THE MARFAN SYNDROME AND RELATED PHENOTYPES

Delineation of various phenotypes and analysis of the fibrillin gene (FBN1) for putative mutations

BY

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A thesis submitted for the Degree of Doctor of Medicine

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This thesis is dedicated to my parents, Félix and Huguette whose "ruach", humour, wisdom and love will always be major forces in my life. Thanks for sprinkling my childhood with stories like "Toto, mange ta soupe", and for all those wonderful philosophical sayings like "le chameau ne voit jamais sa bosse" and, most importantly, "ou bien ou rien". And thanks for everything else.

"What is not given is lost"

Dominique Lapierre

"Genius is childhood rediscovered at will"
Charles Baudelaire

ABSTRACT

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Marfan syndrome is an autosomal dominant connective tissue disorder involving multiple organ systems which, if untreated, shortens life expectancy mainly because of cardiovascular complications. It has an incidence of at least 1 in 5,000 live births. The phenotype represents a continuum, one end of which merges with the normal population. The disease is caused by mutations in the FBN1 gene, which encodes the fibrillin-1 protein. Fibrillin, a component of extracellular matrix microfibrils, has a highly repetitive structure including forty-seven EGF-like motifs, seven cysteine-rich TGF-β1 BP motifs and two hybrid motifs.

The clinical and molecular study of patients with unequivocal Marfan syndrome, or an undiagnosed connective tissue disorder that has some features in common with Marfan syndrome, forms the basis of this thesis. The clinical features of these forty-eight patients are described. The phenotype of six Marfan patients in whom a FBN1 mutation was determined, patients with Shprintzen-Goldberg syndrome or Furlong syndrome (two Marfanoid-craniosynostosis disorders), and two children with congenital aneurysms of the great vessels, are presented.

The molecular screening of 44% of the FBN1 gene coding sequence for putative mutations is detailed. Five novel heterozygous single base pair changes in the FBN1 gene were identified in five separate Marfan syndrome families. These were G2113A, G2132A, T3163G, G3458A and A7868C. These result in the amino acid substitutions A705T, C711Y, C1055G, C1152Y and H2629P, respectively. Three of the characterised mutations, C711Y, C1152Y and C1055G result in replacement of cysteine by another amino acid; the latter two occur within EGF-like motifs in exon 27 and 25, respectively. The A705T mutation occurs at exon 16 adjacent to the GT splice site. The H2629P mutation occurs immediately adjacent to one of the conserved cysteines in the second-to-last EGF-like domain. The A705T and C711Y mutations at exon 16 and 17, respectively, are the first documented in the second TGF-β1 BP-like motif. Polymorphisms, believed to be normal variants, were identified in exons 15 and 28 of FBN1 in nine patients.

STATEMENT

This work contains no material which has been accepted for the award or 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 has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Lesley Carole Adès

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This research would not have been possible without the co-operation of the patients and their families, to whom I am indebted.

GLOSSARY OF ABBREVIATIONS

α1(I)	alpha one chain of type I collagen
α2(I)	alpha two chain of type I collagen
α1(III)	alpha one chain of type III collagen
α^{32}P-dCTP	alpha ^{32}P-2'-deoxycytidine 5'-triphosphate
A	adenine
AMP	associated microfibril protein
APS	ammonium persulphate
bp	base pair(s)
BME	beta-mercaptoethanol
C	cytosine
cb	calcium binding
CCA	congenital contractural arachnodactyly
cDNA	complementary deoxyribonucleic acid
COL1A1	gene for alpha one chain of type I collagen
COL1A2	gene for alpha two chain of type I collagen
COL3A1	gene for alpha one chain of type III collagen
cm	centimetre
°C	degrees centigrade
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxynucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DHEA-S	di-hydro-epiandrosterone sulphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
ECG	echocardiography
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EtBr	ethidium bromide
FBN1	fibrillin gene (chromosome 15)
FBN2	fibrillin gene (chromosome 5)
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
FLP	fibrillin-like protein
fmol^{3}H-R1881	femta(10^{-12})molar tritiated H-methyltrienolone
FSH	follicular stimulating hormone
g	gravitational force
G	guanidine
hGH	human growth hormone
H-NMR	H-nuclear magnetic resonance
IPTG	isopropyl-beta-D-thiogalactopyranosid
IU	international units
kb	kilobase pair(s)

kD	kiloDalton(s)
LA	left atrium
LH	luteinising hormone
LVED	left ventricle end diastole
LVES	left ventricle end systole
M	molar
MAGP	microfibril associated glycoprotein
MIM	Mendelian Inheritance in Man
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mm	millimetre
mm Hg	millimetre of mercury
mM	millimolar
mRNA	messenger RNA
$(NH_4)_2SO_4$	ammonium sulphate
NaOH	sodium hydroxide
nm	nanometre
NR	normal range
°C	degrees Celsius
OD	optical density
OI	osteogenesis imperfecta
ORF	open reading frame
p	short arm of chromosome
p <	probability less than
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF1, 2, 3	pepsin-resistant fragment 1, 2, 3
polyA stretch	consecutive occurrence of several adenine nucleotides
pmol/l	picamole per litre
PTC	premature termination codon
q	long arm of chromosome
RA	right atrium
RNA	ribonucleic acid
rpm	revolutions per minute
RR	reference range
RT-PCR	reverse transcription polymerase chain reaction
Sarkosyl	N-lauroylsarcosine
SD	standard deviation
SDS	sodium dodecyl sulphate
SSCP	single stranded conformational polymorphism
T	thymidine
TBE	tris-borate EDTA
TE	tris EDTA
TEMED	N,N,N,N tetramethylethylenediamine
TGF-β1 BP	transforming growth factor beta one binding protein
Tris	tris(hydroxymethyl) aminomethane
UTR	untranslated region
V	volt(s)

W	Watt(s)
WSCR	Williams Syndrome Chromosome Region
µCi	microCurie
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
µU/ml	microUnits per millitre
U	units
X-Gal	gal-5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside

CONTENTS

CHAPTER ONE

LITERATURE REVIEW

1.1 Background

1.1.1 Historical Perspective and Introduction

First described in 1896 by the French paediatrician, Antoine Marfan, the clinical manifestations of the Marfan syndrome have since been redefined, modified and expanded. Marfan's description of a five and a half year old girl with arachnodactyly and dolichostenomelia highlighted the skeletal anomalies of the condition (Marfan, 1896). Two decades later, the cardinal ocular feature of ectopia lentis, and involvement of the cardiovascular system were also recognised in association with the skeletal changes. Aortic involvement in Marfan syndrome was noted in 1943 (Etter and Glover, 1943) but it was not until 1955 that aortic disease was shown to account for most deaths (McKusick, 1955).

Marfan syndrome is now recognised as an autosomal dominantly inherited, pleiotropic connective tissue disorder characterised by cardiovascular, ocular and skeletal abnormalities (McKusick, 1956). It occurs at an incidence of one in 5,000 liveborns in the population; 10% to 30% of these cases are sporadic and are presumed to result from new mutations in parental germ cells (Pyeritz and McKusick, 1979; Dietz and Pyeritz, 1995). The Marfan syndrome shows high penetrance. Variability in the clinical expression of the disease is common both between and within families. The basis for this variability remains unknown. It is believed that Rachmaninoff, Paganini, and Abraham Lincoln all had Marfan syndrome (Manusov and Martucci, 1994).

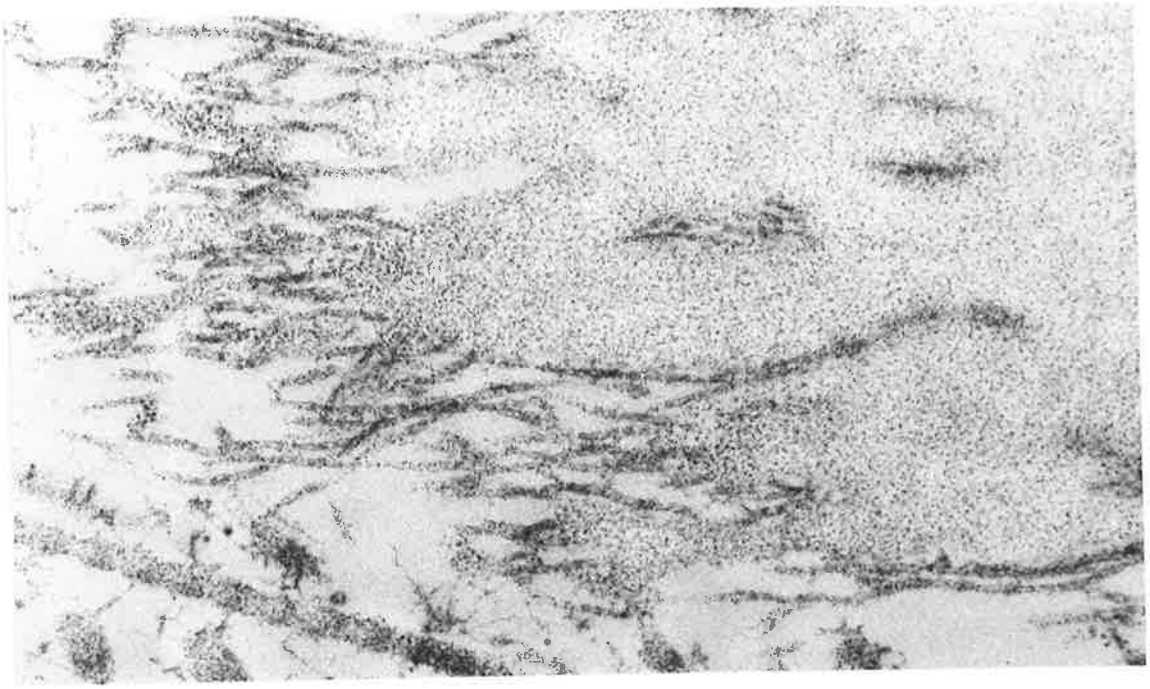
The skeletal features of the syndrome include tall stature, dolichostenomelia, scoliosis, arachnodactyly, pectus carinatum and/or excavatum, pes planus and joint hypermobility. Cardinal ocular manifestations include ectopia lentis, myopia and retinal detatchment. The major life-threatening complications arise from the cardiovascular abnormalities, such as dilatation of the ascending aorta, aortic dissection and aortic and mitral valvular insufficiency. Prior to the advent of cardiovascular surgery, the average age at death of an affected person was 32 years (Murdoch et al., 1972).

1.1.2 Elastic Fibres, Elastin and Microfibrils

Initial studies to determine the pathogenesis of Marfan syndrome involved histological examination of aortas from affected individuals. Abnormal findings included fragmentation of elastic tissue in the tunica media of the aorta (Derouette et al., 1981), other arteries (Takebayashi et al., 1988), and skin (Tsuji, 1986), biochemical studies of aortic elastin (Perejda et al., 1985), and an increased urinary excretion of elastin metabolites in affected patients (Gunja-Smith and Boucek, 1981). These findings suggested a defect in the formation of elastin fibres and initially, attention was directed towards the elastin gene. At first, a candidate gene approach failed to exclude elastin as the candidate gene, due to a lack of polymorphisms in or around the gene. Studies in a Finnish family using a rare polymorphism eventually excluded elastin as a candidate gene in that population (Huttunen et al., 1989). Interestingly, William's syndrome, a condition clinically unrelated to the Marfan syndrome, but one in which there is a defect of aortic tissue (recognised clinically as supravalvular aortic stenosis), has recently been shown to be due to mutations in the elastin gene, located on the proximal region of chromosome 7q (Olson et al., 1993). The focus for the pathogenesis of the Marfan syndrome then turned to the second major component of elastic fibres, namely the microfibrils.

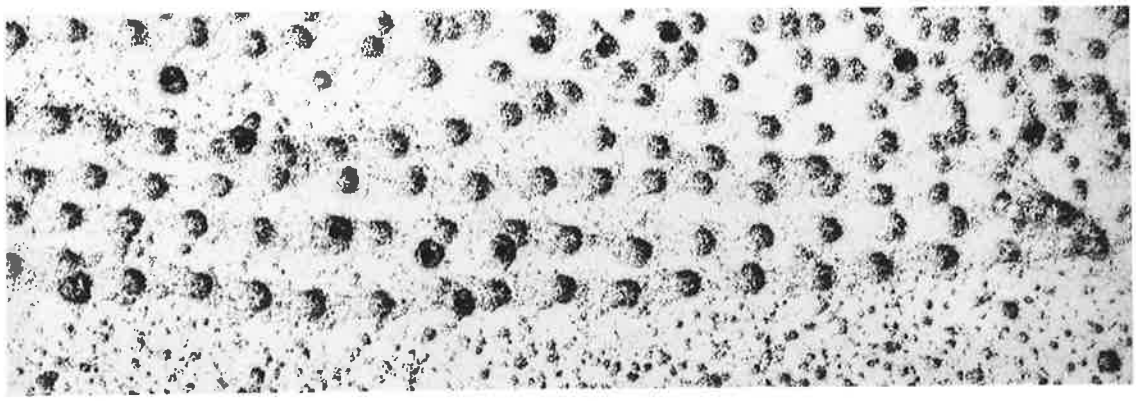
Elastic fibres are complex structures consisting of the base elastin protein and 10 to 12-nm fibrillar structures (Cleary and Gibson, 1983). In the aorta, elastic fibres are woven together to form lamellae that lie between layers of smooth muscle cells and completely surround the vascular lumen. The elastic lamellae are interconnected by radially oriented interlaminar fibres that transfer stress throughout the vessel wall. Unlike collagen fibres, which have variable fibres and a clear banding pattern, individual microfibrils have a uniform diameter and display no periodic banding pattern. These 10-12-nm diameter fibres comprise a discrete, widely distributed and pleiomorphic fibre system in human tissues. They form a variety of structures, including long rods, lamellae and meshworks (Cleary and Gibson, 1983). In longitudinal sections, microfibrils display characteristic light and dark staining patterns, giving the microfibril a beaded appearance (Figure 1.1 (a) and (b)). Microfibrils are made up of several glycoproteins, including microfibril-associated glycoprotein (MAGP) (Gibson et al., 1991) and fibrillin (Sakai et al., 1986). The fibres serve as a scaffolding for the deposition of elastin during elastogenesis and become incorporated into the elastic structures of many tissues (Cleary and Gibson,

Figure 1.1 (a) Electron Microscopy View of Elastic Fibres



The rod-like structure (lower left) represents collagen while the lightly staining amorphous background represents elastin. The microfibrils appear as beaded linear molecules.

Figure 1.1 (b) Rotary Shadowing of Microfibrils with Polyclonal Antibody to MAGP



The polyclonal antibody MAGP binds to the beaded domains. The beads are considered to represent the microfibrils, and the intervening "string" represents fibrillin and possibly also other secondary connective tissue proteins.

(photographs courtesy of M Gibson)

1983; Cleary, 1987).

The gross organisation of elastic fibres and microfibrils varies in the different connective tissues. Microfibrillar bundles at the dermal-epidermal junction have little or no association with elastin and have been named "oxytalan" fibres. Connecting the bundles of microfibrils at the dermal-epidermal junction with the deeper dermal elastic fibres, are "elaunin" fibres or bundles of microfibrils with small, varying amounts of amorphous elastin (Cotta-Pereira et al., 1976). Microfibrils are abundant in elastic and non-elastic tissues altered in Marfan syndrome, including the aortic media, periosteum, perichondrium, cartilage, tendon, muscle, cornea, ciliary zonule, alveolar wall, the renal glomerulus, the dural sheath, and skin. The ciliary zonule is composed solely of microfibrils, with no other structural elements (Raviola, 1971).

The fibrillins, MAGP and associated microfibril protein (AMP) are all thought to contribute to microfibrils (Gibson et al., 1991; Horrigan et al., 1992). Fibrillin is now recognised to represent a small family of structurally related proteins including the products of the fibrillin-1 and fibrillin-2 genes, and FLP, a fibrillin-like protein (Ramirez et al., 1993; Rosenbloom et al., 1993). Other proteins that reside in elastic fibres without contributing to microfibril formation, include emilin, lysyl oxidase and elastin (Bressan et al., 1993; Kagan et al., 1986). Microfibrillar-associated proteins, such as vitronectin, amyloid P component and the microfibril associated protein (MAP) may interact with some microfibrils, but not others (Dahlbäck et al., 1990a; Breathnach et al., 1991; Kobayashi et al., 1989). Proteoglycans may be associated with microfibrils, and versican has been specifically localised in skin to microfibrils (Baccarani-Contri et al., 1990; Zimmerman et al., 1994).

During fetal development, microfibrils are the first component of elastic fibres to appear in the extracellular matrix, arranged as aggregates in a parallel array. Elastin is then deposited as amorphous material within these bundles of microfibrils, which then coalesce to form mature elastic fibres (Mecham and Heuser, 1993). Elastin deposition and formation of mature elastic fibres continues throughout embryogenesis up to early childhood, after which elastogenesis ceases almost completely. Thereafter, there is very little turnover of elastic fibre components.

A note on the conventions adopted in this thesis

Wherever the term "fibrillin" is used in the text, it refers exclusively to the protein implicated in the pathogenesis of Marfan syndrome. Where comparisons are made between different fibrillin proteins, then fibrillin-1 (otherwise annotated as Fib 15 or FBN1) refers to the protein involved in Marfan syndrome, whilst fibrillin-2 (otherwise annotated as Fib 5, or FBN2) refers to the protein implicated in the pathogenesis of congenital contractural arachnodactyly (Zhang et al., 1994). The nucleotide numbering sequence for FBN1 was originally based on the partial cDNA sequence (Maslen et al., 1991). This system was superseded when the complete cDNA sequence was elucidated (Pereira et al., 1993a). The nucleotide numbering sequence used in this thesis assumes that the 5' exon (exon A) identified by Corson et al. (1993) (see Section 1.1.6.1) is the start site of transcription for the predominant mRNA transcript. The codons are numbered from the first ATG sequence whose context fits the consensus site for translation initiation. Nucleotide A of the ATG sequence is located at position -2694 (Maslen numbering), which corresponds to position +1 (Pereira numbering). Unless otherwise stated, all mutations referred to in the thesis concur with the Pereira nomenclature. The amino acid abbreviations in the text are detailed in Appendix 1A. Wherever the MIM annotation occurs in the text, this refers to the Mendelian Inheritance in Man catalogue (McKusick, 1992). Unless otherwise stated, all the clinical photographs presented in this thesis, including radiographs and CAT (computerised axial tomography) scans, have been prepared exclusively from the research patients.

1.1.3 Fibrillin

In 1986, fibrillin, a 350-kD collagenase-resistant single-chained glycoprotein was isolated from the culture media of human dermal fibroblasts, and provided evidence for this protein as the major structural component of microfibrils. Fibrillin was specifically immunolocalised to microfibrils in a number of human tissues with the use of several monoclonal antibodies (Sakai et al., 1986). These same antibodies reacted avidly with ciliary zonules, which lack elastin and are composed almost exclusively of microfibrillar fibres (Raviola, 1971).

The isolation and characterisation of fibrillin pepsin fragments PF1, PF2, and PF3 from human amnion provided some of the first clues to the structure of fibrillin monomers and

microfibrils (Maddox et al., 1989). A monospecific antiserum to PF2 and a monoclonal antibody to PF3, together with immunoelectron microscopy and immunoblotting showed that both antibodies were specific for fibrillin. The fragments PF1 and PF2 appeared as short rod-like structures under rotary shadowing electron microscopy, whereas the PF3 fragment (a disulphide-bonded, crosslinking region containing an aggregation of fragments of between 6 to 10 molecules) appeared as a complex globular structure with projecting arms (Maddox et al., 1989). Thereafter, fibrillin was purified from the medium of human dermal fibroblasts and ligament cells in culture.

Rotary shadowing electron microscopy and velocity sedimentation studies showed that fibrillin is an extended molecule 148 x 2.2 nm in size. Analysis of the amino acid composition showed that fibrillin contains approximately 14% cysteine, of which one-third appears to be in the free reactive sulphydryl form (Sakai et al., 1991). This suggests that disulphide crosslinks may join the fibrillin monomers to each other or to other proteins in the mature microfibrils. Pulse-chase experiments using chick aorta organ cultures demonstrated that fibrillin molecules form intermolecular disulphide-bonded aggregates within 1-4 hours after secretion (Sakai et al., 1991).

