enzymology results obtained (Table 6.1). This eventually led to heavy selection of clinically normal cats with very low 4S/ β hex ratios as probable MPS VI heterozygotes, which in retrospect were both L476P/D520N and D520N/D520N genotypes. The expected incidence of 1 in 4 clinically affected MPS VI cats (L476P homozygotes) was not observed until identification of the L476P mutation, which identified both L476P/D520N compound heterozygotes and L476P/normal heterozygotes using PCR based mutation analysis. However a large range in leukocyte 4S/ β hex ratios was still obtained from these cats, which was only clarified when the D520N mutation was discovered.

Despite an outwardly normal physical appearance, L476P/D520N and D520N/D520N cats demonstrate biochemical and histological evidence of MPS VI disease, with abnormally low leukocyte 4S/ β hex ratios, mild DSuria, and lysosomal storage in chondrocytes and leukocytes (Table 6.7). Cultured fibroblasts from D520N/D520N and L476P/D520N cats show mild elevations in [^{35} S]-sulphate storage, and expression of the mutant D520N 4S in Chinese Hamster ovary cells results in normal amounts of a normal sized but unstable protein (Yogalingam *et al.*, 1998). Expression of the L476P allele resulted in low yields of an inactive 4S precursor protein (Yogalingam *et al.*, 1996). Lysosomal storage in only several cell types in L476P/D520N and D520N/D520N genotypes suggests that the residual 4S activity of this unstable protein is sufficient to prevent the lysosomal accumulation of DS in most tissues, compared with the extensive lysosomal storage in connective tissues in L476P/L476P cats.

The only apparent clinical outcome of these findings was the high incidence of degenerative joint disease in L476P/D520N cats (see summary in Table 6.7). The clinical and radiographic features of the joint disease in L476P/D520N cats were not characteristic of

other known causes of arthritis in cats including rheumatoid arthritis, periosteal proliferative polyarthritis and feline idiopathic polyarthritis (Bennett and Nash, 1988; Bennett and May, 1995; Graeme Allan, personal communication), and it was therefore concluded that these changes were attributable to the mutant 4S genotype. Excessive calcification observed in the stifle of one cat is also likely to be seen in hypervitaminosis A, however the subchondral bone changes and remodelling in both the shoulder and stifle of this cat were atypical and similar to the changes observed in the other L476P/D520N cats (Graeme Allan, personal communication).

Degenerative joint changes have been reported in several adult MPS VI patients (John Hopwood, unpublished observations), although to our knowledge, there is only one published report of severe arthritic changes in the hips and shoulders of a 27 year old MPS VI patient at postmortem (Keller *et al.*, 1987). Initial diagnosis in less severe MPS VI patients is often bilateral Perthes disease (Spranger *et al.*, 1970; Pilz *et al.*, 1979) and bilateral hip replacements have been performed in some patients (Pilz *et al.*, 1979). The lack of description of gross joint pathology may be due to the small numbers of MPS VI patients who survive long enough for possible degenerative joint changes to develop, and also the difficulty in obtaining joints for examination. Due to increased longevity of bone marrow transplanted MPS patients and identification of more patients with less severe phenotypes due to improved diagnostic methods, greater numbers of MPS patients are surviving for longer periods of time. New management problems with these patients are emerging, but the future course of their disease is largely unknown. Our results from the mild MPS VI cat model suggest that degenerative joint changes in MPS VI patients may be an important clinical problem, and that chondrocytes have the greatest 4S requirements of all cell types. This site of pathology should therefore be an important target for gene product or gene replacement therapies. In addition, in the light of results from L476P/D520N and D520N/D520N cats, it would also be of interest to systematically screen younger patients with degenerative joint disease of undetermined cause, for qualitative changes in urinary GAG excretion.

A wide range in disease severity is observed in human MPS VI patients, ranging from dwarfism with death usually in the second decade (Whitley, 1993; Neufeld and Muenzer, 1995), to less severely affected patients with almost normal skeletal growth and lifespan

(Quigley and Kenyon, 1974; Pilz *et al.*, 1979; Paterson *et al.*, 1982; Tonnesen *et al.*, 1991). The same degree of phenotypic variation in MPS animal models has not previously been described, with similar disease severity observed in MPS VI cats found in apparently unrelated Siamese cats (Haskins *et al.*, 1979b; Breton *et al.*, 1983), long-haired Siamese (Di Natale *et al.*, 1992), and domestic long-haired (Beekman, 1993) cat breeds from different locations including Italy. Animals with a severe phenotype are more likely to be identified than those with subtle clinical changes such as in L476P/D520N compound heterozygotes.

The availability of a feline MPS VI model with both mild and severe phenotypes will enable studies to better understand the molecular, biochemical and histological basis for genotype to phenotype correlations. A better understanding of this correlation has been important in patient diagnosis, prognosis, management and evaluation of therapies. In particular, knowledge of the genotype to phenotype relationship has been invaluable in assessing efficacy of bone marrow transplants (Hopwood *et al.*, 1993). MPS VI patients have less than approximately 10% residual 4S activity in leukocytes and fibroblasts compared with normal controls (Hopwood *et al.*, 1986; Jin *et al.*, 1992; Litjens *et al.*, 1996), and recent studies have demonstrated some correlation between this 4S activity, genotype and disease phenotype (Brooks *et al.*, 1991b; Wicker *et al.*, 1991; Litjens *et al.*, 1996). When each feline 4S genotype was examined as a group (all with a fairly uniform genetic background), there was a clear correlation between marginal increases in leukocyte 4S activity, genotype and disease phenotype, however it would not have been possible to determine the same relationship from individual animals.

As stated in previous chapters, the pathogenesis of abnormal bone growth typical of most of the MPS types is unknown, however the characteristic severe lysosomal storage in chondrocytes indicates that they are closely implicated in this abnormal process. The severity and extent of lysosomal inclusions in chondrocytes in the three abnormal genotypes closely correlated with abnormal skeletal growth and joint disease, resulting in three different phenotypes: L476P/L476P genotype with severe skeletal and joint disease, L476P/D520N with normal skeletal appearance but moderate joint disease, and D520N/D520N with both normal skeletal appearance and normal joints. This suggests that partial correction of storage in chondrocytes is sufficient for skeletal growth to proceed normally, however morphology of osteogenic cells was not examined in L476P/D520N and

D520N/D520N cats. It also suggests that if leukocyte 4S/ β hex ratios directly reflect the enzyme levels in other cell types, then theoretically only small increases in enzyme activity in cell types involved in bone growth would lead to normal skeletal growth. Evaluation of chondrocyte and osteogenic cell morphology in growth plates from L476P/D520N and D520N/D520N genotypes may provide valuable insights into GAG turnover or 4S requirements in various regions of the growth plate, as assessed by the degree of lysosomal vacuolation. This may assist in understanding the pathogenesis of the skeletal disease in L476P homozygotes.

Lack of correction of chondrocyte pathology and persistence of degenerative joint disease in L476P/L476P cats which underwent ERT (Chapter 5), and negligible levels of enzyme in cartilage in distribution studies (Chapter 4) is evidence of poor diffusion of rh4S through cartilage matrix, with insufficient levels of enzyme reaching chondrocytes. Mannose-6-phosphate receptor mediated correction of storage in cultured chondrocytes indicates that chondrocytes have the appropriate cell surface receptors for enzyme uptake once sufficient enzyme levels are available (Fillat *et al.*, 1996). There is a need to improve targeting of therapies to chondrocytes, and the mild MPS VI models in L476P/D520N and D520N/D520N cats provide the opportunity to study this cell type *in vivo* in isolation from other pathologies. Following targeting of enzyme to chondrocytes, more rapid changes in the degree of lysosomal vacuolation is anticipated in these genotypes due to lower levels of intracellular storage, which will assist in development of these techniques.

The gradual reduction in severity of vacuolation in chondrocytes towards the inner zones in rib cartilage in L476P/D520N compound heterozygotes and D520N homozygotes presumably reflects reduction in GAG metabolism and hence storage. Reduced chondrocyte metabolism and different matrix GAG types and concentrations are possible reasons for the different pattern and degree of vacuolation of chondrocytes observed in articular cartilage, however this is purely speculative.

Chondrocytes are normally responsible for production and continued maintenance of the surrounding cartilage matrix. Cartilage is composed of a meshwork of predominantly type II collagen fibrils and proteoglycans that interact to form a matrix that can normally withstand mechanical stresses. Mutations in the type II procollagen gene have been shown to cause

primary osteoarthritis in humans (Ala Kokko *et al.*, 1990) and transgenic mice (Helminen *et al.*, 1993). Thinner type II collagen fibres associated with osteoarthritis has also been observed in transgenic mice expressing abnormal type IX collagen (Nakata *et al.*, 1993) adding to the evidence that type IX collagen plays a role in regulating type II collagen fibrillogenesis and matrix integrity (Diab, 1993).