The presence of fibrillin, the major component of microfibrils, in the major tissues affected in Marfan syndrome, suggested that fibrillin might be the primary candidate gene defective in the Marfan syndrome.

1.1.4 Identification of the Genetic Defect in Marfan Syndrome

In 1990, Finnish researchers reported linkage of the Marfan syndrome to the long arm of chromosome 15 (Kainulainen et al., 1990). Subsequent studies further defined the locus and showed that there was no evidence for genetic heterogeneity (Kainulainen et al., 1991). This was followed by the cloning of the partial cDNA of fibrillin (Maslen et al., 1991; Lee et al., 1991) and then the assignment of fibrillin cDNA sequences to the region, 15q21.1 (Lee et al., 1991; Magenis et al., 1991). Polymorphic markers within the gene were shown to be closely linked to the Marfan syndrome without recombination (Dietz et al., 1991a; Lee et al., 1991). The linkage analyses and localisation of the gene coding for fibrillin (FBN1) to the same locus, were complemented by identification of the first *de novo* missense mutation in the fibrillin-specific cDNA in two unrelated

sporadic Marfan syndrome patients. (Dietz et al., 1991b).

1.1.5 Protein Structure of Fibrillin

1.1.5.1 General overview

The complete cDNA sequence encoding fibrillin is now known (Pereira et al., 1993a). The predicted primary structure of fibrillin is shown schematically in Figure 1.2. The majority of the protein consists of tandem arrays of cysteine-rich motifs. The amino and carboxy-terminal regions show no homology to any known protein and these are labelled as unique domains in Figure 1.2.

The deduced pre-fibrillin molecule is a polypeptide of 2,871 amino acids (Pereira et al., 1993a). A schematic representation of the fibrillin chains (fibrillin-1 and fibrillin-2) is shown in Figure 1.3. Excluding the signal peptide, the fibrillin molecule has five structurally distinct regions called A, B, C, D and E (Figure 1.3). Two of these regions (B and D) are exclusively made of cysteine-rich sequences, the majority of which adhere to the epidermal growth factor (EGF) peptide motif (Figure 1.3).

Analysis of the cDNA sequence revealed a total of 47 tandem domains with homology to human EGF; four of them are of the generic type (Maslen et al., 1991; Pereira et al., 1993a). Three are located adjacent to the amino terminal sequence, and the fourth is adjacent to region C (Figure 1.3). The remaining 43 EGF-like repeats contain a calcium binding (cbEGF-like) motif (Pereira et al., 1993a). There are seven domains containing eight cysteines and these are termed TGF-β1 BP (transforming growth factor-β1 binding protein) motifs. The two other domains which also contain eight cysteines are known as hybrid motifs. Fibrillin has three unique domains: a highly basic amino-terminal domain comprising a 29 amino acid stretch of largely basic residues (region A), a proline-rich domain (region C) near the amino-terminal domain of the protein, and a lysine-enriched carboxy-terminal domain (region E) (Figure 1.3). Each of these regions are sequences of unique composition. The protein also has a number of putative sites for N-linked glycosylation, the majority of which reside in region D where a putative cell attachment signal (RGD site) is also noted (Figures 1.2) (Pereira et al., 1993a). Further details of the specific regions of the fibrillin protein are outlined in sections 1.1.5 below.

Figure 1.2 Primary Structure of Fibrillin-1

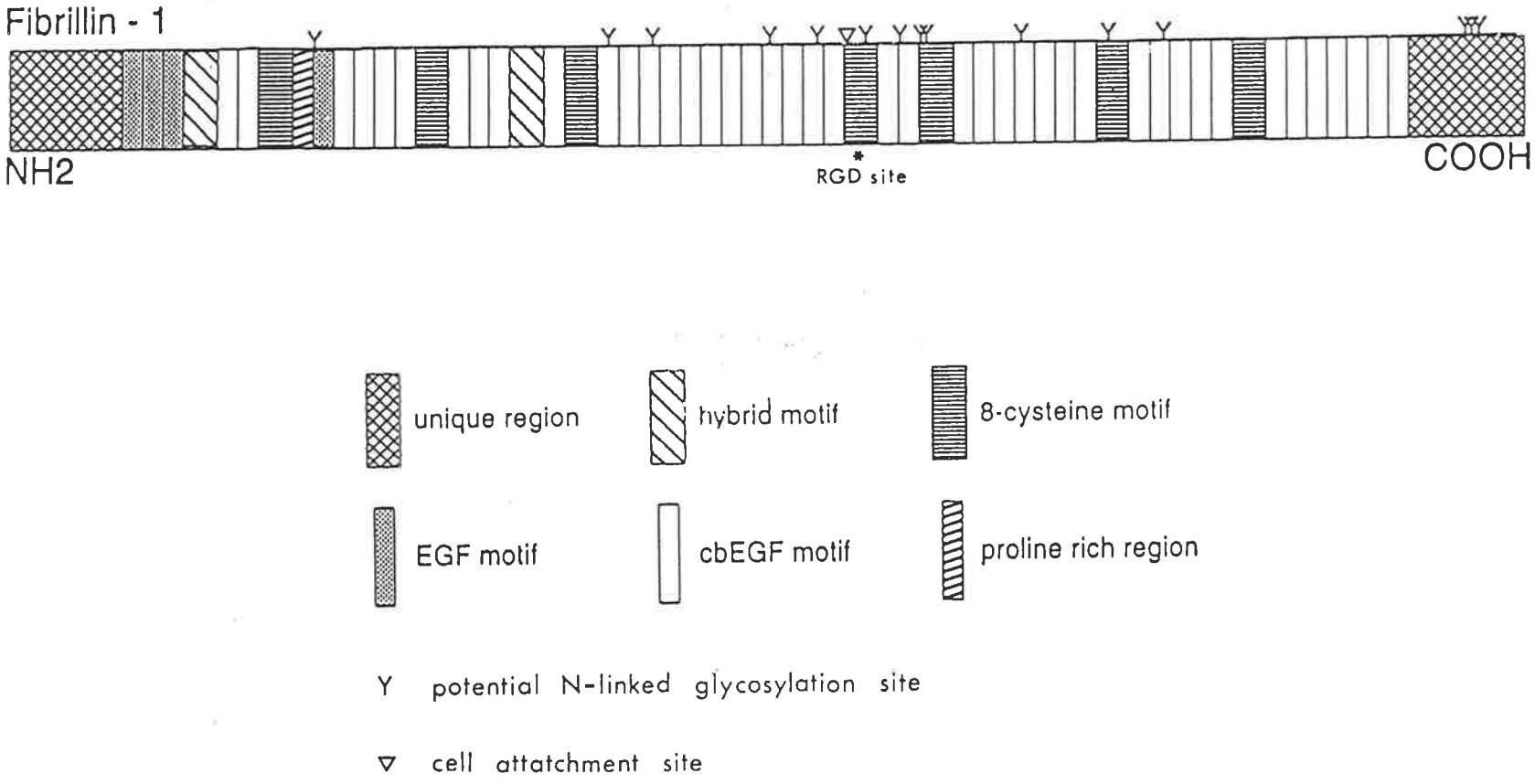
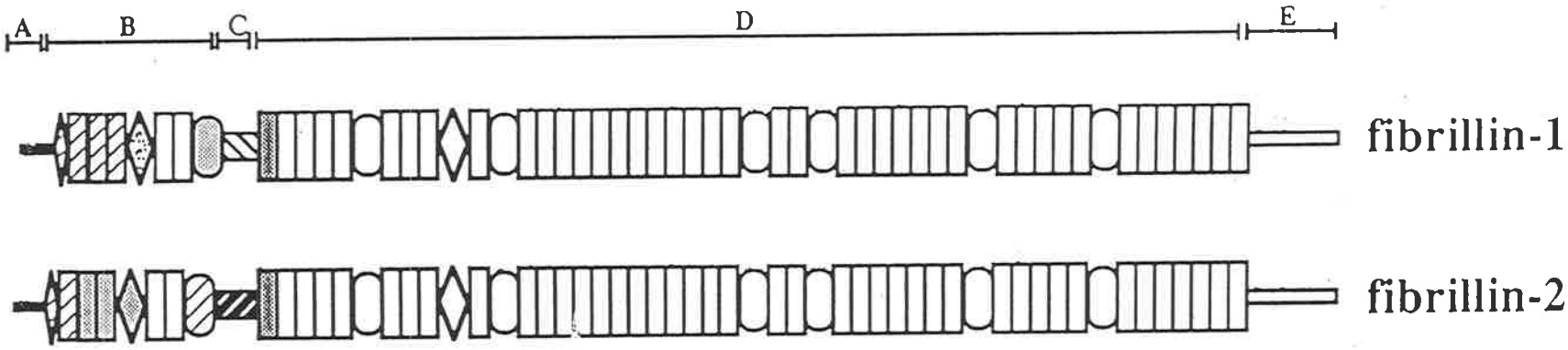


Figure 1.3 Schematic Representation of the Fibrillin Chains (Fibrillin-1 and Fibrillin-2)



EGF-calcium binding: $D\,X^{N}_{D}E\;C\;X_{(6)}C\;X_{(4)}C\;X\,N\,X_{2}\,G\,X_{3}\,C\;X\;C\;X_{2}\,G\,X_{(8)}C\;X$

TGF-β binding protein: $D\,X\,R\,X_{3}\,C\;{}^{Y}_{F}X_{(8)}C\;X_{(12)}C\,C\,C\;X_{(10)}C\;E\,X\;C\;P\,X_{9}\;L\;C\;P\,X_{(14)}$

The boundaries of the five structurally distinct regions (A to E) are shown above the figure. White rectangles and ovals indicate the sequences homologous to the EGF-calcium binding and TGF-β1 binding protein motifs, whose consensus sequences are shown beneath the figure. The other rectangles signify generic EGF-like sequences.

(adapted from Dietz et al., 1994)

Fibrillin molecules bind calcium, but only when intrachain disulphide bonds are intact (Corson et al., 1993). By analogy to other proteins, such as *Drosophila* Notch and protein S, which also contain cbEGF-like repeats, calcium binding may stabilise protein conformation and mediate protein-protein interactions (Dahlbäck et al., 1990a; Fehon et al., 1990). Conformational stability for fibrillin is predicted to be important for the proper assembly of microfibrils, and for the long-term stability of microfibrils against proteases.

1.1.5.2 The proline-rich region

The amino-terminal end of the fibrillin protein comprises a nonrepeated region of 47 amino acids, 21 of which (45%) are proline and none are cysteine (Corson et al., 1993). This region contains a peptide, YLYPSREPPRV, that is present at the amino terminus of PF1 (Maddox et al., 1989). At position -451 an asparagine occurs within a context (N-V-T) suitable for glycosylation. At the extreme end of the amino-terminal domain, the sequence appears to be unique, with no significant homology to other regions of the molecule or to other known sequences within current databases (Corson et al., 1993). The isolated expression of the extreme amino terminus of fibrillin upon a normal genetic background can recreate a Marfan-like cellular phenotype (Dietz et al., 1994). These data suggest that the amino-terminal end of the molecule plays a critical role in the polymerisation of fibrillin aggregates, in keeping with the head-to-tail model of microfibrillar asembly (Sakai et al., 1991).

The proline-rich region C is of potential interest for microfibrillar assembly. By analogy to proteins such as dystrophin, which has an overall extended conformation interrupted by proline-rich sequences (Ervasti and Campbell, 1991), region C may allow the fibrillin molecule to bend. It is unlikely that this domain forms a surface loop since about 30% of the residues in region C are hydrophobic (Pereira et al., 1993a). Bending of the fibrillin molecule may facilitate the interactions thought to occur at the ends of the molecule as it aligns in a head-to-tail fashion during microfibril assembly (Sakai et al., 1991).

The fibrillin homologue, fibrillin 5 (FBN2) has a very similar structural domain to fibrillin but instead of this proline-rich region, it has a glycine-rich motif (Ramirez et al., 1993). Whether these differing domains are important in clarifying the different roles of these two proteins has yet to be determined.

1.1.5.3 The RGD (cell attachment) sequence

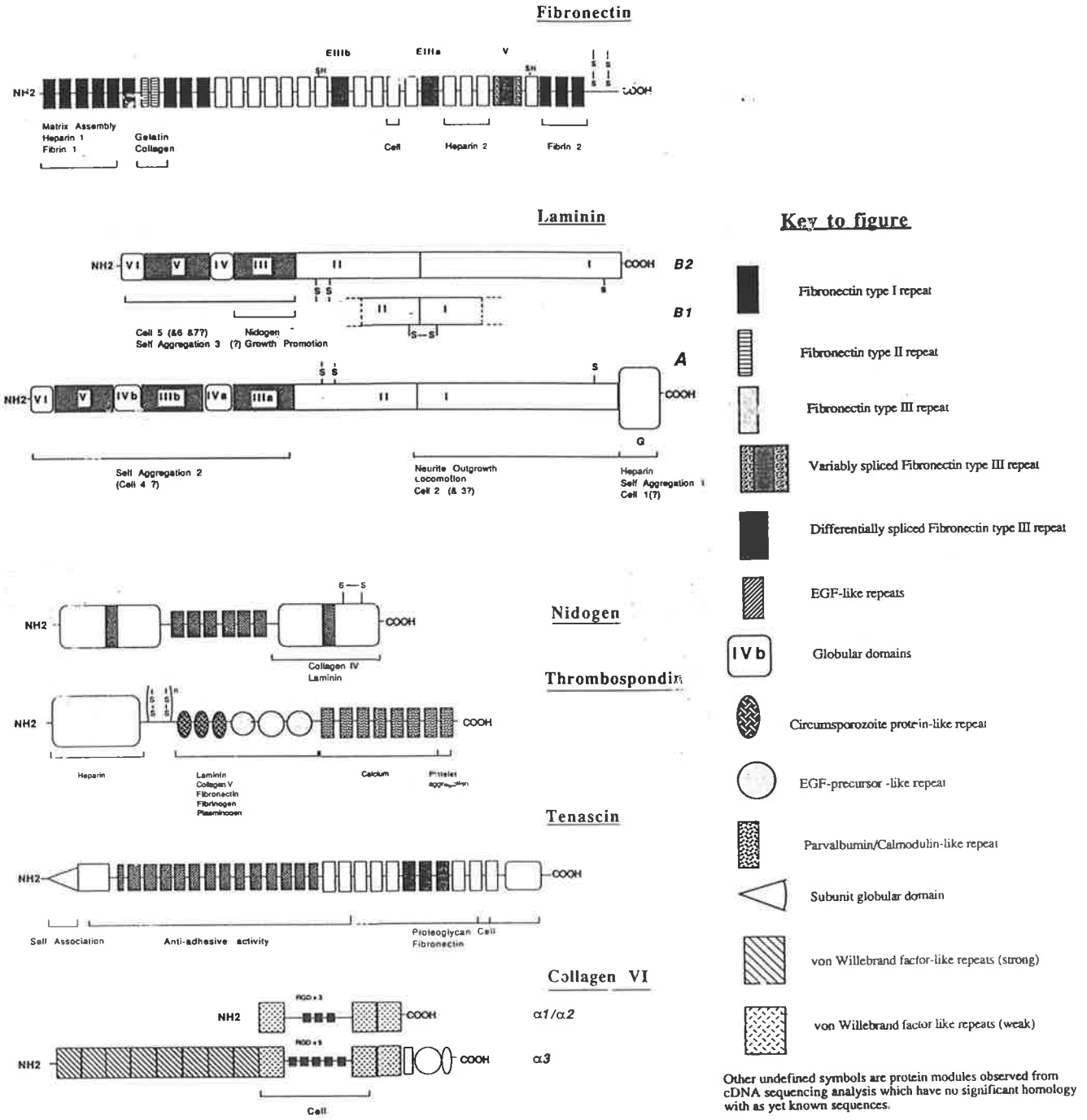
Fibrillin has a single arginine-glycine-aspartate (RGD) sequence located within one of the 8-cysteine TGF-β1 BP motifs, at amino acids 1541-1543 (Maslen et al., 1991). Many adhesive proteins present in extracellular matrices and in the blood contain this RGD tripeptide as their cell recognition site. These proteins include fibronectin, vitronectin, osteopontin, type I collagen, thrombospondin, fibrinogen and von Willebrand factor (Ruoslahti and Pierschbacher, 1987). The RGD sequence has been established as the basic unit of a widespread cellular recognition system (Ruoslahti and Pierschbacher, 1986). Despite the similarity of the cell attachment sequence in the various adhesive proteins, cells can recognise them individually. This specificity is provided by a number of receptors, integrins, each of which is capable of recognising only a single RGD-containing peptide ligand, or in some cases a limited number of ligands (Ruoslahti and Pierschbacher, 1987). The adhesion proteins and their receptors constitute a recognition system that provides cells with anchorage, traction for migration, and signs for polarity, position, differentiation, and possibly growth. The structural elements of some of these extracellular matrix adhesive proteins are shown in Figure 1.4. Although both the EGF receptor and the N-terminal part of β-transforming growth factor precursor contain an RGD sequence, the potential physiological significance of this sequence in these proteins has not been established.

1.1.5.4 EGF-like motifs

The most abundant pattern of the fibrillin molecule is of calcium binding (cb) epidermal growth factor (EGF)-like motifs. These motifs are approximately 40 amino acids in length and contain six cysteine residues in a highly conserved spacing, together with a number of other amino acids in well-conserved intervening positions (Corson et al., 1993). The six cysteine residues form three intramolecular disulphide linkages (between the 1st and 3rd, 2nd and 4th, and 5th and 6th cysteines).

This motif is highly homologous to one found in the precursor molecule of EGF and numerous other proteins (Appella et al., 1988) including those with a presumed role in development, like the Notch protein of *Drosophila* (Wharton et al., 1985; Kelley et al., 1987; Hartley et al., 1987) and uEGF-1 of sea urchin (Hursh et al., 1987). The cbEGF-like motif has been shown to bind calcium (Dahlbäck et al., 1990b; Handford et al., 1991; Selander-Sunnerhagen et al., 1992). Within the 5' region of the fibrillin molecule,

Figure 1.4 Structural Elements of Extracellular Matrix Adhesive Proteins



The general theme of adhesive matrix proteins appears to be a reiteration and amplification of structural motifs. A series of the most frequent recurrent motifs is shown.

(after von der Mark and Goodman, 1993)

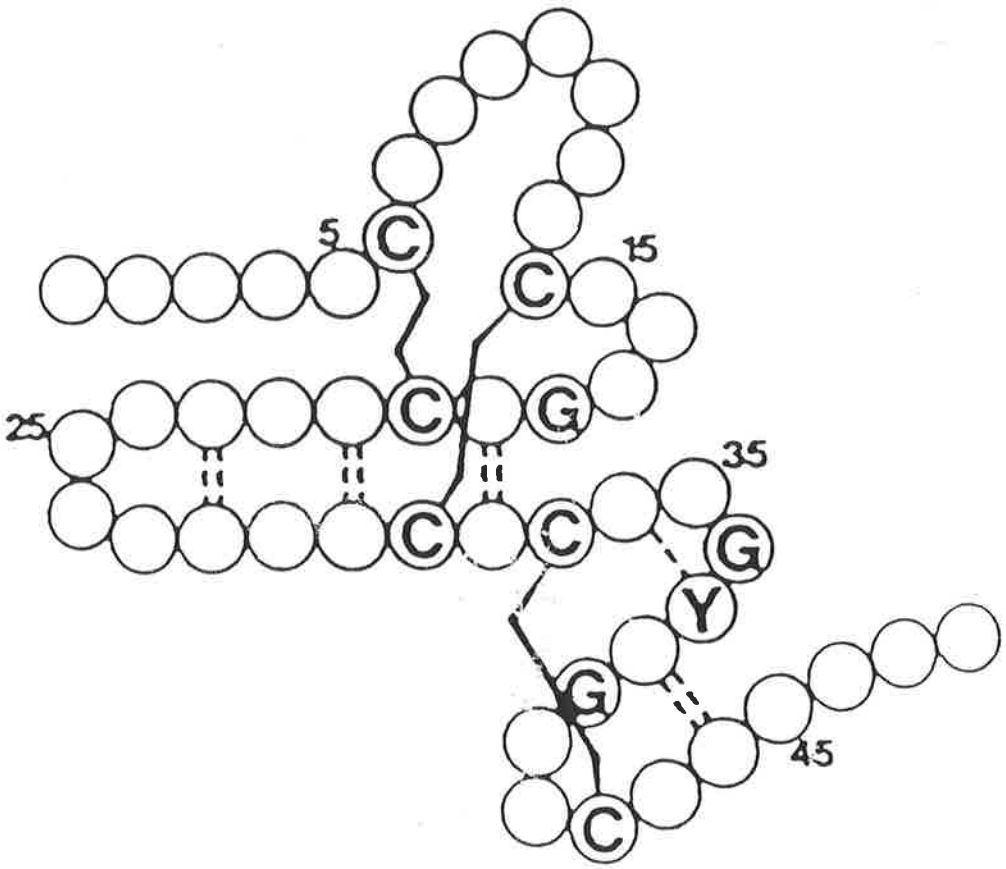
the motif is repeated nine times; one additional repeat has a similar structure but lacks the amino-terminal aspartic acid and asparagine that has the potential for β-hydroxylation (Stenflo et al., 1987), which have been implicated as being important in calcium binding (Handford et al., 1991; Selander-Sunnerhagen et al., 1992). There are three additional repeats clustered near the amino-terminal end of the fibrillin molecule that exhibit the characteristic six-cysteine pattern but lack the putative calcium binding consensus sequence and most of the other conserved residues found within the cbEGF-like motifs (Corson et al., 1993). These are designated by the term EGF-like motif. In total, there are 43 cbEGF-like motifs and 4 EGF-like motifs in the fibrillin molecule. The secondary structural features of human EGF are shown in Figure 1.5.

The biological function of these domains is thought to be dependent on calcium binding which allows for crucial protein-protein interactions (Selander-Sunnerhagen et al., 1992). The replacement of one cysteine is predicted to have an obligate effect on the conformation of an individual repeat and on the function of a tandem array.

1.1.5.5 TGF-β1 BP motifs

Another motif, which contains eight cysteines in a well-conserved spacing, is interspersed among the cbEGF-like motifs and recurs seven times in the fibrillin molecule (Corson et al., 1993). This motif is approximately 70 amino acids long. Three of the eight-cysteine containing motifs appear consecutively near the centre of the fibrillin molecule. Six of these motifs are interspersed among the cbEGF motifs of region D (Figure 1.3); they are encoded by exons 16/17, 24, 37/38, 41/42, 50/51 and 57 (Pereira et al., 1993a). It is not yet known whether the cysteine residues in these motifs are free or involved in intrachain disulphide bonds. However, the cluster of three consecutive cysteines in the centre of the motif may implicate free cysteines here and this cluster could conceivably have a role in interchain disulphide bonding (Pereira et al., 1993a; Dietz et al., 1994). The potential cell-attachment sequence of fibrillin is within one of these 8-cysteine motifs (Figure 1.2). So far, these eight-cysteine repeats have been found in only one other gene, the one coding for the TGF-β1 BP (Kanzaki et al., 1990). TGF-β1 BP is the third component of the large latent TGF-β1 complex. The consensus amino acid sequence of the TGF-β1 BP motif of fibrillin is shown in Figure 1.3.

Figure 1.5 The Secondary Structural Features of Human Epidermal Growth Factor



Disulphide bridges are denoted by solid lines and hydrogen bonds by dashed lines. The molecule comprises two subdomains (residues 1-32 and residues 33-48). Residues 19 to 23 and 28 to 32 form an antiparallel β-sheet with a β-turn involving residues 24 to 27. The carboxy-terminal β-sheet involves residues 37 to 38 and 44 to 45. Some highly conserved amino acids are marked. These are represented by the standard one letter code.

(after Cooke et al., 1987)

The TGF-βs have dramatic effects on growth and differentiation of most cell types, and they are synthesised by many different cell types. These facts indicate that the activation of TGF-βs from their high molecular latent complexes must be an important regulatory step in vivo (Kanzaki et al., 1990). Despite this knowledge, little is known of the nature and function of the TGF-β1 BP motifs.

1.1.5.6 The hybrid motifs

A novel third, and cysteine-rich, motif consisting of approximately 65 amino acids, occurs twice within the fibrillin molecule. This has been called the hybrid motif because it appears to comprise both TGF-β1 BP and EGF-like sequences and is unique to fibrillin (Pereira et al., 1993). The evolutionary origin of the hybrid motif is unknown. Homology between the hybrid and the TGF-β1 BP motifs is stronger in the amino-terminal portion; a weaker homology exists between the hybrid and EGF-like motifs in the carboxy-terminal portion. In 40 of the 47 EGF-like repeats in fibrillin, the amino acid on the carboxyl side of the glycine residue in the consensus sequence, -C-X-C-X-X-G-X_9-C-X, is tyrosine or phenylalanine, which is present in the hybrid sequence. The analagous postion in the TGF-β1 BP motif is usually occupied by proline, followed by glycine. One notable divergence within the amino-terminal portion is the presence of only two cysteines at the position homologous to the characteristic central CCC triplet of the TGF-β1 BP motif (Corson et al., 1993).

1.1.5.7 The carboxy-terminal region

The carboxy terminus is a cysteine-poor region (only 2 of the 184 amino acid residues are cysteines) that is unusually rich in lysine and arginine residues (Maslen et al., 1991).

1.1.6 The Fibrillin Gene

The fibrillin gene is relatively large (approximately 110 kb) and highly fragmented: it is organised into 65 exons. The 9.6 kb of transcript has now been characterised and the intron/exon boundaries of the fibrillin gene have been defined (Pereira et al., 1993a; Corson et al., 1993). Fifty of the 57 cysteine-rich motifs are encoded by single exons. Another fibrillin gene on chromosome 5 (FBN2) is organised in a structurally similar fashion to FBN1. The cloning of this second fibrillin-like transcript was followed by the genetic linking of this locus to congenital contractural arachnodactyly, a rare disorder

that shares some of the skeletal manifestations of Marfan syndrome (Lee et al.,1991). The discovery of two fibrillins and their association with overlapping phenotypes strongly suggest that these molecules have related functions in the extracellular matrix. It has been suggested that most of the ancestral fibrillin gene arose by multiple duplications of a cbEGF-like coding exon (Pereira et al., 1993a). After the inclusion of the eight-cysteine coding exon, rearrangements between this and the cbEGF-like coding exon created the units for the other cysteine-rich motifs in fibrillin. Subsequently, the ancestral gene duplicated and relocated to different chromosomes to give rise to FBN1 (chromosome 15q15-q21) and FBN2 (chromosome 5q23-q31).

The full-length human pre-fibrillin mRNA comprises 9,662 nucleotides, with 5' and 3' untranslated sequences of 134 and 916 nucleotides, respectively, and an open reading frame (ORF) of 8,613 nucleotides or 2,871 amino acids (Pereira et al., 1993a). The methionine at nucleotide position -2694 (Maslen nomenclature) is the predicted initiation site for translation (Corson et al., 1993).

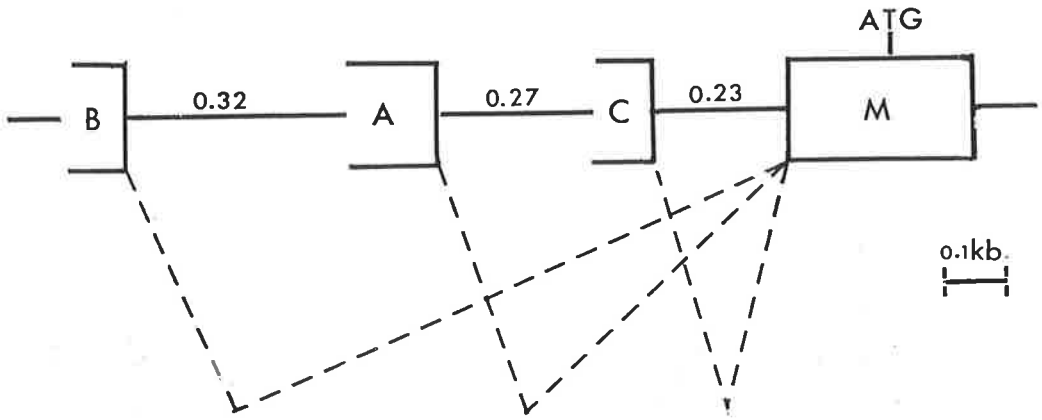
1.1.6.1 Alternatively spliced exons at the 5' end of FBN1

Cloning of the 5' end of the FBN1 gene revealed the presence of alternative exon utilisation in the 5' region (Corson et al., 1993). One of three small exons (called A, B or C) is spliced to a shared downstream exon (M) (Figure 1.6). Exon A, the middle of the 3 exons, is the most commonly included exon. Both exons A and B contain small ORFs which are in-frame with the rest of the cDNA. The ORF of exon C is interrupted by an in-frame termination codon six triplets upstream of the splice junction. This might imply, at least for transcripts including exon C, the utilisation of the ATG (at position -2694; Maslen nomenclature) within the common exon M as the translational start site. The start site of transcription has not been confirmed (Corson et al., 1993).

1.1.6.2 The promotor region

Sequencing of the region directly upstream of the three alternatively spliced exons described, failed to reveal any CCAAT or TATA box promotor elements. These three exons are within a CpG island that extends over 1.8 kb (Corson et al., 1993). CpG islands overlap the 5' ends of all housekeeping genes and many other widely expressed genes involved in the regulation of growth and development (Gardiner-Garden and Frommer, 1987).

Figure 1.6 Alternatively Spliced Exons at the 5' end of the FBN1 Gene



The three most 5' exons of the FBN1 gene (B, A, C) are shown above as open boxes. The broken lines show alternative splicing of these exons to a shared exon (M) that contains an in-frame ATG codon.

(after Corson et al., 1993)

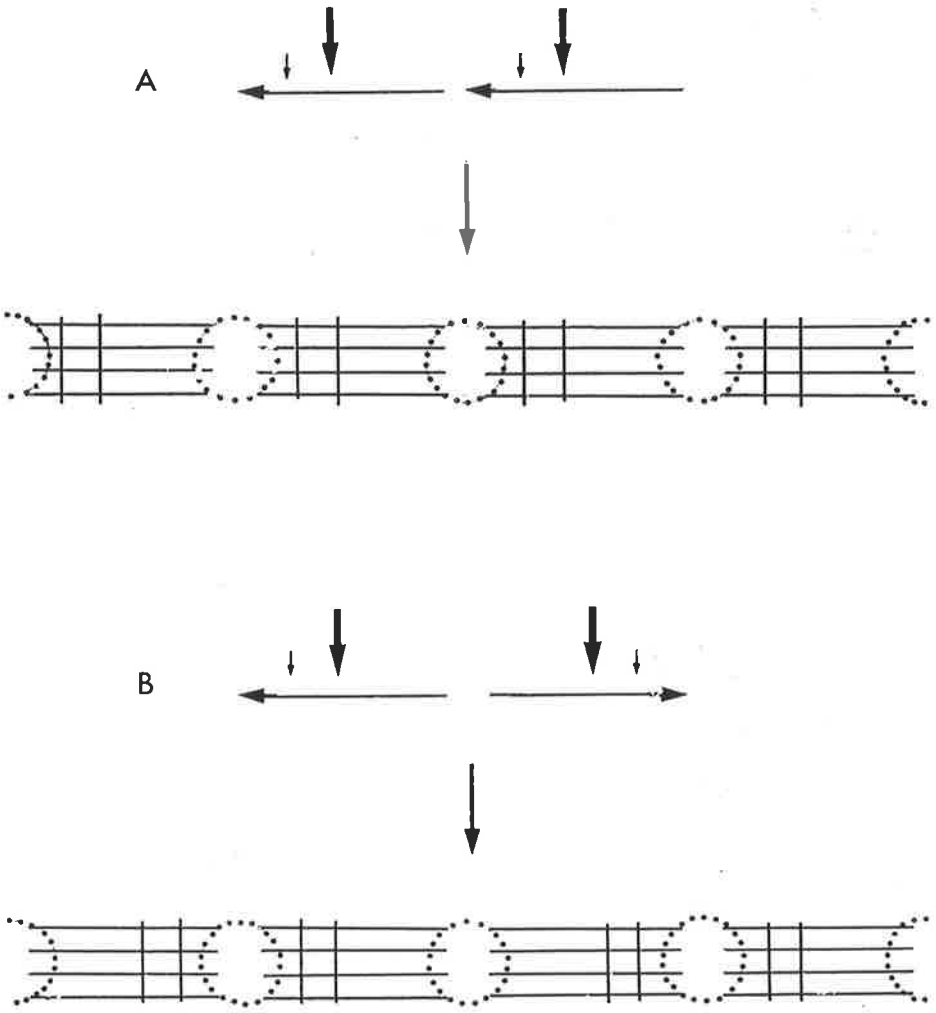
1.1.7 Microfibrillar Structure and Assembly

Microfibrils have proven very difficult to analyse because they are highly insoluble. Ultrastructural analyses have demonstrated the complex architecture of fibrillin microfibrils (Kielty et al., 1991; Keene et al., 1991; Wright and Mayne, 1988). They exhibit a pronounced beaded morphology with a diameter of 10-12 nm and an average, but variable, periodicity of 55 nm. The diameter of the beaded structures are between 15-22 nm and the average periodicity of these globular portions varies from 33 to 94 nm between different microfibrils. The periodicity is usually constant in any one microfibril (Keene et al., 1991; Kielty et al., 1991; Sakai et al., 1991). Electron microscopy rotary shadowed images of fibrillin molecules and microfibrils are shown in Figures 1.1 (a) and (b).

The fibrillin-specific monoclonal antibody 69 which reacts with PF3, was shown to consistently label only one side of the beaded structures of microfibrils, demonstrating that these regions are asymmetric (Keene et al., 1991). The periodicity of the binding of several fibrillin-specific monoclonal antibodies was consistent with a head-to-tail alignment of the fibrillin monomers in microfibrils (Sakai et al., 1991). These are subsequently organised into the microfibril superaggregates recognisable as tubular 10-nm fibres on electron microscopy (Keene et al., 1991). Two possible models have been suggested for the alignment of fibrillin molecules in microfibrils (Sakai et al., 1991). One model proposes that multiple fibrillin molecules assemble in a parallel head-to-tail alignment to form microfibrils. The second model proposes the assembly of multiple fibrillin molecules in an anti-parallel, head-to-head and tail-to-tail alignment. These models are represented diagramatically in Figure 1.7. Additional evidence supporting the head-to-tail model comes from cross-linked peptides that have been isolated and shown to contain amino acid sequences from both the amino and carboxy terminal domains of fibrillin (Sakai et al., 1993).

Electron microscopy of microfibrils in stretched ligament tissue has shown that the periodicity of fibrillin specific antibody staining increased from 52 to 75 nm (Keene et al., 1991), highlighting the flexibility of fibrillin monomers. This larger antibody labelling period observed on microfibrils when tissues are stretched was the first evidence supporting a possible molecular basis for the role of microfibrils in tissue extensibility. The treatment of tissues with a disulphide bond reducing agent leads to the disappearance

Figure 1.7 Two Possible Models for the Alignment of Fibrillin Molecules in Microfibrils



Fibrillin monomers, represented as lines with arrowheads at the amino-terminal ends, assemble through unknown steps into microfibrils, represented as periodic circles and groups of lines. The number of molecules assembled into microfibrils is unknown. The heavy arrow marks the relative position of a fibrillin-specific monoclonal antibody mAb 201; the fine arrow, a hypothetical position of mAb 69. The broken circles represent potential sites of interaction between fibrillin and other requisite structural proteins. The vertical lines represent antibody banding patterns.

A: shows a parallel, head-to-tail linear arrangement of fibrillin molecules, and matches the known immunohistochemical data

B: shows an anti-parallel arrangement of fibrillin molecules and results in two antibody banding periods, which is inconsistent with immunohistochemical data

(after Sakai et al., 1991)

of microfibrils. Reduction of beaded microfibrils also leads to loss of morphology, whereas nonreducible cross-links maintain the aggregates. Only small amounts of fibrillin monomers can be solubilised from fetal tissues by reducing agents, suggesting that only newly synthesised fibrillin is released by reduction and that fibrillin is normally further cross-linked by nonreducible bonds (Sakai, 1990). Fibrillin is secreted as a monomer and then very rapidly assembled into disulphide bonded aggregates.

Candidate domains in fibrillin that may participate in intermolecular disulphide cross-linking include the amino and carboxy terminal ends. The proline-rich domain in fibrillin has been proposed to function as a bend or hinge, facilitating interactions required for assembly (Pereira et al., 1993a; Zhang et al., 1994). Calcium binding may also be important in the assembly of fibrillin. Calcium has been shown to promote the deposition of fibrillin into the extracellular matrix of fibroblasts in culture (Aoyama et al., 1993). In addition, the removal of calcium with a chelating agent has been shown to alter the conformation of beaded fibrils (Kielty and Shuttleworth, 1993).

1.1.8 Fibrillin Biosynthesis, Metabolism and Extracellular Assembly

Relatively little is known about fibrillin biosynthesis, metabolism and extracellular assembly into the microfibrillar network, but studies of fibrillin immunofluorescence and [^{35}S] cysteine pulse-chase studies from dermal fibroblasts in normal individuals and in Marfan syndrome have shed some light on these processes.

The first biochemical data implicating fibrillin in the pathogenesis of Marfan syndrome came from the consistent and specific demonstration of decreased immunofluorescence of anti-fibrillin antibody-stained dermal biopsies and cultured fibroblasts in affected patients compared to controls (Hollister et al., 1990). This and other data (Godfrey et al., 1990a) showed that the microfibrillar abnormalities cosegregated with the Marfan phenotype in families and were not found in unaffected family members. These immunostaining results were observed in about 90% of individuals with an unequivocal clinical diagnosis of Marfan syndrome (Hollister et al., 1990). In the same study, 25% of patients in a group with "other connective tissue disorders" also exhibited abnormal fibrillin immunofluorescence, indicating that these microfibrillar findings were not specific to Marfan syndrome. Importantly, however, none of the disorders in this latter group could be confused clinically with Marfan syndrome. Patients with mitral valve prolapse

syndrome and annuloaortic ectasia who have some of the cardiovascular manifestations of Marfan syndrome, but no ocular or skeletal involvement, had normal immunofluorescence findings. Similar studies were conducted in an unusual patient with unilateral Marfan syndrome (presumably due to somatic mosaicism), in whom the deficiency of microfibrils was shown to be limited to the affected side of the body (Godfrey et al., 1990b). On the basis of these data, it was hypothesised that molecular defects of one or more constituent structural glycoproteins might produce deficient or functionally incompetent microfibrils.

The fibrillin glycoprotein is secreted within 4 hours of synthesis and deposited into the cell-associated matrix as a smaller protein (320 kD) that presumably arises by proteolytic cleavage. The deposited protein, either alone or in conjunction with other proteins, aggregates to form both the microfibrillar network associated with elastic fibres and the isolated microfibrillar network present throughout the body (Cotta-Pereira et al., 1976). Fibrillin then becomes incorporated into poorly soluble fibrillar structures and stabilised by inter- and intramolecular disulphide bonds.

Results of [^{35}S] cysteine pulse chase studies on dermal fibroblasts from Marfan syndrome patients suggest that fibrillin is synthesised as a precursor protein (profibrillin), secreted from the cell, and processed extracellularly to a lower molecular weight product (fibrillin). Findings also suggest that most of the fibrillin found in the extracellular matrix is secreted and deposited directly into the matrix. Studies of fibrillin synthesis, secretion and aggregation into the extracellular matrix by dermal fibroblasts have shown abnormalities in 22 of 26 (84%) Marfan syndrome fibroblast cell lines (Milewicz et al., 1992). One-third of the cell lines synthesised about half the normal amount of fibrillin, consistent with a single null allele or with production of a fibrillin with different electrophoretic mobility that was not identifiable. An additional third of cell lines synthesised fibrillin molecules that were poorly assimilated into the extracellular matrix. One- third of cell lines showed defects in the ability of cells to incorporate fibrillin into the pericellular matrix. These defects did not alter the amount of fibrillin synthesised or the efficiency of secretion of the synthesised molecules, suggesting that conformational requirements for fibrillogenesis may differ from those for secretion.