In vitro inhibition of type II collagen fibrillogenesis by the small DS containing cartilage proteoglycan decorin, decorin core protein, and free CS GAG (Vogel *et al.*, 1984; Kuijjer *et al.*, 1985; Schonherr *et al.*, 1995) has led us to speculate that impaired DS degradation in L476P/L476P and L476P/D520N chondrocytes leads to abnormal extracellular matrix concentrations of either free GAG or small proteoglycans in the articular cartilage. This may then cause altered collagen fibrillogenesis and matrix structural integrity, resulting in degenerative joint disease. Abnormal subchondral bone almost certainly results in the more severe degenerative joint disease observed in L476P/L476P cats. Degenerative joint disease has also been described in MPS I and MPS VII dogs (Haskins *et al.*, 1984, 1991a; Shull *et al.*, 1982, 1984), and DS degradation is also impaired in both of these MPS types. Detailed ultrastructural examination of cartilage from L476P/L476P and L476P/D520N genotypes should clarify this hypothesis, and any differences may also help to improve understanding of the abnormal skeletal growth in L476P/L476P cats and may further knowledge of cartilage structure and factors that maintain normal matrix function.

Although an extensive pedigree was unavailable, Family 2 and Family 3 MPS VI cats were originally thought to be unrelated (Haskins *et al.*, 1979b). Our findings of the L476P allele in the American Family 2 and 3 MPS VI cats, and an Italian MPS VI cat from Siamese ancestry suggests a high incidence of this allele in the general Siamese cat population. In addition the presence of the L476P allele in Family 2 and Family 3 cats clarifies previous observations made in these two families (Haskins *et al.*, 1979b). Previously observed differences in residual 4S activity between the two families (Family 2 = Family b; and Family 3 = Family a; McGovern *et al.*, 1985) may have been due to phenotypic variation between cats or may have been due to methodological limitations. The lack of dimerization of partially purified liver 4S from Family 3/a heterozygotes is consistent with our observations with recombinant feline 4S which has only been observed to be present as a monomer (Yogalingam *et al.*, 1996). Both of these observations are in contrast to previous studies

where it was concluded that feline liver 4S was present as a homodimer (McGovern *et al.*, 1982). Preliminary molecular analysis of DNA and RNA from the Italian MPS VI cat using Southern and Northern blot analysis suggested a gross deletion or rearrangement of the 4S gene, although these findings were not conclusive (De Luca *et al.*, 1993). Identification of the L476P allele in this cat now clarifies these previous observations.

In one of the original feline MPS VI papers, in addition to describing MPS VI in three unrelated families of Siamese cats, a clinically normal Family 3 obligate heterozygote male with leukocyte 4S activities consistently in the affected range was also observed (Haskins *et al.*, 1979b). By examining the pedigrees presented in several papers (Haskins *et al.*, 1979b; McGovern *et al.*, 1985), this cat was identified as "190 male", and he was used extensively in further breeding. This cat had no radiographic features of MPS VI, no corneal clouding, and lacked excessive urinary GAG and coarse leukocyte granulation. Since the D520N mutation originated in our Family 3 heterozygote founder cats imported from the USA (Figure 6.2), it is likely that this cat was also an L476P/D520N compound heterozygote. The clinical findings reported were consistent with our observations of L476P/D520N cats, as some individuals had no abnormal radiographic changes, leukocyte granulation was variable and mild in some cats, and total urinary GAG was also indistinguishable from normal. Only one USA Family 3 L476P/normal heterozygote was tested by genotype analysis, therefore failure to detect the D520N allele was not surprising. Genotype analysis of more USA Family 3 cats is needed to determine if this mutation is still present within the original USA Family 3 colony. Further screening for the L476P and D520N alleles in other established MPS VI cat colonies, new feline MPS VI cases and Siamese cats in general would be helpful to clarify previous observations made in MPS VI cats and to establish if these mutations are common in the general Siamese cat population. A very useful and rapid screening method for L476P/D520N and D520N/D520N cats was the presence of obvious leukocyte inclusions on routine blood films despite a normal physical appearance.