1.2 Marfan Syndrome: The Clinical Entity

Marfan syndrome is a heritable disorder of connective tissue, with manifestations involving the ophthalmologic, musculoskeletal and/or cardiovascular systems. It is one of the most common genetic disorders, estimated to affect one in 5,000 people. Marfan syndrome has no predilection for race or sex. It is rare for any one patient to exhibit all the cardinal manifestations of the disease. The Marfan clinical spectrum varies from mildly affected individuals with minimal morbidity, to those with the severe lethal neonatal form of the disease. There is marked clinical variability in the distribution and severity of manifestations both within and between families. The lack of a reliable detection test for the disorder, combined with a high rate of sporadic mutations and the marked clinical variability, may be responsible for an underestimation of disease prevalence. Diagnosis of the more mildly affected individual, especially if a sporadic occurrence, can be difficult. Diagnostic criteria have been developed (Pyeritz et al., 1979; Beighton et al., 1988) to aid in the evaluation of the patient who may have Marfan syndrome (Table 1.1). The diagnosis is based on a careful personal and family history and physical examination, with specific attention to the systems involved.

1.2.1 Cardiovascular Features

In over 90% of cases in which a cause of death is identifiable, the cardiovascular system is involved and the most common cause of death is from aortic dissection (Murdoch et al., 1972). With the advent of echocardiography, the reported incidence of cardiovascular involvement has increased to more than 95% of all patients with Marfan syndrome. Physical examination is inadequate to recognise the cardiovascular abnormalities in Marfan syndrome, and echocardiography, computerised axial tomography or magnetic resonance imaging is necessary. The most frequent cardiovascular manifestations include aortic root dilatation, aortic dissection and aortic regurgitation. Mitral valve prolapse and mitral regurgitation occur frequently (80%).

The associated complications of aortic root dilatation (Figure 1.8(a) and (b)), and the rate of progression of aortic root dilatation, are difficult to predict. Echocardiographic criteria for aortic root dilatation (Brown et al., 1975) and a nomogram that correlates aortic root diameter with height in healthy subjects whose height is greater than the 95th percentile, have been established (Reed et al., 1993). Dissection of the adult aortic root generally

Table 1.1 Diagnostic Manifestations of the Marfan Syndrome

Skeletal	
	Pectus excavatum and/or carinatum
	Dolichostenomelia not due to scoliosis
	Arachnodactyly
	Scoliosis, thoracic lordosis/ kyphosis
	Tall stature, especially compared to unaffected first degree relatives
	High narrow arched palate, dental malocclusion
	Protrusio acetabulae
	Congenital flexion contractures, joint hypermobility
Ocular	
	Ectopia lentis*
	Flat cornea, elongated globe, retinal detatchment, myopia
Cardiovascular	
	Dilatation of the ascending aorta*
	Aortic dissection*
	Aortic regurgitation, mitral regurgitation due to mitral valve prolapse, mitral valve prolapse
	Abdominal aortic aneurysm, dysrhythmia, endocarditis
Pulmonary	
	Spontaneous pneumothorax, apical bleb
Skin and integument	
	Striae distensae
	Inguinal hernia, other hernia (umbilical, diaphragmatic, incisional)
Central nervous system	
	Dural ectasia*
	Lumbosacral meningocoele, dilated cisterna magna

(after Beighton et al., 1988). Asterisks indicate major manifestations

Figure 1.8 (a),(b) CAT Scan of the Thoracolumbar Aorta in Marfan Syndrome

(a)

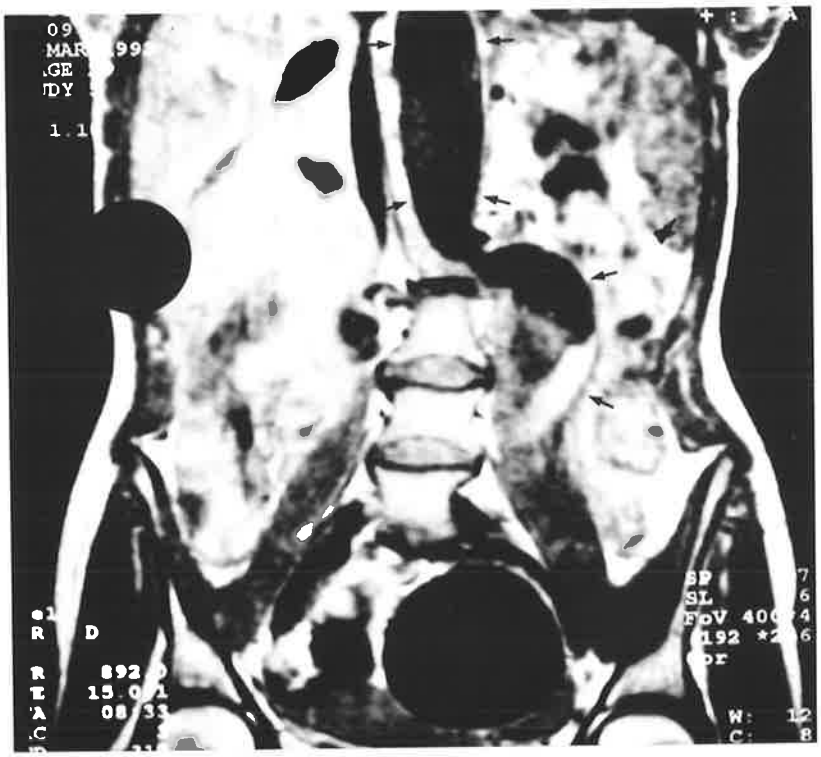


Figure 1.8 (a). Anteroposterior view showing diffuse aortic dilatation (patient 25), indicated by arrows

(b)

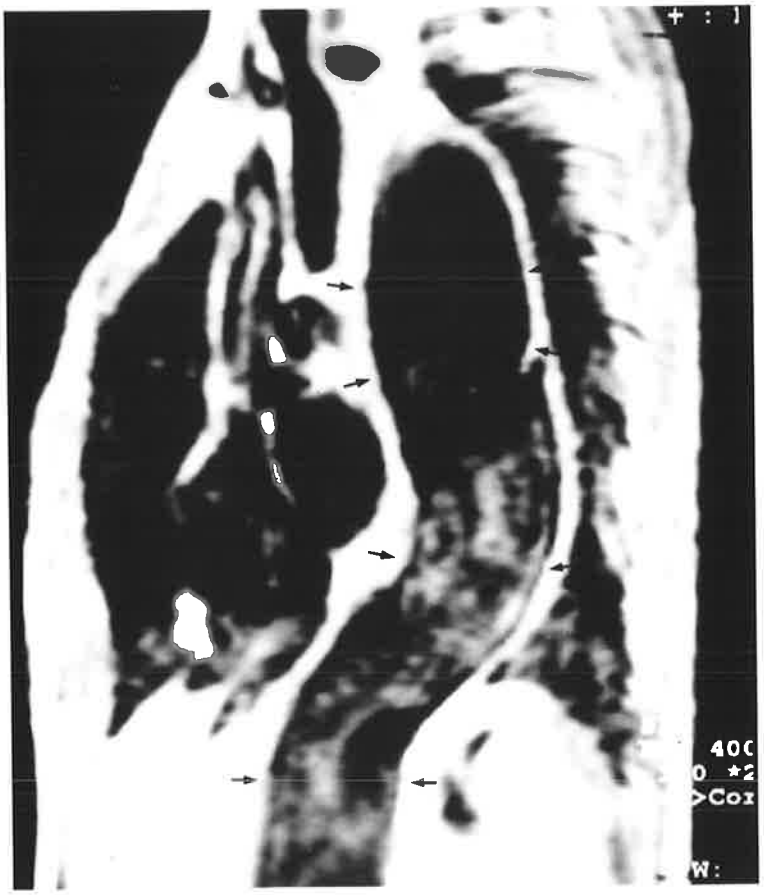


Figure 1.8 (b). Lateral view showing diffuse aortic dilatation (patient 25), indicated by arrows

does not occur before the maximal dimension at the sinuses of Valsalva reaches 55-60 mm (Dietz et al., 1994) but 20% of dissections occur with a normal aortic root size (less than 40 mm) (Bosner et al., 1993). In childhood, such a correlation of aortic size and the risk for dissection has not been clearly established. Severe mitral regurgitation develops in one of every eight patients with Marfan syndrome by the third decade (Lima et al., 1985). Less common cardiovascular features include tricuspid valve prolapse, pulmonary artery dilatation and dissection, descending thoracic or abdominal aortic aneurysm, and primary cardiomyopathy.

1.2.2 Ocular Features

The ocular pathology is characterised by preadolescent and severe myopia, and lens dislocation (Maumenee, 1981). Superior dislocation of the lens (Figure 1.9) occurs in up to 80% of patients. Surgical removal of the lens is reserved for situations when the displaced lens directly obstructs the path of light. High myopia and/or ectopia lentis can predispose to refractive errors, glaucoma and retinal detatchment.

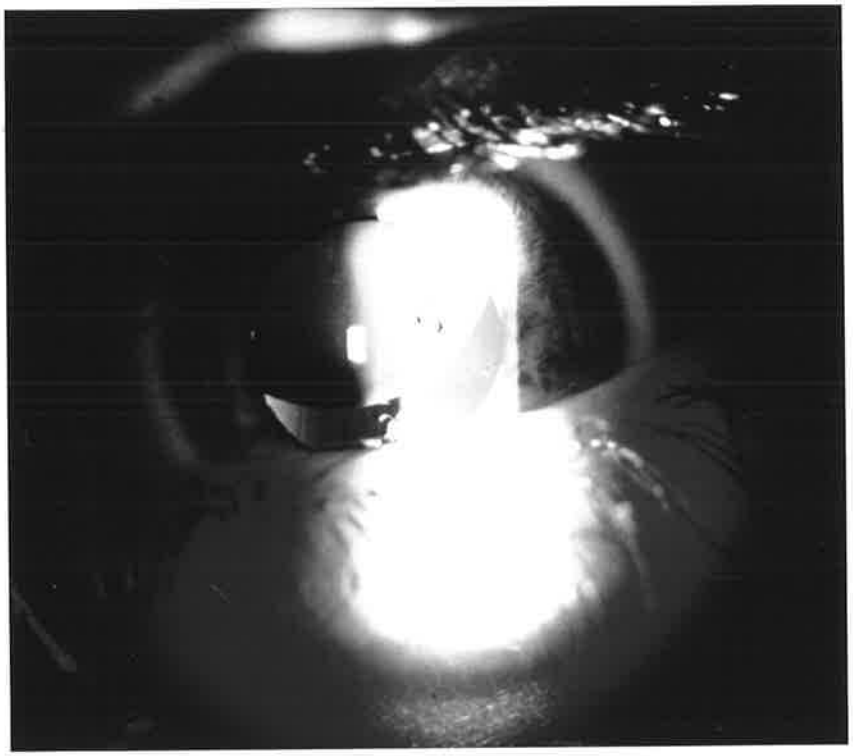
1.2.3 Skeletal Features

Skeletal involvement is characterised by overgrowth of the long bones (Magid et al., 1990). This produces disproportion of the limb length in comparison to the trunk, and is responsible for an overall reduction in the upper to lower segment ratio. It has been suggested that the periosteum may normally provide a physical restriction to the rate and extent of bone growth, and that this structural inhibition might be released if the periosteum is abnormally lax. Other manifestations include tall stature, arachnodactyly, joint contractures, pes planus, pectus excavatum and/or carinatum, scoliosis, and a highly arched and narrow palate with dental overcrowding. Joint hyperextensibility is common. The consequences of these abnormalities may include joint dislocation, chronic pain, difficulty with ambulation, and cardiorespiratory compromise.

1.2.4 Other Features

Less common manifestations in Marfan syndrome include herniae, spontaneous pneumothorax, striae distensae, dural ectasia, protrusio acetabulae and mild learning disability with or without hyperactivity.

Figure 1.9 Superior Dislocation of the Lens in Marfan Syndrome



Visualisation of upwardly dislocated lens by slit lamp examination

1.2.5 Diagnosis

The current diagnostic guidelines were formulated by a group of experts who participated in a Workshop held during the 7th International Congress of Human Genetics in Berlin in 1986. In the absence of a family history, current diagnostic guidelines require the involvement of the skeleton and at least two other organ systems, with at least one "major" manifestation (Table 1.1). In the presence of an unequivocally affected first degree relative, there must be involvement of at least two organ systems; a "major" manifestation is preferred but not required. It was also recommended that homocystinuria be specifically excluded by urinary amino acid analysis in the absence of pyridoxine supplementation (Beighton et al., 1988).

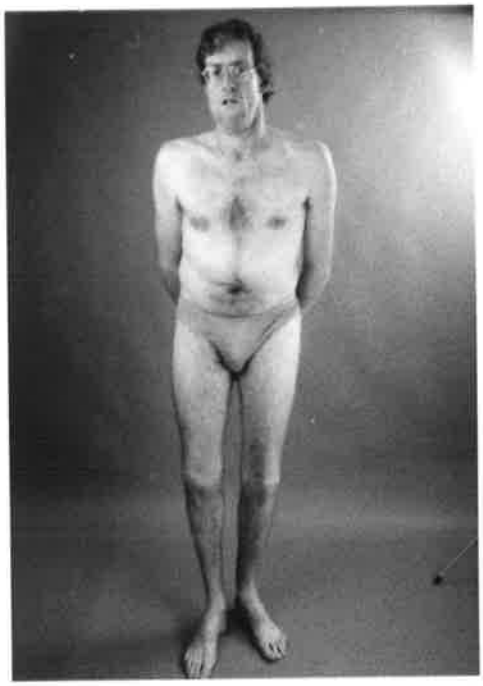
1.2.6 Differential Diagnosis

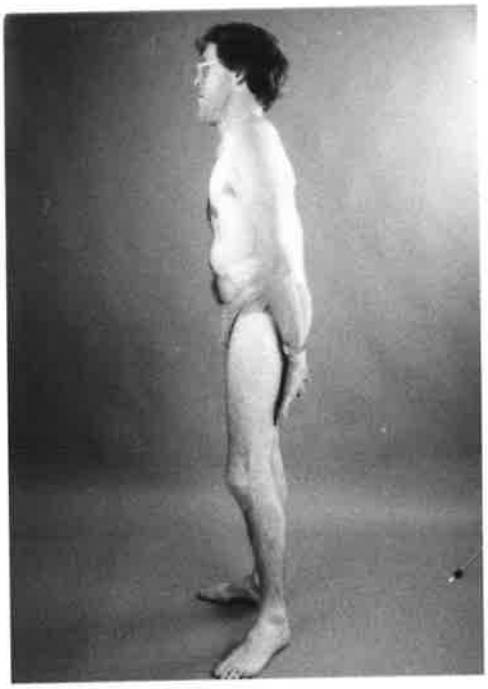
The Marfan syndrome occupies one end of a phenotypic continuum, which at the other end blurs into the general population. Some patients who do not have Marfan syndrome will display several "typical" features (Glesby and Pyeritz, 1989). In other families, successive generations may be affected by some of the more severe manifestations, yet those patients do not have Marfan syndrome either; examples include autosomal dominant ectopia lentis (Tsipouras et al., 1992a), familial aortic dissection (Nicod et al., 1989), and familial annuloaortic ectasia (Kontusaari et al., 1990). Molecular biology has begun to shed some light on our understanding of how these phenotypically distinct disorders relate to Marfan syndrome.

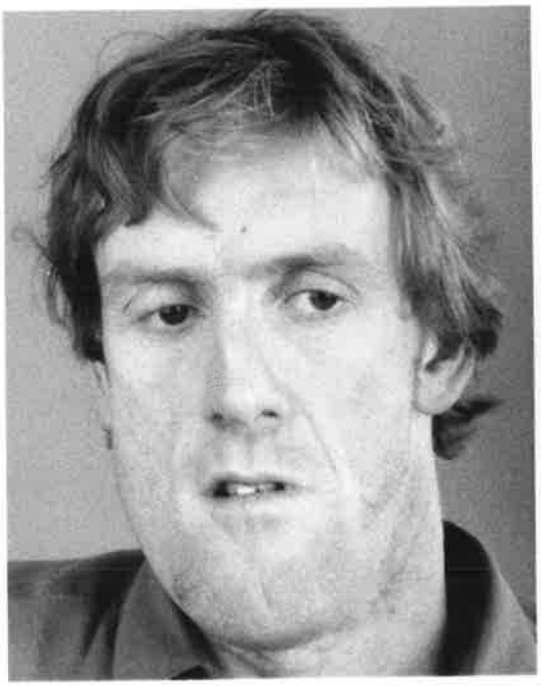
In evaluating any patient with a suspected diagnosis of Marfan syndrome, several other conditions should be considered in the differential diagnosis. Homocystinuria (MIM 236200) is due to a deficiency of cystathionine β-synthase. Affected individuals (Figure 1.10) have tall stature, dolichostenomelia, scoliosis, anterior chest deformity, and ectopia lentis. Homocystinuria can be differentiated from Marfan syndrome by the additional features of mental retardation and vascular thromboses, and by its autosomal recessive inheritance. Sporadic cases may be difficult to diagnose. Diagnosis is confirmed by the finding of homocystine in the urine and plasma of an affected individual. False negative results may occur in the presence of pyridoxine supplementation. Stickler syndrome (MIM 108300) shares several features with Marfan syndrome, including autosomal dominant inheritance, tall stature, retrognathia, midfacial hypoplasia, joint problems,

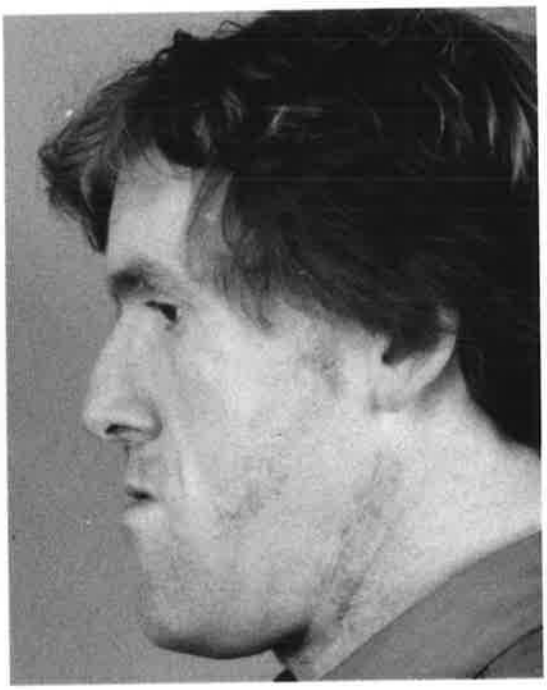
Figure 1.10 Clinical Features of Homocystinuria (Patient 48)

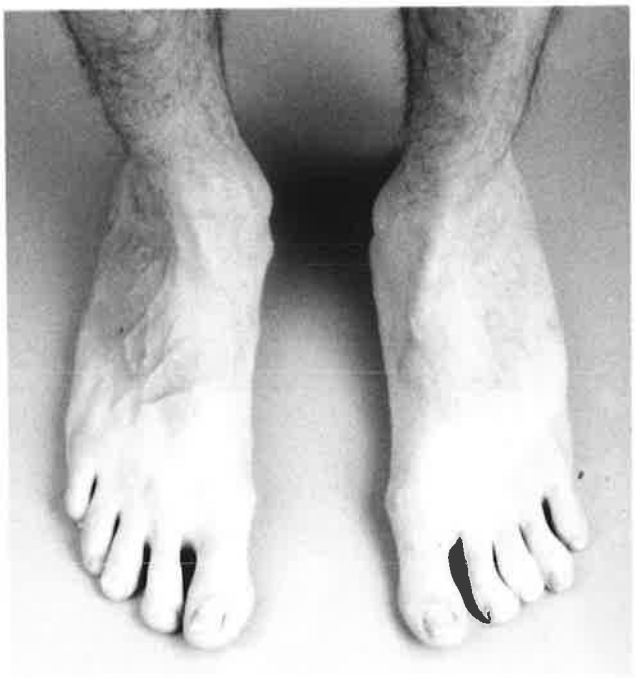
(see overleaf)











retinal detatchment, myopia, and mitral valve prolapse. It can usually be differentiated by the characteristic vitreoretinal degeneration, and by a family history of cleft palate or deafness segregating with the skeletal and ocular abnormalities. Some cases of Stickler syndrome are due to mutations at the type II procollagen locus (Francomano et al., 1987; Ahmad et al., 1991).