The fact that the two mutant alleles are inherited independently rules out the possibility of the D520N allele contributing to the clinical variability observed in L476P homozygotes. In addition, detection of some phenotypic changes in L476P/D520N and D520N/D520N genotypes had no impact on the normal control groups used in the ERT studies in the previous chapter, as the majority of control animals were D520N/normal heterozygotes,

with data from L476P/normal heterozygotes also used occasionally. In any case, it appears that most data and samples from L476P/D520N and D520N/D520N cats could also be used in the control group, with the exception of cartilage, leukocytes and possibly also total urine GAG. Histomorphometric analysis of the L5 vertebra was also performed on several immature and adult L476P/D520N cats, and normal bone mineral volumes were observed, however the trabecular structure in one adult L476P/D520N appeared altered (Sharon Byers, unpublished observation). It is possible that subtle differences in bone structure may be present in either or both of these genotypes, and needs to be evaluated further.

By comparing the extent of lysosomal storage in tissues from L476P homozygotes with L476P/D520N compound heterozygotes, only small changes in enzyme levels dramatically affects the degree of lysosomal storage and disease outcome. Also, the phenotypic variation observed in L476P/L476P homozygotes may be due to environmental or other genetic factors affecting levels of 4S enzyme gene expression. Therefore, the apparent Kupffer cell storage in only one 6 month old L476P/D520N cat and radiographic evidence of degenerative joint disease in only one D520N/D520N cat may reflect the severe end of the disease spectrum in these two genotypes. It was unfortunate that the joints in the D520N/D520N cat were not examined at postmortem, however this cat was euthanased prior to identification of the D520N mutation.

Hip dysplasia is rare in cats (Kolde, 1974; Hayes *et al.*, 1979; Rabin *et al.*, 1994), but has been observed in young heterozygotes from one of the original MPS VI breeding colonies in the USA (Jezyk, 1981). No dysplasia was observed in 11 L476P/normal and 9 D520N/normal heterozygotes, but bilateral hip subluxation was present in 1 out of 25 L476P/D520N compound heterozygotes in our colony. It is speculative whether the hip dysplasia originally observed in "heterozygotes" (Jezyk, 1981) was in fact due to the D520N allele and occurred in L476P/D520N compound heterozygotes, however the low incidence in our colony suggests other genetic factors causing the hip dysplasia may have been involved in the original colony.

The presence of normal levels of total GAG at most ages, but mild DSuria and very low 4S activity in L476P/D520N compound heterozygotes and D520N homozygotes emphasises the importance of qualitative urinalysis to detect human MPS VI patients. Less severely

affected human MPS VI patients have previously been observed with normal total urine GAG, but DSuria following electrophoresis of urine GAG (Paterson *et al.*, 1982; Tonnesen *et al.*, 1991). In addition, a 44 year old patient (JW) has been observed with low level DSuria, leukocyte granulation characteristic of MPS VI, and very low residual 4S activity in cultured skin fibroblasts consistent with a diagnosis of MPS VI. However the only clinical problem was slight photophobia (John Hopwood, unpublished observations). This lack of clinical pathology despite biochemical evidence of MPS VI appears similar to our observations in D520N homozygote cats. It also suggests that leukocyte granulation as well as DSuria, is an important marker for biochemical evidence of MPS VI disease, even in the absence of clinical symptoms. Prominent leukocyte granulation is also observed in MPS VII.

Leukocyte ultrastructure in L476P/L476P cats appeared consistent with previous observations made in MPS VI cats, including those from "Family 3" (Haskins *et al.*, 1979b; Alroy *et al.*, 1989). However, eosinophils from clinically affected MPS VI cats have previously been reported to contain both eosinophilic and basophilic granules using a Wright-Giemsa stain by light microscopy (Alroy *et al.*, 1989), which is different from our observations of a complete lack of staining in eosinophils in L476P/L476P cats. The origin of the MPS VI affected blood is not stated and it may be possible that these cats had a different genotype resulting in slightly less severe modifications of the eosinophil specific granules. It is difficult to compare ultrastructural features in detail from this paper with the L476P/L476P cats described here to support this hypothesis. Alternatively, differences may be due to differences in stain preparations.