The skeletal features of Marfan syndrome occur in Ehlers-Danlos syndromes types II (MIM 130010) and III (MIM 130020), multiple endocrine neoplasia type III (MIM 162300), Klinefelter syndrome and congenital contractural arachnodactyly MIM 121050). With regard to ocular findings, retinal detatchment occurs in Ehlers-Danlos syndrome type VI (MIM 225400), and ectopia lentis is common in Weil-Marchesani syndrome (MIM 277600) and occurs as isolated autosomal dominant (MIM 129600) and recessive traits (MIM 225200 and 225100).

Among Mendelain disorders affecting the cardiovascular system, Ehlers-Danlos syndrome type IV (MIM 130050) causes rupture of large arteries due to defective type III collagen. Aneurysms rarely form but if present, usually involve the abdominal aorta, not the ascending (thoracic) aorta which is primarily affected in Marfan syndrome. A defect in type III procollagen was reported in one family prone to thoracic and abdominal aortic aneurysms (Kontusaari et al., 1990). After this discovery of a family that included four individuals affected with aneurysms of various arteries, it was hypothesised that mutations in the COL3A1 gene could be responsible for a major part of isolated aortic aneurysms (Kuivaniemi et al., 1991). A larger study of 54 probands failed, however, to reveal additonal missense mutations in COL3A1 that cosegregated with the aneurysm phenotype (Tromp et al., 1993), suggesting that the initial family was affected with a variant of Ehlers-Danlos syndrome type IV.

Some of the conditions mentioned above, including autosomal dominant ectopia lentis, familial aortic aneurysms, congenital contractural arachnodactyly and other conditions that warrant consideration in the differential diagnosis of Marfan syndrome such as annuloaortic ectasia and the mitral valve prolapse syndrome, are expanded upon further in Section 1.4.1.

1.2.7 Intrafamilial Variability in Marfan Syndrome

One unresolved clinical dilemma is the molecular basis for the marked intrafamilial variability that has been documented in Marfan syndrome. Many FBN1 gene mutations segregating with disease in kindreds with classic Marfan syndrome have now been reported. In one such large kindred, the phenotype varied widely with respect to age of onset, tissue distribution, and severity of manifestations (Dietz et al., 1992a). The only two individuals with an identical phenotype were monozygotic twins. These data suggest the presence of genetic modifiers influencing the clinical expression of an FBN1 gene defect. Such modifiers would vary with genetic background, explaining divergent phenotypes in and between families. Environmental modifiers may conceivably also affect phenotypic expression.

1.2.8 Genetic Heterogeneity in Marfan Syndrome

That genetic heterogeneity exists in Marfan syndrome is currently a contentious issue. Heterogeneity has been suggested by the report of a family in which Marfan syndrome segregated independently of the fibrillin gene and eight other chromosome 15 markers (Boileau et al., 1991). More recently, the same researchers claim to provide definitive evidence for the assignment of a second locus for Marfan syndrome to the chromosomal region 3p24.2-p25 (Collod et al., 1994) based on the family first reported by them in 1991. These findings have been forcefully challenged, firstly because the clinical presentation of this kindred does not meet the diagnostic criteria for Marfan syndrome and secondly, because the assignment of "affected" status by the authors is arbitrary (Dietz et al., 1995a). Definitive evidence for genetic heterogeneity in Marfan syndrome is unresolved and deserves further investigation.

1.2.9 Presymptomatic and Prenatal Diagnosis

Since the cardiovascular complications of Marfan syndrome are life-threatening, presymptomatic identification of these patients is extremely important. Presymptomatic diagnosis has been undertaken in some families (Rantamäki et al., 1994; Wang et al., 1995a), and linkage analysis and chorionic villus sampling has been used to perform prenatal diagnosis at 11 weeks' gestation (Godfrey et al., 1993a). Various strategies have been employed, including the use of the polymerase chain reaction and fluorescence labelled primers to establish linkage data in informative families (Wang et al., 1995a), or the use of polymorphic intragenic and tightly linked flanking FBN1 markers together

with haplotype segregation analysis (Rantamäki et al., 1994). Confirmation of the diagnosis is of benefit to the medical management of these patients. Recent work has demonstrated the usefulness of β-adrenergic blockade in slowing the rate of aortic root dilatation in Marfan syndrome (Shores et al., 1994). It has been suggested that the earlier this therapy is instituted in the course of the disease, the greater the benefit. In addition, the use of linkage analyses in informative families can exclude the diagnosis of Marfan syndrome (Rantamäki et al., 1994). In clinically equivocal cases, or cases where a diagnosis of Marfan syndrome has been incorrectly assigned, exclusion of the diagnosis has clear psychological benefits for the patient and also averts the need for the routine use of expensive diagnostic testing in follow-up. The use of polymorphic markers in the diagnosis of Marfan syndrome does not however, eliminate the diagnostic problem in sporadic cases (up to one third of all cases) and in small or uninformative kindreds. In these cases, for the diagnostic analysis to be reliable, identification of the specific FBN1 mutation is necessary. Molecular confirmation of the diagnosis of neonatal Marfan syndrome by localisation of a specific FBN1 mutation, may have significant implications in the medical management of these infants, and in the genetic counselling of the parents. Although genetic linkage analyses have suggested overwhelmingly that there is little, if any, heterogeneity in Marfan syndrome (Kainulainen et al., 1991; Tsipouras et al., 1992), the report of a family with the clinical diagnosis of a "Marfan-like" disease (Boileau et al., 1993), that is not linked to fibrillin (Collod et al., 1994), must be considered when performing linkage analyses. However, if a clinical diagnosis of Marfan syndrome is unequivocal, then the possibility of heterogeneity is remote.

1.2.10 Clinical Management

A multidisciplinary approach to management is essential. This should be directed towards the prevention of life-threatening complications, the optimisation of quality of life, the provision of ongoing psychological support and genetic counselling. Patients are treated with long-term β-adrenergic blocking agents to help retard the progression of aortic root dilatation by decreasing heart rate, blood pressure and vessel wall stress. They should receive antibiotic prophylaxis for dental and surgical procedures to protect against bacterial endocarditis. When the aortic root diameter exceeds 55 mm, elective prophylactic aortic root replacement with a composite graft is recommended. The operative mortality rate has been less than 2% in all elective and emergency operations, and the 5-year survival has been over 85% (Pyeritz, 1986). Haemodynamically

significant mitral valve insufficiency ultimately develops in about 25% of patients (Lima et al., 1985) and is treated with either surgical valve repair or replacement. All patients with Marfan syndrome require lifetime surveillance.

Patients should refrain from physical activities involving acceleration and deceleration, and contact sports should be limited. Activities that increase the heart rate or blood pressure and isometric exercise should be avoided, whilst participation in limited low-impact exercises should be encouraged.

Pregnancy results in increased haemodynamic stress on the aorta. Of the reported cases of aortic dissection during pregnancy, most have occurred in women with aortic regurgitation or marked aortic root dilatation. It is recommended that women with mild or no aortic root dilatation have children early in life, to be monitored as a high-risk pregnancy, and to be surveyed with an echocardiogram every six to eight weeks (Smith et al., 1989; Goldsmith, 1992).

All first degree relatives of an affected patient should be counselled, screened with echocardiography to diagnose cardiovascular involvement, and have slit-lamp examination to exclude ectopia lentis. Many patients with Marfan syndrome face lifestyle, employment and insurance restrictions, frequent medical care and financial burden. These factors may significantly affect self-esteem, interpersonal relationships and quality of life. These and the more specific issues related to the genetics and transmission of the condition, reproductive options, and the availability of prenatal or presymptomatic diagnosis, should be addressed in a genetic clinic. Other resources, providing literature and support to Marfan syndrome families, include the local and international Marfan syndrome support groups.

1.3 FBN1 Gene Mutations: Their Characterisation and Mechanisms of Action

Currently (November, 1995) 55 mutations in the fibrillin gene have been identified in patients with Marfan syndrome (Dietz and Pyeritz, 1995). Apart from the first *de novo* mutation identified in two unrelated affected individuals, and a recent report of a second recurrent mutation in unrelated Marfan individuals (Nijbroek et al., 1995), all mutations have been private or unique in every family (Peltonen et al., 1992; Milewicz, 1994).

This probably reflects a relatively high rate of new mutation, and impaired reproductive fitness in Marfan syndrome due to early morbidity and mortality. Although there is some clustering of mutations around cysteine residues in EGF-like domains, mutations have been identified with approximately equal frequency along the length of the gene, and include point mutations, in-frame deletions, skipped exons and frameshift errors. The wide distribution of both the type and location of mutations along the FBN1 gene makes diagnostic screening using DNA analysis extremely difficult.

1.3.1 FBN1 Gene Mutation Detection

Various techniques have been used to screen the FBN1 gene for mutations. Overall, the general experience has been of a disappointingly low yield of identifiable mutations, ranging from 10-20% of analysed Marfan syndrome patients (Dietz et al., 1991b, 1992a, 1992b, 1993a; Kainulainen et al., 1992; Hewett et al., 1993; Hewett et al., 1994a; Kainulainen et al., 1994). There is only one contrasting study wherein the entire FBN1 gene was screened for nine probands with classic Marfan syndrome using mutation detection enhancement (MDE) gel heteroduplex analysis. A mutation was identified in seven of nine (78%) of patients samples (Nijbroek et al., 1994). To date, this improved yield of mutations has not been reproduced in a larger number of patients, nor has it been reported by any other groups.

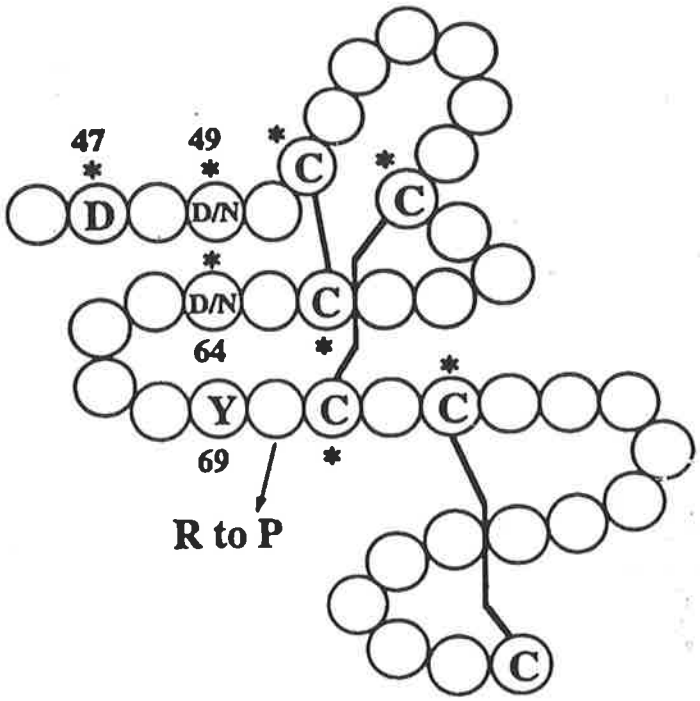
The low level of mutation detection is in apparent contradiction with the biochemical and linkage analyses which have provided clear evidence for abnormal fibrillin metabolism in the majority of Marfan syndrome fibroblast strains (Milewicz et al., 1992; Aoyama et al., 1993; Aoyama et al., 1994) and for localisation of the disease phenotype to the site of FBN1 on 15q. The reasons for this remain unknown. Contributing factors might include absent or low levels of cytoplasmic transcript of the mutant allele, or the presence of large intragenic deletions that escape detection by RT-PCR. In addition, the sensitivity of SSCP as compared to other methods of mutation detection will need to be evaluated for this particular gene. The availability of genomic sequence (Pereira et al., 1993a; Corson et al., 1993), can now be exploited to devise mutation search schemes at the genomic level. To date, protein biosynthesis studies have provided the best window on the biological consequences of fibrillin mutations at the cellular level, and may be the most promising for the development of diagnostic tests.

Two essential groups of mutations in the FBN1 gene have been defined, namely missense mutations, and those leading to a truncated protein. These are discussed in the sections below, in conjunction with postulates regarding their respective mechanisms of action.

1.3.2 Missense Mutations

Most of the identified mutations causing Marfan syndrome substitute cysteine residues in cbEGF-like domains (Dietz et al., 1992a; Tynan et al., 1993; Kainulainen et al., 1994). These mutations cause an obligate disruption of the conformation, and possibly also the calcium-binding property of a repeat. Specific residues within the EGF-like domains have been shown to be critical for calcium binding (Kanzaki et al., 1990; Handford et al., 1990; Handford et al., 1991). The structural features of fibrillin cbEGF-like domains are illustrated in Figure 1.11. These features include an antiparallel β-sheet conformation, six predictably placed cysteine residues, aspartic acid (D) and aspartic acid or asparagine (N) residues at the positions corresponding to residues 47 and 49 of the first cbEGF-like domain of human coagulation factor IX, respectively, D or N at the position corresponding to residue 64, and an aromatic residue at position 69 (Handford et al., 1991; Mayhew et al., 1992). The D/N at position 64 is found within the consensus sequence that promotes the β-hydroxylation of these residues. The ligand requirements for calcium binding to EGF-like domains have been determined through analysis of the first cbEGF-like domain in human coagulation factor IX (Mayhew et al., 1992). The wild type and several mutant sequences were chemically synthesised and studied by H-NMR spectroscopy, in the presence and absence of calcium. Residues 47 and 64 were shown to directly contribute ligands to the calcium ion. There are 4 naturally occurring mutations causing Marfan syndrome that do not substitute cysteine residues in cbEGF-like domains; all substitute residues in fibrillin 1 corresponding to positions 47 and 64 in factor IX (Dietz et al., 1993b; Hewett et al., 1993; Kainulainen et al., 1994). A fifth mutation substitutes a highly conserved glutamic acid (E) with a lysine (K) residue at a position corresponding to residue 50 in factor IX (Kainulainen et al., 1994). Although the previously discussed data suggest that calcium binding may be critical to the normal function of fibrillin, and that a majority of missense mutations causing Marfan syndrome may act by disturbing this process, the precise role of disrupted domain conformation and altered calcium binding in the pathogenesis of the disorder has yet to be elucidated. Quantitative pulse-chase studies, examining fibrillin metabolism in cell lines with known mutant phenotypes have offered some insight to this question (Aoyama et al., 1993).

Figure 1.11 Structure of Fibrillin cbEGF-like Domain



The structure of fibrillin cbEGF-like domain as modelled after the solution structure of native EGF: The disulphide linkages (bold lines) between the six predictably spaced cysteine (C) residues create an antiparallel β-pleated sheet conformation. The positions of the aspartic acid (D), asparagine (N), and aromatic tyrosine (Y) residues that are considered critical for calcium binding are indicated. The numbers indicate the corresponding positions in the first cbEGF-like domain of coagulation factor IX. The sites of naturally occurring mutations are indicated by asterisks. The position of the only mutation that does not substitute a highly conserved residue (arginine (R) to proline (P)) is shown by the arrow.

(after Dietz et al., 1994)

The cell lines showed normal levels of fibrillin synthesis (compared to control cell lines), but nearly all the cell lines carrying mutations that substituted cysteine residues in cbEGF-like repeats showed a dramatic delay in the secretion of fibrillin, and reduced fibrillin deposition into the extracellular matrix. The addition of a reducing agent to the control cells reproduced this cysteine-substitution phenotype. Delayed secretion could represent a failure of conformational folding, or the production of abnormal protein-protein complexes. The correct folding of individual cbEGF-like repeats and of a given cluster of repeats appears to be critical for normal intracellular trafficking of fibrillin. In contrast, mutations predicted to have an isolated effect on calcium binding impair extracellular incorporation. This cellular phenotype was reproducible when control lines were deprived of calcium. Abnormal conformation or altered calcium binding both lead to a failure of incorporation of fibrillin into the extracellular matrix. The mechanisms remain unclear but may include impairment of intermolecular interactions or increased susceptibility of abnormal fibrillin monomers or multimers to the activity of proteases.

1.3.3 Mutations Leading to a Truncated Protein

A second group of mutations are predicted to result in the formation of shortened fibrillin monomers. Defects include splice-site mutations leading to single exon skipping and the loss of an internal repeat, genomic deletions causing the loss of multiple repeats, and frameshift or nonsense mutations that lead to the production of monomers with carboxy-terminal truncations. All types of defects can be associated with classic Marfan syndrome (Kainulainen et al., 1992; Dietz et al., 1993a,b; Godfrey et al., 1993a; Tynan et al., 1993; Kainulainen et al., 1994). The synthesis of microfibrils is believed to involve an ordered aggregation of fibrillin monomers, possibly in association with other proteins. It has been postulated that the presence of shortened fibrillin polypeptides could interfere with intermolecular interactions and with the phasing of polymerising multimers. The abnormal structure of such aggregates could directly impair their functional integrity, or predispose them to premature degradation.

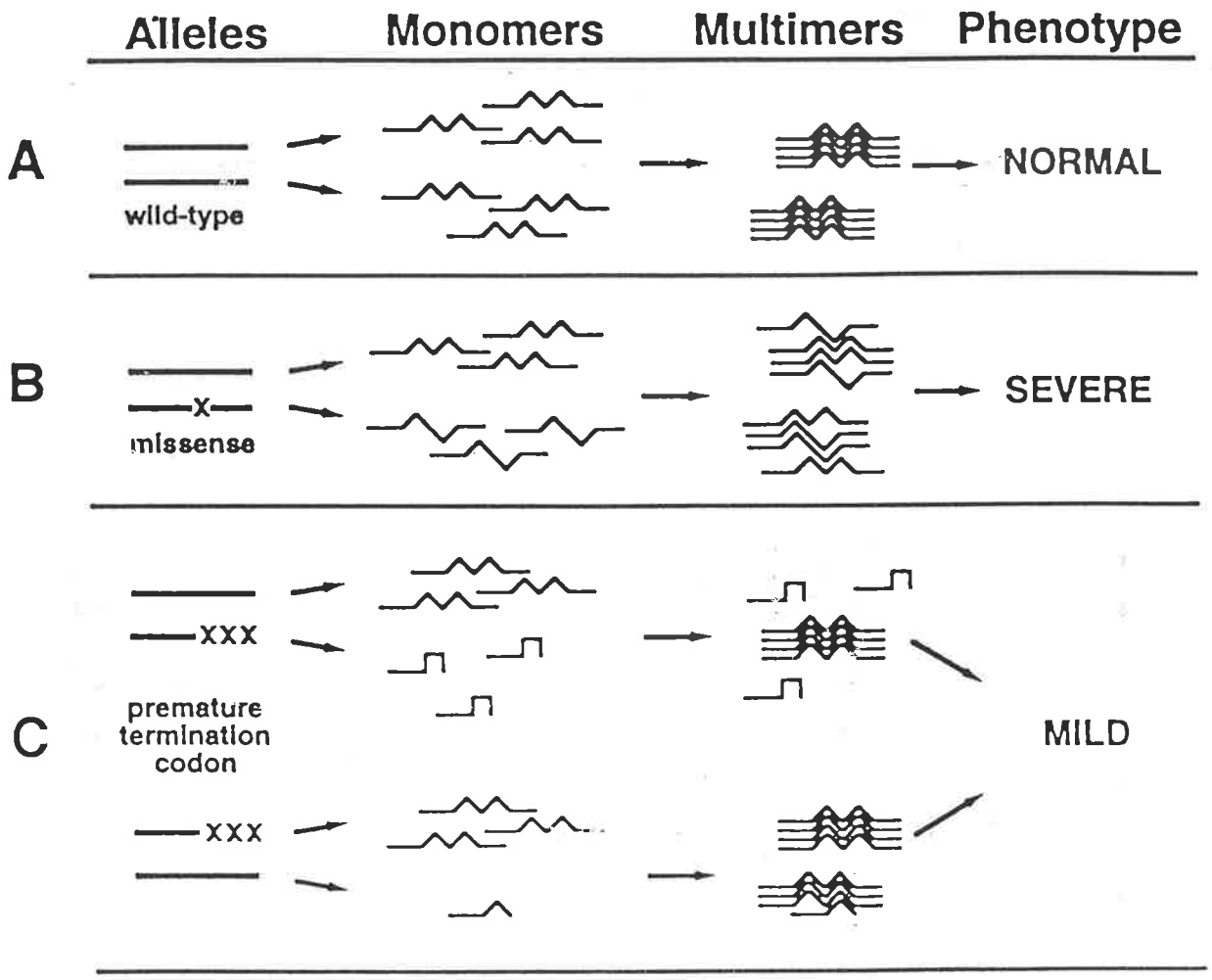
It has been shown that the presence of a premature termination codon prior to the penultimate exon of a gene, is generally associated with a severe reduction in the level of mutant transcript (Cheng et al., 1990; Urlaub et al., 1989). Two models have been put forward to account for this phenomenon. The nuclear scanning model suggests that an as yet uncharacterised nuclear factor has the ability to read the frame of mature mRNA. If a

premature termination codon is encountered, the mRNA is degraded. The translational translocation model proposes that extrusion of mRNA through the nuclear pore is physically coupled to, and partly dependent on, the cytoplasmic translation machinery. If a premature termination codon is encountered, the transcript will not pass to the cytoplasm and will ultimately be degraded.

The first mutation of this kind identified in FBN1, was a four base-pair insertion (Dietz et al., 1993b). This frameshift mutation leads to the formation of a premature termination codon and is associated with a mutant transcript level that is only 6% of that observed from the wild type allele. Unlike all patients with missense mutations and normal transcript levels, this patient had a very mild phenotype that did not meet the established diagnostic criteria for Marfan syndrome. These data suggested a dominant negative pathogenesis of the Marfan syndrome. The dominant negative model presumes that the presence of an abnormal protein that impairs processing of the wild type product, rather than a relative deficiency of the normal peptide, is critical to the expression of the disease phenotype (Herskowitz, 1987). The mild phenotype in this patient can be explained in one of two ways (Dietz et al., 1993b). The predicted truncated peptide from the mutant allele may be so different from the normal monomer, that it does not participate in multimer formation. Alternatively, the severely reduced mutant transcript level predicts very low amounts of mutant peptide and leads to a preponderance of normal multimer and an accompanying mild disease phenotype (Figure 1.12).

Such "dominant negative" mutations could exert their inhibition by producing a mutant molecule that participates with the product from a normal allele in the formation of a structurally and functionally abnormal multimer. This effect has been observed with type I collagen (COL1A1 and COL1A2) defects in osteogenesis imperfecta (OI) type II, a lethal disorder (Willing et al., 1992; Byers and Steiner, 1992). Alternatively, if the mutation produces a nonfunctional allele, the disease phenotype may result from reduction of a gene product to 50% of normal level, generated by the constitutively expressed wild type allele, as has been seen in OI type I, a clinically milder form of the disease. Additionally, the mutant product may interrupt the machinery necessary for normal protein metabolism, including interactions with other proteins or transport mechanisms.

Figure 1.12 A Dominant Negative Model for Marfan Syndrome



(A): Two normal copies of the fibrillin gene lead to homogeneous populations of normal monomer and multimer, and a normal phenotype. (B): Missense mutations that substitute one amino acid for another lead to equal populations of normal and mutant monomer, a homogeneous population of abnormal multimer, and severe disease. (C): Mutations that cause a premature termination codon, and a mild phenotype. The truncated protein could be so dissimilar to the wild-type product that the two can no longer interact. Alternatively, the reduced RNA level could predict a very low amount of mutant monomer. In either situation, there would be a preponderance of normal multimer, and mild disease.

(after Dietz et al., 1993b)

Further information has come from the study of two other reduced-transcript mutants, and from a quantitative biosynthetic study of cell lines carrying these mutant alleles (Dietz et al., 1993a,b). In one patient, a nonsense mutation in exon 51 led to a complex exon skipping phenotype and a corresponding mutant transcript level of 25%. In the other patient, a splice-site mutation caused the skipping of exon 2, resulting in a frameshift in exon 3 and a premature termination codon in exon 4. The mutant transcript level was 16% of that observed from the wild type allele. Both patients had a classic severe form of Marfan syndrome. These data suggest that a relatively small amount of mutant peptide can cause disease. Conceivably, a critical threshold, between 6 and 16% of wild type levels, may be necessary to cause sufficient disruption of microfibrillar assembly to produce the classic Marfan phenotype.

Several conclusions can be drawn from the mutation analysis. The disruption of the secondary structure of EGF-like domains (eg disruption of disulphide pairing by mutating one of the cysteines) can cause the phenotypic manifestations of Marfan syndrome. Mutations that disrupt the calcium-binding motif in the EGF-like domains can produce the disease. Selective deletion of one of these domains can also result in the disorder. Finally, truncation of the protein, even within the unique carboxy-terminal domain, can produce the disease. Surprisingly, no large genomic rearrangements leading to a non-expressed or null allele have been identified, raising the possibility that a true null allele does not cause the Marfan syndrome.

1.4 Genotype-Phenotype Correlations

In most inherited diseases, it has not been possible to predict the phenotype caused by a specific mutation. The task is especially complex for FBN1 which codes for a polypeptide chain participating extracellularly in the formation of multimeric assemblies, such as microfibrils. In the case of type I collagen, it seems that the closer to the C-terminus a mutation is situated, the more severe the resulting phenotype (Byers, 1993), probably because the folding of the individual polypeptide chains into a collagen triple helix is initiated at the C-terminal end.

Attempts have been made to correlate clinical phenotype with the corresponding biochemical fibrillin protein defect or putative FBN1 gene mutation in Marfan syndrome

and related phenotypes, although this approach remains limited by several factors. Firstly, the mutation detection rate using currently available techniques for the genomic screening of FBN1, still only yields mutations in some 10-20% of clearly affected Marfan syndrome individuals. Currently, there is no definitive correlation between the position of a mutation and the severity of the resultant Marfan phenotype or specifically affected organ systems. One possible exception is the recent report of FBN1 mutations associated with the neonatal presentation of severe disease that are clustered in the central portion of region D (see Section 1.4.1.1) (Kainulainen et al., 1994; Milewicz and Duvic, 1994). Secondly, although the results of biochemical metabolic labelling studies of dermal fibroblasts have demonstrated fibrillin defects in 85-90% of individuals with Marfan syndrome, the findings are not specific to Marfan syndrome alone, and may also be completely normal in some 10-15% of cases (Milewicz et al., 1992; Schaefer and Godfrey, 1995)

Other limiting factors include the low number of patients carrying the same mutation, the great diversity in genetic background (and of potential genetic and/or environmental modifiers) in and between families, and the inability to study protein expression and interactions at different stages of early fetal development.

1.4.1 Spectrum of Phenotypes Associated with Fibrillin Gene Mutations

Defects in the fibrillin gene have been linked to isolated cases of neonatal Marfan syndrome, familial ectopia lentis, variants of the mitral valve prolapse syndrome (MASS phenotype), common forms of aortic aneurysm and isolated skeletal features of Marfan syndrome.

1.4.1.1 Neonatal Marfan syndrome

The severe neonatal type of Marfan syndrome leads to death in early infancy (Pyeritz, 1993). These infants usually die of congestive cardiac failure. Clinical findings and fibrillin immunofluorescence patterns in cultured dermal fibroblasts, have helped to distinguish neonatal Marfan syndrome patients from those with classic disease. The analysis of cells from these neonatal cases has shown diminished incorporation of fibrillin into the extracellular matrix (Superti-Furga et al., 1992; Raghunath et al., 1993).

Thirteen fibrillin gene mutations have been published in patients with neonatal Marfan syndrome. Of these, twelve are clustered in exons 24 through to 32 of the FBN1 gene (Kainulainen et al., 1994; Milewicz and Duvic, 1994; Wang et al., 1995b; Putnam et al., 1995a, 1995b). Examination of the phenotype associated with mutations in this region leads to several observations. Firstly, missense mutations in exons 24, 25 and 26 and splicing errors that delete exon 32 result in lethal Marfan syndrome. These are the only identified FBN1 mutations described in patients with neonatal Marfan syndrome, with the one exception of a neonatal case due to compound heterozygosity for FBN1 mutations (Karttunen et al., 1994). Although these numbers remain small, the findings suggest that neonatal Marfan syndrome is associated with mutations in this central region of the FBN1 gene. As part of the research undertaken towards this thesis, a novel mutation in exon 25 of the FBN1 gene has been identified in an infant with neonatal Marfan syndrome (see Section 4.3).

Interestingly, all published reports of sporadic cases with contractures and severe cardiovascular disease (but not classified as neonatal Marfan syndrome), also have mutations in the region encompassing exons 24 to 32 of the FBN1 gene. One such patient (P002) with severe cardiovascular disease and congenital contractures did not have a mutation within this region, suggesting that some of the causative mutations in these individuals may be located outside of this restricted region of the FBN1 gene. (Putnam et al., 1995b). Only one patient with a mutation in this region has been classified as having classic Marfan syndrome (C1117Y) (Tynan et al., 1993). All of the mutations in this region are sporadic, suggesting that these mutations lead to severe disease and decreased fitness. The known FBN1 gene mutations in surrounding exons (21, 36, 38) result in classic manifestations of the Marfan syndrome (Wang et al., 1995a; Tynan et al., 1993). The severe phenotype associated with mutations in this region of the gene suggests a critical function for these domains of the fibrillin protein. The domains encoded by exons 25 to 36 are found midway in the protein and constitute the longest stretch of EGF-like domains in the protein (Figure 1.13). Exon 24 encodes an eight-cysteine domain found immediately amino-terminal to this stretch of EGF-like domains. The relative location of the EGF-like domains and the eight-cysteine domain are conserved in this region between FBN1 and TGF-β1 BP (Kanzaki et al., 1990). TGF-β1 BP is a protein that is found associated with TGF-β1 in the extracellular space. It plays a role in the assembly and secretion of TGF-β1, and is thought to target TGF-β1 to particular extracellular matrix

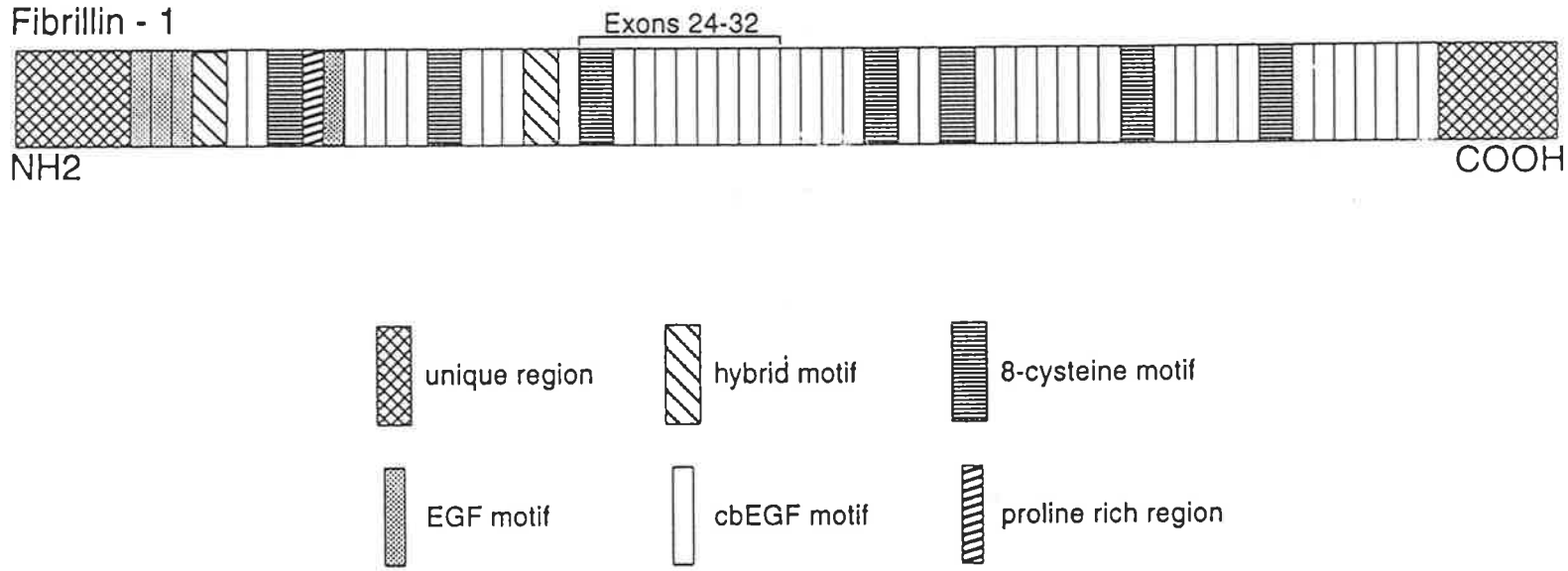
sites (Taipale et al., 1994). TGF-β1 binds to cell surface receptors, where it is thought to have an important role in controlling the production and structure of the extracellular matrix, as well as affecting cell growth, morphology and differentiation (Kingsley, 1994). The homology between fibrillin and TGF-β1 BP raises the possibility that fibrillin binds TGF-β1 during development. Mutations that result in the severe Marfan phenotype map into this fibrillin/TGF-β1 BP homologous region. Possibly, disruption of the extracellular targetting of the action of TGF-β1 during development may explain the more severe Marfan phenotype.

The interaction of fibrillin with TGF-β1 could explain why some fibroblast cell lines from patients with neonatal Marfan syndrome have decreased production of decorin, a small chondroitin-dermatan sulfate proteoglycan found in the extracellular matrix (Pulkkinen et al., 1990; Raghunath et al., 1993; Superti-Furga et al., 1992). Decorin binds TGF-β1 and neutralises the activity of the growth factor (Yagamuchi et al., 1990). TGF-β1 stimulates the synthesis of decorin by various cell types (Bassols and Massague, 1988). This evidence suggests that decorin is an effector molecule in a negative feedback loop that regulates TGF-β1 activity (Ruoslahti and Yagamuchi, 1991). The altered expression of decorin by dermal fibroblasts from patients with neonatal Marfan syndrome may reflect abnormal regulation of TGF-β1 due to interaction of TGF-β1 with mutant fibrillin proteins.

Alternatively, the abnormal fibrillin produced from a mutated allele derived from the central region of FBN1 may disrupt microfibril formation. FBN1 exons 24 to 32 may encode a region of the fibrillin protein that is critical for multimerisation into stable microfibrils. Since the mutations known to produce neonatal Marfan syndrome, including the exon splicing errors, maintain the reading frame of the protein, the production of a mutant full length fibrillin protein may be necessary to disrupt microfibril formation.

Because of the relatively small number of cases of neonatal Marfan syndrome, and the non-neonatal presentation of other mutations within this region, the apparent genotype-phenotype correlation described above requires confirmation. Definition of the phenotype associated with mutations in exons 24 to 32 of FBN1 is important, since the identification of mutations within this region may have prognostic and diagnostic implications. The

Figure 1.13 Schematic Representation of FBN1 Showing the Primary Region Encoding Mutations in Neonatal Marfan Syndrome



(after Putnam et al., 1995b)

analysis of a discrete region of the FBN1 gene would be both feasible and affordable with currently available techniques. Individuals with features of neonatal Marfan syndrome could be screened for mutations in this region to confirm the diagnosis. Identification of a sporadic mutation in a neonate has implications for genetic counselling, since perinatal lethal Marfan syndrome can also result from compound heterozygous mutations (Karttunen et al., 1994). In addition, the identification of a sporadic mutation in exons 24 to 32 of FBN1 could be used both prognostically and in the clinical management of these patients. Infants with mutations in this region have more severe cardiovascular manifestations of Marfan syndrome, and may benefit from more aggressive medical therapy, such as the early institution of β-adrenergic receptor blockers.

1.4.1.2 Autosomal dominant ectopia lentis

This rare condition is characterised by isolated lens displacement, associated with myopia, astigmatism, and in some cases, visual loss. An autosomal recessive form also exists in which the lens dislocation is associated with deformity and displacement of the pupil (Colley et al., 1991). Ectopia lentis in the autosomal dominant form is thought to result from a defect in the suspensory ligament of the lens. This condition has been linked to the FBN1 gene locus in three affected kindreds (Tsipouras et al., 1992a; Edwards et al., 1994). FBN1 gene mutations have been reported in ectopia lentis (Kainulainen et al., 1994; Lönnqvist et al., 1994). It is not known yet whether there is allelic heterogeneity for this condition.

1.4.1.3 Annuloaortic ectasia, mitral valve prolapse syndrome and the MASS phenotype

Two other disorders that overlap with the Marfanoid phenotype include annuloaortic ectasia (Nicod et al., 1989) and the mitral valve prolapse syndrome (Devereux et al., 1983). There has been an attempt to encompass this whole clinical spectrum by the designation of the so-called MASS phenotype (ie, mitral valve prolapse associated with either aortic (usually mild, non-progressive aortic root dilatation), skeletal, or skin manifestations) (Glesby and Pyeritz, 1989). Initial studies of families with dominantly inherited mitral valve prolapse or annuloaortic ectasia have not shown linkage to the FBN1 gene (Milewicz, 1994). The relation of these other distinct syndromes to fibrillin is as yet undetermined, although one FBN1 mutation has been characterised in a patient

with the MASS phenotype (Dietz et al., 1993b).

1.4.1.4 Familial aortic aneurysms

Aortic aneurysms are relatively common in the general population, and abdominal aortic rupture and dissection account for 1-2% of deaths in men over 50 years of age (Collin, 1985). The mortality from ruptured abdominal aortic aneurysms is 85-95% when rupture and bleeding occur prior to surgical intervention (Crawford et al., 1981). The mortality is $<10\%$ with elective surgery.

Many aortic aneurysms are familial. In one report, 29% of asymptomatic brothers of patients with ruptured abdominal aortic aneurysms had dilatations of the aorta detectable by ultrasound examination (Bengtsson et al., 1989). Another report found that 15% of 542 patients undergoing surgery for abdominal aortic aneurysms had a first-degree relative with an aneurysm (Darling et al., 1989). Mutations in the type III procollagen (COL3A1) gene have been reported in several patients with type IV Ehlers-Danlos syndrome, a generalised connective tissue disorder that is frequently associated with arterial aneurysms and rupture of other hollow organs such as the intestine (Superti-Furga et al., 1989; Tromp et al., 1989a,b; Kuivaniemi et al., 1990). More recently, mutations in the COL3A1 gene were shown to cause familial aortic aneurysms in a large 4 generation kindred in whom the aneurysms segregated in an autosomal dominant manner (Kontusaari et al., 1990).

Most recently, a family has been reported in which ten individuals in two generations were affected with varying degrees of ascending aortic disease, ranging from mild aortic root enlargement to ascending aneurysm or dissection (Francke et al., 1995). The segregation pattern of the aortic disease is consistent with autosomal dominant inheritance. Although isolated minor skeletal and ocular features similar to those seen in Marfan syndrome were found or reported in some affected individuals, no lens dislocation, pectus deformity, scoliosis, dolichostenomelia or arachnodactyly was present. Molecular genetic and biochemical analyses of fibrillin were performed, and a G1127S mutation in an EGF-like domain of the fibrillin gene was identified in nine of the ten affected individuals. The mutation is likely to be responsible for the aortic disease in this family, and is believed to produce an autosomal dominantly inherited weakness of elastic tissue, which predisposes to ascending aortic aneurysm and dissection later in life.

This is the first report of a FBN1 mutation segregating in a family with aortic disease in whom there was no clinical support for a diagnosis of Marfan syndrome.

1.4.1.5 Isolated skeletal features

There is a single report of a three generation family with a FBN1 gene mutation that results in isolated skeletal features of Marfan syndrome (Milewicz et al., 1995). No-one in the family had cardiovascular or ocular complications characteristic of Marfan syndrome. The thirteen year-old proband had tall stature, pectus carinatum, scoliosis, arachnodactyly and pes planus. In most of the other affected individuals in the family, the only skeletal manifestation was tall stature (height > 95th percentile for age and sex).