The lack of eosinophil staining observed in blood films of L476P/L476P cats is probably due to extreme modifications of the specific granules observed ultrastructurally. In normal cats, these granules consist of a densely osmiophilic crystalline core, appearing as concentric rings, surrounded by a less dense matrix (Ward *et al.*, 1972). The eosinophil major basic protein, which strongly binds to eosinophilic dyes due to charge, has been immunolocalised to the core of the eosinophil specific granule in humans (Peters *et al.*, 1986) and guinea pigs (Lewis *et al.*, 1978). GAGs have also been cytochemically demonstrated in immature eosinophil specific granules (Parmley *et al.*, 1982). Although the interactions between these two granule components are poorly defined, it seems probable that modification of the

GAGs in the L476P/L476P eosinophil granules, due to impaired GAG metabolism, affects the charge of the eosinophil major basic protein in the granule core and results in lack of binding by eosinophilic dyes.

Basophil specific granules contain mostly 4-sulphated chondroitin and DS in humans (Metcalf *et al.*, 1984) and guinea pigs (Orenstein *et al.*, 1978), therefore it is likely that the greatly increased basophilia observed in L476P/L476P basophils is due to increased storage of 4-sulphated GAG in the specific granules. It is also noteworthy that total arylsulphatase activity in different leukocyte cell types has been observed to vary greatly, with the highest activity observed in eosinophils, then basophils and then neutrophils, in an approximate ratio of 8:2:1 (Tanaka *et al.*, 1962). Essentially no activity was observed in lymphocytes. Values for each cell type were extrapolated from differential cell counts from patients with elevated leukocyte numbers. These observations correlate with the ultrastructural observations of the most severe modifications being present in L476P/L476P eosinophils.

6.5 Summary and conclusions

The origin of both 4S mutations conclusively originated in heterozygote cats imported from the USA, and may still be present in the natural cat population. Identification of the L476P allele in American and Italian MPS VI cats with Siamese origins suggests a high incidence of this allele in the general Siamese cat population. The L476P and D520N mutations were inherited independently and were therefore not the cause of variability observed in untreated L476P homozygotes. The clinical, histological and biochemical features of L476P/D520N and D520N/D520N cats indicates that residual enzyme activity in these genotypes is sufficient to prevent the severe MPS VI phenotype seen in L476P/L476P cats. Degenerative joint disease in L476P/D520N cats is probably solely due to consequences of impaired chondrocyte GAG metabolism. Understanding the pathogenesis of this process may provide new insights into cartilage matrix development and organisation. It also indicates that chondrocytes have perhaps the highest 4S requirement of any cell type in the cat, and that degenerative joint disease may be an emerging problem in longer lived MPS patients undergoing therapies. This should therefore be a focus for further developments in therapies. The possibility of MPS VI in human patients with normal physical phenotypes but with degenerative joint disease of unknown cause should also be considered.

7. Conclusions and future work

7.1 Introduction

At the outset of this study little was known about the efficacy of ERT in lysosomal storage disorders, and no ERT studies in MPS animal models had been published. The feline model of MPS VI was chosen as a good model for evaluation of ERT as pathology had previously been shown to closely parallel pathology in human MPS VI patients. In particular, the pattern of skeletal disease was similar in both feline and human MPS VI. In addition, the lack of CNS pathology in MPS VI removed the added difficulty of targeting enzyme across the blood brain barrier, which prevents access of large molecules to the CNS. Bone marrow transplantation as the current therapy in human MPS patients has in general been effective in alleviating soft tissue problems such as airway obstruction and joint stiffness. However only small improvements in long bone growth have been observed in some patients transplanted at a young age, and skeletal pathology has progressed essentially unchanged in these patients. This has lead to development of severe orthopaedic problems particularly involving the spinal column and pelvis, because transplanted patients are longer lived than untreated patients. In addition, a number of risks are associated with the transplantation procedure such as graft-vs-host-disease, which has a high morbidity and some mortality in transplanted patients.

The aim of this thesis was therefore to evaluate the efficacy of ERT with rh4S in the MPS VI cat as a possible alternative therapy for use in humans. The impact of ERT in particular on progression of skeletal pathology, and any adverse effects of therapy were also examined.

7.2 Summary

Enzyme distribution studies with rh4S in normal cats demonstrated enzyme activity in a number of tissues, although a large proportion was cleared by the liver and negligible activity was detected in cartilage and cornea. Preliminary low dose ERT studies in MPS VI

cats with established skeletal pathology indicated that ERT at these dose rates was unable to reverse or alter progression of skeletal disease. However subtle improvements in bone histomorphometric parameters in the cat treated from the youngest age suggested that early onset ERT may improve skeletal response to therapy. These studies also demonstrated that long term ERT had minimal side effects, although the efficacy of therapy may have been reduced in one cat which developed an elevated antibody titre against rh4S (Crawley *et al.*, 1996).