A FBN1 mutation was identified in exon 64 in these individuals. This exon encodes a region of the unique carboxy terminal domain of fibrillin that contains a known recognition motif for proteolytic cleavage by a cellular protease (currently unidentified). The mutation, a C to T transition at nucleotide 8,175, changes an arginine to a tryptophan at codon 2,726. Biochemical analysis of fibrillin synthesis and processing from the proband's cultured dermal fibroblasts demonstrated the disruption of the extracellular processing of profibrillin to fibrillin, identifying the putative cleavage site of this processing. Only one half of the profibrillin was processed to fibrillin by proband's cells by 20 hours and this pool of profibrillin remained unprocessed for up to 40 hours. The amount of fibrillin incorporated into the extracellular matrix by the proband's cells was one half that of the control cells. The unprocessed profibrillin was not incorporated into microfibrils. Ultrastructural analysis of the microfibrils made by the proband's cells was normal.

In several dominant disorders, a mutation that produces nonexpression of one allele causes a milder disease phenotype than missense mutations because of the dominant negative effect of mutations on protein function (Herskowitz, 1987). Mutations in the type I collagen genes (COL1A1 and COL1A2) lead to osteogenesis imperfecta (OI), with OI type II being the most severe and OI type I the least severe disease phenotype (Byers, 1990). OI type II results from missense mutations in the COL1A1 or COL1A2 gene that alters glycines found in the triple helical region of the type I collagen molecule. Most cases of OI type I result from mutations that affect production from one of the COL1A1

alleles and are equivalent to a "null" allele for the COL1A1 gene. This leads to a 50% reduction in the level of type I collagen, but the collagen is structurally normal. Thus, a qualitative abnormality in the protein structure leads to a more severe phenotype than a quantitative abnormality.

The FBN1 gene mutation described in the kindred with isolated skeletal features of Marfan syndrome represents a mild "disease" phenotype and it has been suggested that at the protein level, this mutation may be equivalent to a nonexpressed or "null" FBN1 allele.

1.4.1.6 Congenital contractural arachnodactyly (CCA)

Secondary to efforts to isolate and characterise the fibrillin gene (FBN1) responsible for Marfan syndrome, a second fibrillin gene, FBN2 was identified and localised on chromosome 5q23-31 (Lee et al. 1991). This gene produces a transcript similar in size to that of FBN1 and exhibits a similar, repetitive EGF-like modular structure. The FBN2 locus is genetically linked to a Marfan syndrome-related disorder, congenital contractural arachnodactyly (Lee et al., 1991; Tsipouras et al., 1992a).

Congenital contractural arachnodactyly is an autosomal dominant condition characterised by multiple joint flexion contractures that improve with age. Patients have the skeletal findings of Marfan syndrome (arachnodactyly, dolichostenomelia and kyphoscoliosis) (Viljoen et al., 1991) but lack the cardiovascular and ocular features of the syndrome. In addition, they have very characteristic "crumpled" external pinnae. This condition has a lower incidence than Marfan syndrome, with only some twenty affected families and over 13 isolated cases reported (Hall, 1990). It was originally thought that this disorder was a mild allelic form of Marfan syndrome, but it is more likely a genetically distinct disorder caused by defects within the Fib 5 gene product, FBN2. Causal mutations in this gene in two unrelated affected patients have recently been identified and characterised (Putnam and Milewicz, 1995; Wang et al., 1995c).

In light of the finding of FBN2 gene mutations in two patients with CCA, and the clearly established FBN1 gene mutations in Marfan syndrome, several hypotheses may be advanced. Firstly, different microfibrils may have different composition, and thus diverse functions in distinct tissues or at different developmental stages. Fibrillin-2,

which is expressed earlier than fibrillin-1, may serve as the first nucleation centre directing the subsequent deposition of fibrillin-1. The substantially lower incidence of congenital contractural arachnodactyly compared with Marfan syndrome reflects the low viability of FBN2 mutations, and implies that fibrillin-2 plays a more critical role than fibrillin-1 during the early stages of morphorganogenesis. Conceivably, the fibrillins may form homo- or heteropolymers of differing ratios, depending upon the specific genetic program of the cell type. In some tissues, one fibrillin may be more abundant than the other, while in other tissues they may be more equally expressed. In tissues where the fibrillins are more equally expressed, a defect in one fibrillin may be compensated for by the other fibrillin. In tissues where one fibrillin is more prevalent than the other, such a compensatory process may not occur because of its more critical structural role.

The mutations described in the preceding sections show that FBN1 mutations can result in several different phenotypes: classical Marfan syndrome, ectopia lentis, neonatal Marfan syndrome, isolated skeletal features of Marfan syndrome, and isolated familial aortic aneurysms. Based on the neonatal cases reported there seems to be a clustering of these particular mutations at the beginning of the longest stretch of EGF-like motifs. The idea that the site of the mutation along the polypeptide chain is more important than the nature of the mutation is in keeping with analyses of different phenotypes associated with mutations in the EGF-like repeats of the Notch locus in *Drosophila* (Kelley et al., 1987). These analyses have shown that mutations causing certain phenotypes are clustered in different regions of the polypeptide chain rather than spread throughout the EGF-like repeats. Although these corrollaries help in our understanding of the apparent clustering of FBN1 mutations in neonatal Marfan syndrome, detailed structural analyses aimed at clarifying the specific functional significance of each region within FBN1 is needed before an understanding of genotype-phenotype correlations is truly possible.

1.4.2 Spectrum of Phenotypes Associated with Fibrillin Protein Defects

The varied clinical features of Marfan syndrome have led to speculation that isolated features of the disorder, or related syndromes, may be due to mutations in the FBN1 gene.

1.4.2.1 Idiopathic scoliosis

Scoliosis is one of the features of Marfan syndrome. This raises the possibility that

individuals with isolated adolescent idiopathic scoliosis may have defects in fibrillin. Collaborative studies are underway, to study fibrillin processing by fibroblasts explanted from the ligamentum flavum of individuals with idiopathic scoliosis. Biochemical assessment of fibrillin synthesis and processing has identified a small subset of patients (4 out of 23, ie 17%) who have a defect in fibrillin processing (Milewicz, 1994). The specific defect identified in these patients was a failure of fibrillin to incorporate into the pericellular matrix surrounding the cells. This preliminary data suggets that fibrillin defects may have a role in the causation of idiopathic scoliosis. As yet, no FBN1 mutations have been identified in this group of patients.

1.4.2.2 Bovine Marfan syndrome

The occurrence of Marfan syndrome in cattle is well documented (Besser et al., 1990). Dermal fibroblasts from affected calves and age-matched controls have been assessed by metabolic labelling studies of fibrillin (Potter et al., 1993). These studies showed that cells from affected calves failed to incorporate fibrillin into the pericellular matrix whereas cells from normal calves demonstrated normal incorporation of fibrillin. These results suggest that the bovine Marfan syndrome is also caused by mutations in fibrillin, leading to defective incorporation into microfibrils. The search for a putative mutation in FBN1 in the affected cattle has begun, with no reported mutations to date.

1.4.3 Extending the Search

Given the information presented above, it is reasonable to speculate that fibrillin gene defects may play an aetiological role in patients with other isolated or atypical features of Marfan syndrome. Included in this category, are patients with isolated thoracic aortic aneurysms, annuloaortic ectasia, the mitral valve prolapse syndrome, patients with so-called "idiopathic" pneumothoraces, and possibly also those patients with one of the rare Marfanoid-craniosynostosis syndromes. The role of fibrillin in the pathogenesis of these disorders has yet to be elucidated, but clearly warrants further investigation.

1.4.3.1 Marfanoid-craniosynostosis syndromes

This subgroup of extremely rare conditions includes the Furlong syndrome and Shprintzen-Goldberg syndrome, amongst others. These two disorders are described here because of their relevance to six patients studied as part of my research (see Section 3.3.1 and Appendix 3A).

Shprintzen-Goldberg syndrome (MIM 182212) is characterised by many features of a generalised connective tissue dysplasia including craniosynostosis, arachnodactyly, camptodactyly, micrognathia, exophthalmos, abnormal pinnae, pectus carinatum or excavatum, mitral valve prolapse, abdominal herniae and mental retardation (Sugarman et al., 1981). Distinctive skeletal and radiographic abnormalities have also been described (Adès et al., 1995). Immediately prior to submission of this thesis, a novel sequence variant at codon 1223 (C1223Y) in exon 29 of the FBN1 gene in a seven-year-old girl with Shprintzen-Goldberg syndrome was reported. A second sequence alteration at codon 1148 (P1148A) in exon 28, was also reported by the same authors in an eleven-year-old girl with typical features of Marfan syndrome together with craniosynostosis, dysmorphism, umbilical hernia and mental retardation (Dietz et al., 1995b). The P1148A sequence alteration has been found in a variety of clinical settings (see Section 4.8.5 for discussion); its pathologic significance is unclear at present.

Furlong syndrome comprises Marfanoid features, craniosynostosis and normal intelligence (Furlong et al., 1987). Other features include facial dysmorphism, ptosis, myopia, a high arched or cleft palate, mitral valve prolapse, aortic root dilatation, recurrent inguinal herniae, hypospadias and spondylolisthesis. The genetic aetiology of these two disorders is unknown but because of the many shared features between these two conditions and the Marfan syndrome, the fibrillin group of proteins clearly warrant consideration.

1.5 Bovine and Other Animal Models

Ultimately, the possible genotype or phenotype correlations established by the characterisation of mutations and the study of fibrillin metabolism can be tested by the production of animal models using the powerful approach of gene targeting in mouse embryonic stem cells (Capecchi 1989). These collective studies may have wide-ranging implications for the prevention, diagnosis, and management of Marfan syndrome.

1.5.1 Bovine Model

The first potential animal model of Marfan syndrome was recently identified by veterinarians at Washington State University (Besser et al., 1990). Bovine Marfan syndrome resembles human Marfan syndrome in that affected cows have skeletal

anomalies (dolichostenomelia, joint hypermobility), ocular abnormalities (ectopia lentis), and cardiovascular lesions (aortic root dilatation and rupture). Indirect immunofluorescence studies have shown a deficiency of fibrillin-containing microfibrils in the affected cattle (Potter et al., 1993). Recently a cDNA for bovine FBN1 has been isolated (Tilstra and Byers, 1993).

1.5.2 Murine Model

Four microfibrillar genes have been cloned in the mouse. They are the recognised murine homologues of MAGP, FBN1, FBN2 and a fourth fibrillin-like gene (Bonadio et al., 1990). Transgenic mice carrying mutant FBN1 constructs are being produced (Pereira et al., 1993b).

1.6 Towards a Diagnostic Test

Despite recent advances, the diagnosis of Marfan syndrome is still based on clinical judgement. Genetic linkage studies are now possible for presymptomatic or prenatal diagnosis (Godfrey et al., 1993a), but informativeness of markers and the need for family history and availability of relatives are limiting factors. In reality, linkage studies are only helpful in a small proportion of the total number of individuals with the condition. About one third of patients with the Marfan syndrome are sporadic, arising from new dominant mutations. In this sub-group, direct analysis of fibrillin for a causal mutation, would be the only feasible molecular study available. Once a fibrillin mutation has been identified, this would confirm a diagnosis of the Marfan syndrome. Failure to find an FBN1 gene mutation would not exclude the diagnosis. Multiple factors, including the size of the fibrillin message (mRNA 10 kb), the fact that most, if not all, families and patients will have unique mutations, and the time and cost necessary for current screening techniques make direct mutation screening impractical for the diagnosis of equivocal cases. It is precisely this group of patients who would benefit from a direct test since without it, these individuals are subject to lifelong clinical surveillance.

The availability of immunofluorescence studies has precipitated numerous requests for fibrillin analysis as an aid in the diagnosis of Marfan syndrome in cases where the clinical features alone are insufficient to permit a certain diagnosis. Recently, quantitative immunoflourescence studies have been attempted in order to address this issue.

Computer-enhanced image analysis was used to establish "normal" and "abnormal" (Marfan) parameters of fibrillin immunofluorescence in dermal fibroblast cultures from 20 patients with a clinically unambiguous diagnosis of the Marfan syndrome and 20 control subjects (Schaefer et al., 1995). Results of these studies showed a median fibrillin fluorescence of more than 21% from control individuals, while the fluorescence in patients with Marfan syndrome was 6% with a confidence interval of less than 15%. These findings were statistically significant ($p < 0.01$). A clear bimodal distribution of immunofluorescence with no overlap, was seen between the Marfan patients and controls.

In addition, eleven Marfan syndrome patients and five control subjects randomly selected from individuals previously analysed by immunohistochemistry (Hollister et al., 1990; Godfrey et al., 1990a) were analysed using the above image analysis technique. A comparison was then made between the image analysis results and those previously published based on microscopy observation alone. In both instances, all control individuals were identified within normal parameters of immunofluorescence staining. More than 80% of Marfan syndrome patients were found to have reduced fibrillin immunofluorescence in fibroblast cultures. The image analysis technique was as accurate in identifying abnormal fluorescence as was direct microscopic observation. Although the consistency and reliability of these results holds promise as an additional tool for the clinician's use, several points should be taken into consideration. The sample numbers analysed were relatively small, the results have yet to be reproduced by others, and like all other methods used in isolation for the prediction of affection status for Marfan syndrome, the image analysis technique clearly is associated with some false negative results.

1.7 Future Directions

In order to gain greater knowledge of the biology of normal and mutant fibrillin, the protein and its interactions will need to be studied in vivo. The application of transgenic technology will provide insight into the biochemical and developmental significance of the normal protein and the consequence of expressing an altered gene in a system that mimics the physiologic complexity of the human system. The use of techniques of homologous recombination in embryonal stem cells should elucidate whether there are

differences in qualitative versus quantitative mutations of fibrillin. Transgenic techniques should allow a comparison of the consequences of identical mutations in the two fibrillin chains.

The development of murine and nonmurine models for Marfan syndrome should provide a means to evaluate conventional medical therapies such as the time of initiation, dosage, or, route of administration of pharmacologic agents that slow the rate of aortic root dilatation. Animal research will also be necessary to test novel gene therapy strategies. If the dominant negative hypothesis is confirmed, then selective inhibition of expression from the mutant allele may help to diminish the clinical burden of individuals with Marfan syndrome. The emergence of antisense or ribozyme strategies for such purposes, together with methods for direct gene delivery to the cardiovascular system, may ultimately allow for the correction of defective FBN1 gene expression by somatic gene therapy.

The defect in Marfan syndrome is expressed throughout the body (Sakai et al., 1986). Part of the phenotypic variability among families may be due to some fibrillin mutations having a deleterious effect in some but not all tisues. Familial annuloaortic ectasia, for example, may be due to fibrillin mutations that are expressed primarily in the aorta. There is no available technology that would permit effective gene therapy of a ubiquitous protein such as fibrillin. Furthermore, many components of the extracellular matrix are involved during embryogenesis and later growth, and turn over extremely slowly. Unless gene therapy was performed early in development, defective microfibrils would contribute to an abnormal phenotype throughout gestation. Thus, a generalised cure for Marfan syndrome is well beyond current technical abilities.

As a first step towards this goal, a laboratory animal model of Marfan syndrome needs to be developed. This is likely to occur in the next few years by inserting or otherwise creating by transgenic techniques a mutation in mouse fibrillin (Merlino, 1991). This will afford researchers the opportunity to study the phenotype of a Marfan mouse, and to investigate pathogenesis from early embryogenesis. If the mouse disease is sufficiently analagous to the human condition, then attempts at generalised somatic gene therapy in the mouse might be directly relevant to humans.

Gene therapy directed at one organ or tissue is somewhat less speculative. Targeted therapy might ameliorate one organ-related set of problems. This raises the question of whether knock-out of the mutant fibrillin in the aortic media of a child with Marfan syndrome could lead to a lessened susceptibility to aneurysm and dissection if only one half the usual amount of fibrillin were produced, but it was all normal fibrillin. The evidence for quantitative (as opposed to qualitative) abnormalities of fibrillin being responsible for a milder phenotype has been demonstrated recently (see Section 1.4.1.5). A number of investigators are attempting to insert genes into vascular tissue (Plautz et al., 1991; Flugelman et al., 1992). If a Marfan mutation were corrected, blocked, or otherwise rectified in the proximal aorta, perhaps the occurrence of potentially lethal problems could be minimised.

1.8 Aims of The Thesis

The aim of the research was to study two patient groups, those with unequivocal Marfan syndrome, and a second group with clinical evidence of a generalised connective tissue disorder with some overlapping features of Marfan syndrome, but in whom the diagnosis was unknown. A finite number of patients were initially included in the research, but this was extended in some instances because of clinical referrals from colleagues. The purposes of the research were to document in detail the clinical features in all these patients, to attempt to make a diagnosis in those patients not considered to have Marfan syndrome on clinical grounds, and to search for putative FBN1 gene mutations in both patient groups. When this project began, only the partial cDNA sequence of FBN1 was known and mutation analysis was based wholly on the reverse transcription of mRNA to cDNA from cultured dermal fibroblasts. However, during the course of the research, the entire genomic DNA sequence became available and the approach to FBN1 mutational analysis was modified once genomic based primers became available.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sources and Consumables

Many of the reagents used were derived from numerous sources and wherever possible, these are stated in brackets in the text. The enzyme *Taq* DNA polymerase was supplied by Boehringer Mannheim and the ^{32}P-containing radionucleotide was obtained from Amersham. Plasticware and pipette tips were obtained from Kartell. Photographic film for agarose gels visualised under ultraviolet light was obtained from Polaroid.

2.1.2 Solution and Equipment Sterilisation

All solutions were made with distilled water and sterilised by autoclaving at 120°C for 15-30 minutes. Microcentrifuge tubes and disposable pipette tips were also autoclave-sterilised.

2.1.3 Precautions Against Ribonucleases

Standard precautions were taken to prevent the ribonuclease degradation of extracted RNA (Sambrook et al., 1989). All reagents and pipettemen were dedicated to RNA work exclusively, and gloves were used at all times when handling these items, their containers, and the 10 ml containing cultured dermal fibroblasts from which RNA was extracted. All RNA work was conducted using fresh disposable gloves after every step of the protocol. Solutions were made up with diethyl pyrocarbonate (DEPC)-treated sterile water and stored at room temperature. Glassware was avoided and all solutions were made up in sterile 50 ml propylene tubes (Falconer) from a previously unopened bag. All RNA samples were kept in screw top eppendorf tubes in a separate, sealed box, were handled on wet ice at all times when in use, and stored at -70°C when not in use.

2.2 General Methods: Sample Preparation

2.2.1 Isolation of Peripheral Lymphocyte DNA

This modified method of Wyman and White (1980) was performed by Jean Spence. DNA was extracted from a total of 116 blood samples for the research. Blood samples were collected in 10 ml EDTA tubes and stored at -20°C. After thawing, 20 ml of cell lysis buffer was mixed with the 10 ml of blood in a 50 ml tube, then left on ice for 30 minutes. The tubes were centrifuged at 2,600 x g at 4°C for 15 minutes. Twenty ml of

the supernatant was removed by aspiration. Cell lysis buffer (20 ml) was added again, and the centrifugation repeated. The supernatant was removed and 3.25 ml of 3 x proteinase K buffer, 500µl of 10 % SDS and 200 µl of proteinase K (at 10 mg/ml) were added and mixed with the pellet. This solution was mixed overnight at 37°C. Phenol (5 ml) was then mixed into the solution for 15 minutes at room temperature, followed by centrifugation at 2,600 x g at 15°C for 10 minutes. The supernatant was transferred to a new tube, and the volume made up to 10 ml with phenol, and mixed for 10 minutes, at room temperature. After centrifugation at 2,600 x g for 10 minutes, the supernatant was transferred to a new tube, and made up to 10 ml with chloroform. The solution was mixed and re-centrifuged. DNA was precipitated by mixing with 300 µl of 3 M sodium acetate (pH 5.2), and adding cold ethanol to 10 ml. After mixing, the tubes were centrifuged and the DNA pellets washed with 70% ethanol, transferred to a 1.5 ml tube, centrifuged briefly at 16,500 x g, then air-dried. The pellet was dissolved in 100 µl of TE, and quantitated spectrophotometrically.