Pathology was observed in a mid-term MPS VI foetus and rapidly progressed in extent and severity after birth, indicating that early onset of therapy may be important to maximise the benefits of therapy. MPS VI kittens could be accurately diagnosed at birth by examination of leukocyte morphology in blood films. A dose responsive effect of ERT from birth in MPS VI kittens was clearly shown, including reversal of lysosomal storage in soft tissues including heart valve which is a major site of pathology in human patients. In addition, ERT was shown to improve skeletal growth, including more normalised bone histomorphometric parameters indicating improved bone quality and structure in response to therapy (Byers *et al.*, 1997a; Crawley *et al.*, 1997). However despite enzyme modifications to improve enzyme penetration into cartilage, ERT was unable to reverse corneal keratocyte and chondrocyte lysosomal storage, resulting in no reduction in corneal clouding and no change in degenerative joint changes in ERT treated cats.

Following evaluation of untreated MPS VI and normal control cats to establish baseline data, some variability was observed in development of disease features in MPS VI cats, however variability in skeletal growth was comparable to that observed in unaffected littermates. In MPS VI cats, variable degrees of impaired growth was observed in different bones, with cervical vertebral lengthening impaired more severely than tibial lengthening. In addition, the discovery of two mutations within the cat breeding colony lead to the identification of three different MPS VI phenotypes (Yogalingam *et al.*, 1996; Yogalingam *et al.*, 1998). L476P/L476P cats with a severe MPS VI phenotype were used in all the ERT studies. D520N/D520N and L476P/D520N cats were found to have similar leukocyte and cultured fibroblast 4S activity to the severe phenotype, however both had normal growth and lysosomal inclusions were only observed in neutrophils and some chondrocytes. The

only clinical outcome of these changes was a high incidence of degenerative joint disease in L476P/D520N cats (Crawley *et al.*, 1998).

7.3 Conclusions and implications

The major conclusion from this work is that ERT with rh4S from birth is able to significantly alter the progression of skeletal disease in the MPS VI cat and it also removes inclusions present at birth and prevents development of lysosomal inclusions in a number of soft connective tissues. This suggests that ERT in human MPS VI patients is likely to lead to significant improvements in quality of life. In particular, ERT is likely to be effective in alleviating cardiorespiratory complications which is usually the ultimate cause of death in MPS patients. In addition, ERT appears to have minimal side-effects and is safe to use during skeletal growth. These are distinct advantages over BMT which can impair growth and has potential complications associated with the procedure. Combined therapy with ERT during growth, followed by BMT at a later age has potential advantages and has recently been demonstrated to improve some parameters to a greater extent than ERT alone in MPS VII mice (Sands *et al.*, 1997).

Diffusion of enzyme into avascular tissues such as cartilage and corneal was minimal. The reversal in lysosomal storage in corneas from some MPS animal models following BMT but not ERT (reviewed in Sands *et al.*, 1997) suggests important differences in enzyme transfer to these avascular sites of pathology. Enzyme transfer from donor derived marrow cells migrating into the avascular cornea is the most likely explanation for the correction of lysosomal storage seen in these BMT animal models. Following ERT, it is not known if cell to cell transfer of enzyme occurs *in vivo* following cellular enzyme uptake. In particular, enzyme levels and enzyme half-life in migrating marrow cells following ERT should be examined. This may be important to elucidate, as any differences with BMT may be important to consider with regard to targeting avascular tissues.

Making the assumption that leukocyte and fibroblast enzymology reflect the enzyme levels in other cell types, it appears that only small increases in residual 4S levels in growth plate cell types normalises skeletal growth in L476P/D520N compound heterozygotes, and prevents development of degenerative joint disease in D520N homozygotes, compared with

L476P homozygotes. Skeletal pathology is severe in some MPS VI patients and these small differences in enzyme levels has positive implications for any therapies being developed. That is, if only small amounts of enzyme can be delivered to the appropriate cell types causing these pathologies, then the clinical phenotype will be corrected. However as observed in BMT studies in LSD animal models and in human patients, the degree of correction will be dependent on the extent of pathology already established prior to therapy. In addition, the presence of lysosomal storage only in chondrocytes and neutrophils in L476P/D520N compound heterozygotes and D520N homozygotes, indicates that these cell types have the greatest 4S requirements compared with the remaining cell types in the cat.

Minimal evidence of elevated antibody titres or antibody mediated problems was observed in MPS VI cats given ERT from birth. Elevated titres have been observed in approximately 15% of ERT treated Gaucher patients, with potentially detrimental effects only present in several of these patients (Ponce *et al.*, 1997). This suggests that there may be only a low incidence of development of antibodies against the administered enzyme in ERT in MPS patients. However the long-term immunological outcome following frequent and repeated administration of enzyme in ERT is still unknown. Patients undergoing ERT with null mutations providing minimal or no residual enzyme protein may experience an increased incidence of elevated titres in response to ERT and probably also gene therapy. Most mutations in MPS VI humans and the mutation detected in MPS VI cats (L476P/L476P) result in some residual 4S activity, however there is a high incidence of the null alleles Q70X and W402X in European MPS I patients (Bunge *et al.*, 1994). These MPS I patients may have a similar immune mediated responses to new therapies, as has been observed with ERT and gene therapy in the null allele MPS I dog (Kakkis *et al.*, 1996; Shull *et al.*, 1996).

7.4 Future work

Future ERT studies in MPS VI cats using recombinant feline 4S may help to improve understanding of the results collected from ERT with rh4S, and whether species specific differences particularly involving immune responses, may affect efficacy of therapy. Altered intracellular targeting of rh4S and increased degradation of administered rh4S has been observed in high titre rats immunised against rh4S (Brooks *et al.*, 1997). Further understanding of this process and whether a similar situation occurs in patients or animals

with much lower titres following ERT will be important to elucidate, as well as the mechanism and control of antibody mediated responses to externally derived enzymes. This is directly relevant to Gaucher patients currently undergoing ERT as well as for ERT anticipated in other LSDs in the future.

Understanding the pathogenesis of the skeletal disease in MPS VI will be important in order to optimise efficacy of new therapies. For example, particular sites within the growth plate may have different levels of enzyme requirements. It may also assist in understanding the pathogenesis of skeletal disease in other MPS disorders. A number of interrelated factors may influence bone growth at the growth plate in MPS VI, including cell function and growth plate matrix structure.

Abnormal cell function may be occurring in one or more regions in the growth plate, such as in the resting chondrocytes or in the proliferative and hypertrophic zones, or in any of the bone producing (osteoblast and osteocyte) or resorbing (osteoclast) cell types. Apart from impaired function of these cell types themselves due to excessive intracellular GAG accumulation, if excessive GAG is present in the surrounding cartilage or bone matrices, this may inhibit or impair normal cell function by mechanisms such as feedback inhibition, or sequestering of growth factors. Any changes in matrix GAG content may also affect collagen fibrillogenesis, and if this occurs in any regions of the growth plate, this may alter matrix organisation leading to abnormal matrix structure. This may then affect mineralisation of trabeculae emerging from the growth plate.

Methods including electron microscopy, *in vitro* cell culture and histomorphometry will be useful to examine various components potentially involved in the pathogenesis of the skeletal dysplasia in MPS VI. Comparison of growth plate pathology from different sites and at different stages of development may reveal differences reflecting the degree of impaired growth in different bones observed in the untreated MPS VI cats. In addition, comparison of growth plate pathology in MPS VI (L476P/L476P homozygotes) with L476P/D520N compound heterozygotes may help to elucidate the differences observed in skeletal growth in these two genotypes.

The ultimate success of ERT is dependent on effective delivery of enzyme to sites of pathology in sufficient amounts at the appropriate time in development of the individual. The optimal dose for maximal response to ERT therefore needs to be examined. An interesting approach to this was withdrawal of ERT in MPS VII mice followed by euthanasia at certain intervals and examination of return of lysosomal storage in certain cell types (Vogler *et al.*, 1996). This provides information about targeting of enzyme to specific cell types and also the enzyme requirements in different cell types. Various dose regimes can be also be employed, such as a constant dose rate as examined in this thesis, or a dose rate proportional to growth rate. That is, starting with a very high dose rate which is reduced with increasing age. In addition, further evaluation of cationized enzyme is warranted, based on the limited but positive results with PL4S compared with unmodified rh4S. Polylysine is available in various lengths, which will affect the charge distribution and also the final size of the cationized enzyme, therefore different results may be observed if this is altered. Increased numbers of animals in each dose group would provide more definitive results than for some of the results collected in this thesis.

Despite a poor understanding of the pathogenesis of skeletal and joint disease in MPS VI, the development of methods to specifically target enzyme to chondrocytes are indicated. L476P/D520N compound heterozygotes may be useful for these evaluations due to milder chondrocyte pathology, and therefore more rapid reversal of lysosomal distension and storage in these cell types following uptake of rh4S. In addition, more objective measurement of articular cartilage thickness would be helpful in evaluating future therapeutic strategies. This may be possible by taking direct measurements from histological sections following a standardised plane of sectioning through particular joints.

Little information is available regarding joint pathology in MPS patients. Our observations in L476P/D520N cats suggest that degenerative joint disease may develop in MPS VI patients surviving for longer periods following BMT or new therapies such as ERT. It also suggests that there may be human patients with degenerative joint disease of unknown aetiology with biochemical evidence of MPS VI. It would be of interest to screen any such patients for DSuria and abnormal leukocyte morphology to determine if the same very mild MPS VI phenotype is present in humans.

Recent ERT studies in MPS VII mice have shown greater improvements in mice given ERT from birth compared with mice in which ERT was delayed until 6 weeks after birth (Sands *et al.*, 1997). Screening of human newborns at birth for LSDs (including MPS VI) is now technically possible (Meikle *et al.*, 1997), however no newborn screening is currently being performed for these patients. Therefore diagnosis is usually delayed until 1-2 years of age in more severely affected patients. Starting ERT at a comparable age to this in the MPS VI cats, using more effective doses than used in our preliminary studies may help to determine how effective ERT may be once pathology is well established. These studies may also be more comparable to the situation of BMT being performed in young MPS patients.

In collaboration with Dr Mark Haskins and Dr Paola Di Natale, our findings of the L476P allele in several American and Italian MPS VI cats from Siamese ancestry suggests a high incidence of this allele in the general Siamese cat population. It would be of academic interest to determine the frequency of this allele in population studies from Siamese cats found in the USA, Australia and Europe.

The studies detailed in this thesis have demonstrated the value of a well characterised animal model for the same disorder in humans, for the evaluation of efficacy of new therapies. These and other studies in MPS animal models have increased understanding about reversability of lysosomal storage following ERT in various tissues including skeletal tissues, problems encountered during ERT and methods of evaluation of therapy efficacy. Further study of these models will also increase understanding of the pathogenesis of disease observed in the MPS disorders. Genetic characterisation of some of these models has also provided important additional information about the severity of the disease mutation, and it also dramatically improved breeding efficiency in our MPS VI cat colony. Animal models of human disease have an important place in current and future medical research.

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ADDENDA



Page 85 Paragraph 4. Delete and replace with "Subtle hindlimb gait abnormalities due to spinal cord compression were first observed in some MPS VI cats as early as 75-80 days of age, but onset was more commonly seen between 4 and 5 months of age (Table 3.2). Changes in hindlimb strength and coordination were most obvious by observing an animals ability to resist being pushed off balance. Other abnormal gait changes included ataxia, hypermetria and a wide based hindlimb stance."

Page 86 Paragraph 2. Delete and replace with "Hindlimb neurological abnormalities progressed in some cats to severe hindlimb paresis or full hindlimb paralysis, with increased hindlimb extensor tone and neurogenic urine retention, however deep pain sensation was still present. Rate of progression of abnormalities was variable, with some cats progressing to severe hindlimb paresis or paralysis over 2 weeks, and other cats over more than 4 weeks. Several cats had unexpected partial reversal of hindlimb neurological deficits (see footnotes Table 3.2). Thoracic limbs appeared neurologically normal in all cats."

Page 87 Footnotes to Table 3.2. Replace with

* 1 cat (239f) had hindlimb paralysis between 4 and ~5 months, but by ~ 5 months had moderate hindlimb paresis.

† 1 cat (163m) with hindlimb paralysis was euthanased at 7 months with neurogenic urine retention.

‡ 1 cats (222f) had hindlimb paralysis at 7 months but improved and had severe paresis at 9 months.

§ 1 cat (223m) had hindlimb paralysis at 9 months and improved to severe paresis at 11 months.

n, number of animals examined.