2.2.2 Extraction of Total RNA

Total RNA was extracted from cultured dermal fibroblasts, using a modified single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987).

The volumes of all added solutions used were per 1 x 10^7 cells. Isolated cells were resuspended in 1 ml of denaturing solution/10^7 cells. This solution D comprised the following reagents: 4M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, and 0.5% N-lauroylsarcosine, stored at room temperature. The stock solution D was heated to 65°C to ensure dissolution of all component reagents. Before use, 7 µl of β-mercaptoethanol (Ajax) was added to every 1 ml of solution D used.

The lysate was passed through a pipette 7-10 times. Each tube was vortexed for 4 minutes and then mixed by inversion. To this, was added 0.1 ml of 2M sodium acetate at pH 4.0. The samples were then spun for several minutes in a Jouan centrifuge (Plasma R1000) at 4,000 rpm and 4°C. The following reagents were added sequentially and mixed by vortexing for 60 seconds: 1 ml water-saturated phenol (refrigerated; USB) and 0.2 ml of 49:1 chloroform/isoamyl alcohol (Ajax). The suspensions were incubated for 15 minutes at 4°C. After centrifugation for 25 minutes at 5,500 rpm and 4°C, the upper

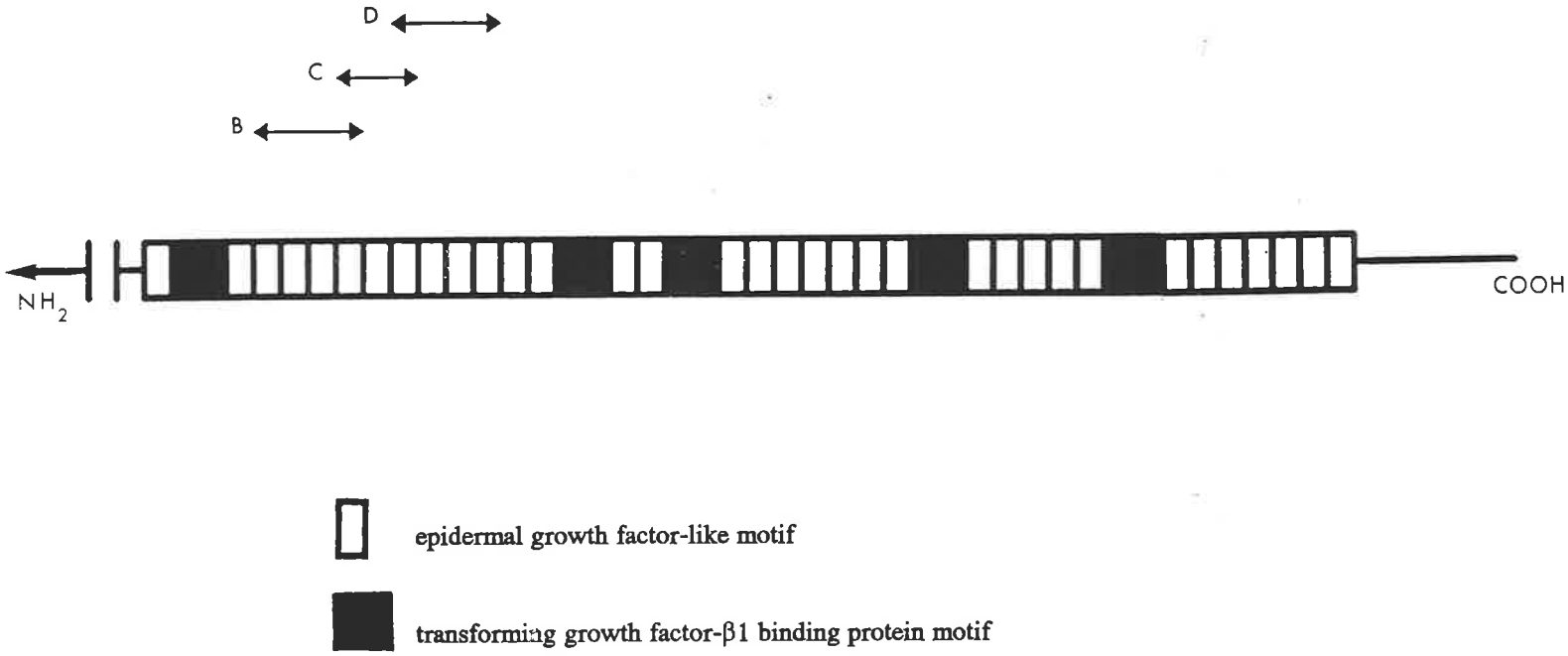
aqueous layer was removed to a fresh tube, and the RNA precipitated from this by adding an equal volume of 100% isopropanol cooled to -20°C over 30 minutes. After centrifugation for 20 minutes at 5,500rpm and 4°C, the supernatant was discarded. This was followed by resuspension of the RNA pellet in another 0.3 ml of solution D. Transfer into a 1.5 ml microcentrifuge tube containing 0.3 ml of 100% isopropanol was followed by incubation at 4°C for 30-60 minutes. The suspension was centrifuged for 10 minutes at 14,000 rpm and 4°C, yielding a small white RNA pellet, and the supernatant was discarded. The RNA pellet was washed in 500 µl of 75% ethanol, then vortexed, and incubated for 10-15 minutes at room temperature to dissolve any residual amounts of guanidinium contaminating the pellet. The sample was centrifuged for 5 minutes at 14,000 rpm, the supernatant discarded, and the pellet vacuum-dried and redissolved in 50 µl of nuclease free water (Promega, PolyATract mRNA Isolation System IV Kit) and 0.25 µl RNAsein (Promega). The purity and quantity of the RNA was ascertained by optical density (see Section 2.2.3). The RNA was stored at -70°C.

2.2.3 Reverse Transcription of RNA and PCR Amplification of cDNA

Primer pairs, spaced at approximately 500-bp intervals, with 100-bp overlap at the 5' and 3' ends, were used to span part of the cDNA sequence of fibrillin (Maslen et al., 1991) (Figure 2.1). The reverse primer of each set was used to prime reverse transcription of the fibrillin cDNA using total cellular RNA, and both forward and reverse primers of a set were used to amplify the fibrillin cDNA fragment by using the Perkin Elmer Cetus GeneAmp RNA PCR kit, Cetus reagents and Perkin Elmer Cetus thermal cycler.

A reverse transcription "mastermix I" was prepared by adding these reagents in the following order and proportions, with their respective final concentrations shown in brackets: 2.0 µl of $MgCl_2$ (5.0 mM), 1.0 µl of 10 x PCR buffer II (1.0 x), 0.5 µl of distilled water, 4.0 µl of dNTP mix (deoxynucleosidetriphosphates dissolved in distilled water and titrated with NaOH to pH 0f 7.0; 1.0 mM), 0.5 µl of RNase inhibitor (1.0 U/µl), 0.5 µl of reverse transcriptase (2.5 U/µl), 0.5 µl of reverse primer (2.5 µM), and 1.0 µl of RNA, to a total sample volume of 20 µl. PCR incubations for reverse transcription using the thermal cycler were as follows: 42°C for 15 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. A PCR "mastermix II" was then prepared with the following reagents: 2 µl of 25 mM $MgCl_2$ (Cetus), 4 µl of 10 x PCR buffer II (Cetus),

Figure 2.1 Location of cDNA-based Primer Pairs for RT-PCR Analysis of FBN1



Graphic representation of the partial cDNA of fibrillin, showing the overlapping primer pairs for the screened cDNA fragments (B, C and D), the amino-terminus (NH_2), the carboxy-terminus, and the structural motifs (after Maslen et al., 1991). The two vertical lines (to left of cartoon) represent the segment of cDNA for which sequence data was unknown at the time that these primer pairs were designed for RT-PCR use.

33 µl of distilled water, 0.25 µl of *Taq* DNA polymerase (Cetus) and 0.5 µl of the forward primer, to a total volume of 39.75 µl. For each sample, 40 µl of mastermix II was added to 10µl of mastermix I, giving a final volume of 50 µl. A drop of paraffin oil was added to each tube, and the samples were microcentrifuged for 30 seconds. PCR incubations for first strand cDNA synthesis of selected cDNA fragments were performed using the thermal cycler for 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds, followed by one cycle of final extension at 72°C for 30 seconds. The cDNA samples were then stored at 4°C until further use.

2.2.4 Spectrophotometric Estimation of Nucleic Acid Concentration

The absorbance at 260 nm was used to estimate the concentration of nucleic acids (Sambrook et al., 1989). The following extinction coefficients were used: 0.05 for double stranded DNA, 0.04 for single stranded DNA and RNA, and 0.02 for oligonucleotides (units are in optical density/$\mu g ml^{-1}$/cm). An OD260:OD280 ratio of 1.8 or greater implied minimal contamination with protein (Sambrook et al., 1989). Spectrophotometry was performed using a Cecil Model CE2010 spectrophotometer.

2.3 Specific Methods

2.3.1 Skin Biopsy Collection

Full thickness 4mm punch skin biopsies were collected from one affected individual from all but one of each family studied, after appropriate informed consent. A total of 36 skin biopsies were obtained. The skin overlying the left deltoid region was prepared by washing with betadine (Faulding Pharmaceuticals), followed by washing with 0.015% w/v chlorhexidine acetate and 0.15% cetrimide aqueous antiseptic solution (Baxter). The skin was then draped with sterile cloth, and 1% lignocaine hydrochloride (Astra) was administered to the site by subcutaneous injection. The biopsy site was sutured with 5-O nylon and sutures were removed after one week. The skin biopsy sample was placed immediately into a sterile container containing OPti-MEMI tissue culture medium (OMI, Gibco, BRL) at room temperature and despatched for subsequent fibroblast culture. In addition, samples of skin (stored in liquid nitrogen) from 12 unaffected individuals were generously provided from the Department of Chemical Pathology for use as controls for the RNA work undertaken (see Sections 2.2.2 and 2.2.3).

2.3.2 Fibroblast Tissue Culture

The skin biopsies were macerated into several small pieces which were adhered to the bottom of a small 25 cm^2 sterile tissue culture flask. The dermal fibroblasts were grown in Opti-MEMI supplemented with sodium bicarbonate, Benzyl Penicillin (CSL, 600 mg/ vial), 2-mercaptoethanol, Phenol Red Solution (Cytosystems) and 5% fetal calf serum (FCS, Trace). The cells were grown until confluent in a 37°C incubator.

Confluent fibroblasts were subcultured by limited trypsin digestion. After removal of the old media and rinsing with sterile Phosphate Buffered Saline (PBS) (Calcium and Magnesium-free, Trace), the flasks were incubated at 37°C for 3-5 minutes with Trypsin EDTA (Trace). Once the cells were seen to float freely, the fibroblasts were pipetted into 1 ml of OMI culture medium to stop the digestion. The cells were pelletted for 5 minutes at 1,000 rpm and the supernatant removed. After resuspension in fresh medium, the cells were placed in a 75 cm^2 flask and flushed with carbon dioxide before reincubation. When this flask was confluent, the cells were subcultured again, and placed in fresh medium in a 175 cm^2 flask before reincubation. Once confluent, the cells were removed by the PBS/Trypsin method described above, transferred into a 10 ml tube containing 1 ml of OMI, and pelletted for 5 minutes at 1,000 rpm. The supernatant was removed, the cells resuspended, and washed in 10 ml of PBS before being pelletted again. This washing process was repeated, the cells were resuspended in a final volume of 10 ml of PBS, and placed on wet ice. A small aliquot part was removed to allow a viable cell count to be made using a haemocytometer.

Since untransformed cells do not withstand repeated passaging, a portion of cells were frozen after several passages for potential future analysis. Three 25 cm^2 flasks contained enough cells to fill 3 x 1 ml screw-top cryotubes. These were pelletted as normal but resuspended in 1 ml each of freezing medium [80% OMI medium, 10% fetal calf serum (FCS), 10% dimethylsulphoxide (DMSO; Ajax)]. The cells were frozen in the neck of a 35 litre liquid nitrogen container for 3 hours before being placed in a metal straw for cryopreservation in liquid nitrogen. When defrosted, the cells were warmed at 37°C and then washed in 5 ml of medium, pelletted, resuspended in fresh medium and then cultured as before.

2.3.3 Fibrillin Pulse-Chase Analysis

Dermal fibroblasts from 5 patients enrolled in the research were selected for fibrillin pulse-chase studies. None of the patients selected for these studies had Marfan syndrome, but all had an undiagnosed severe connective tissue dysplasia (see Section 3.3 (patients 28 and 35), Section 3.3.2 (patients 26 and 45) and Appendix 3A (patient 27; represented as Case 1)). Selection of these patients for the biochemical analysis of fibrillin processing was based on the presence of a constellation of unusual and severe clinical features. The pulse-chase studies were performed by Dr SN Cao, and interpreted by Dr DM Milewicz (Houston) as part of an ongoing collaboration aimed at attempting to determine the underlying biochemical abnormality in this rare subgroup of patients.

2.3.4 Preparation and Electrophoretic Analysis of Fibrillin

Dermal fibroblasts were explanted and maintained in culture according to established methods (Milewicz et al., 1992). To radiolabel synthesised proteins, 250,000 dermal fibroblasts were plated in 35 mm dishes, allowed to attach and spread for 72 hours in Dulbecco-Vogt modified Eagle's medium (DME) supplemented with 10% fetal calf serum. Pulse-chase studies were done using [^{35}S]cysteine to radiolabel synthesised proteins, and the media, cell lysate and cell layer were harvested separately using a previously described protocol (Milewicz et al., 1992). Proteins were dissolved in sample buffer containing sodium dodecyl sulphate (SDS) and β-mercaptoethanol, separated by electrophoresis on a 4% polyacrylamide slab gel containing SDS, and visualised by autoradiography. The location of fibrillin in this gel system has been previously established (Milewicz et al., 1992).

2.3.5 Types I and III Collagen Screening

A subset of 12 patients enrolled in the research had clinical features suggestive of a possible type I or type III collagen defect. One of these patients was classified as having Marfan syndrome, although he had some additional or atypical features; the remainder belonged to a group with features of a connective tissue disorder in whom there was no known diagnosis at the time of commencement of the research. These patients were selected for collagen screening studies. In addition, one further patient was selected for specific study of collagen crosslinking, because of the striking similarity between the arterial abnormalities in this patient and those seen in Menke's disease, a disorder of copper metabolism (Bankier, 1995). This work was conducted as part of an ongoing

collaboration with Dr JF Bateman, AA Chiodo, D Chan and AD Esquivel in the Orthopaedic Molecular Biology Research Unit at the Royal Children's Hospital in Melbourne.

Dermal fibroblasts were used to study the synthesis and secretion of type I, proα(III) and type III collagen according to established protocols (Bateman et al., 1988). Collagen crosslinking was assessed in cultured fibroblasts from patient 35 (see Sections 3.3 and 4.7). Collagen crosslinking was assessed in cultured fibroblasts where collagen matrix formation and maturation was induced by long-term culture for 20 days with ascorbate as previously described (Bateman and Golub, 1994). Collagen was pulse-labelled with 50 μCi [2,3^{3}H-proline] for 24 hours and then chased with isotope-free medium for a further 24 hours. Deposition of labelled collagen into the mature crosslinked collagenous matrix was assessed by a serial extraction with neutral salt buffer (to extract newly synthesised un-crosslinked collagen) followed by extraction with 0.5M acetic acid and then pepsin (to extract the progressively more crosslinked collagens). The proportions of collagens in each of the fractions was quantified by SDS/polyacrylamide gel electrophoresis (Bateman et al., 1988).

2.3.6 Fluorescent In Situ Hybridisation (FISH)

Fluorescent in situ hybridisation was performed in one patient (patient 35) to exclude the possibility of an elastin gene deletion (see Sections 3.3 and 4.7). This work was done by Sharon Bain in the Department of Cytogenetics. The Elastin Williams Syndrome Chromosome Region (WSCR) Probe with D7S427 Chromsome 7 Control Probe (Oncor) were used on cultured dermal fibroblasts prepared by standard cytogenetic methods. The cells were processed according to the Oncor protocol provided using the alternative formamide wash method. A positive signal was indicated by a fluorescent signal present on both chromatids of a metaphase chromosome 7. A nondeleted sample was indicated by the presence of a fluorescent signal on both chromatids of both chromosome 7 homologues.

2.4 The Polymerase Chain Reaction (PCR)

2.4.1 Standard Reaction

Unless otherwise stated, all PCRs (Saiki et al., 1985) for the amplification of genomic DNA were performed using selected intron-based exon-specific primers. A Perkin-Elmer Cetus DNA thermal cycler was used to individually PCR-amplify DNA using primers for the FBN1 exons 15-18 inclusive, 23-32 inclusive and exon 59.

Reactions were carried out under the following conditions: 100 ng of genomic DNA was amplified in a 10 µl volume using 400 µM dNTP mix (comprising the following reagents added in the following order, and stored at -70°C: 33 mM $(NH_4)_2SO_4$ (Ajax), 1.3 mM Tris-HCl (pH 8.8) (Boehringer Mannheim), 0.013 mM EDTA (Ajax), 0.34 µg BSA (Sigma), 2 µl of DMSO (Ajax), 400 µM each of dATP, dGTP, dCTP and dTTP, 20 mM of βME (BDH), $MgCl_2$ (see Table 2.1 for optimal concentrations), 75 ng of both forward and reverse primers, 0.5U *Taq* DNA polymerase (5U/µl) and 0.3 µl of α^{32}P-dCTP (800Ci/mmol) radionucleotide label. A drop of paraffin oil was added to each tube and the tubes were centrifuged at 14, 000 rpm for 60 seconds prior to thermal cycling.

PCR incubations for each exon screened were performed in a Perkin Elmer Cetus thermal cycler for 10 cycles at 94°C for 60 seconds, at 60°C for 90 seconds, at 72°C for 90 seconds, followed by 25 cycles at 94°C for 60 seconds, at 55°C for 90 seconds, and at 72°C for 90 seconds. Samples were stored at 4°C. Each amplified sample was mixed with an equal volume of formamide loading buffer (96% deionised formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol).

2.5 Oligonucleotide Primers

2.5.1 Oligonucleotide Primer Synthesis

The oligonucleotide primers synthesised initially were chosen for the selective screening of various fragments of cDNA of the FBN1 gene. The regions chosen for initial screening were those where several FBN1 mutations had already been identified. These regions were generally less than 500 bp in size, making them good candidate regions to analyse by the SSCP approach (see Section 2.7). The oligonucleotide primers shown in Tables 2.1 and 2.2 synthesised on a Beckman Oligo Synthesiser 1000. Primers for exons

Table 2.1. Genomic screening: Oligonucleotide primer sequences, size of generated PCR fragments (bp), and optimal $MgCl_2$ concentrations (mM)

Exon	Primer Sequence (5'-3')	Size(bp)	$MgCl_2$
15(F)*	TGT CAC TTC ATT TTT AAT AAG TG	212	1.5
15(R)*	GTG ACA GAG GCT GAA CCT C		
16(F)	CTC ATC TGT TTG AAG TGA CAG	257	1.5
16(R)	GGT GGC AGA AGG CTG GC		
17(F)	GAT CTA CCT GTT CTG CAA AC	133	4.0
17(R)	GTA AAT TTT GAA AGG AAT CCT TA		
18(F)	TCA GAA TAT CCT TAC AGT GAG	375	2.5
18(R)	TCT AAG CTA CTC AAA GGC AG		
23(F)	GTT TTA TGA ACT TAC CAG GTT C	333	2.5
23(R)	ACC GAA GCT AAG TGC TCA G		
24(F)	CAG CAA ATT ATT ATG TGT GCA G	418	2.5
24(R)	ATC AAG TAG AGT GCT GAG ATC		
25(F)	CAA GAA CTT CCA ACC TTC ATG	273	1.5
25(R)	TTA AAG GAC GTC CCC TCT C		
26(F)	AAT TAA GGC TGT CCT GAG AC	227	1.5
26(R)	CAT GGA ATC CTT CTC TTT CTG		
27(F)	GGC CCC CAC CTT TAA CAT G	181	2.5
27(R)	GAA AGT CTT TGC TCC TTA C		
28(F)	TGC CAA AGT TGG AAG CTT ATG	225	4.5
28(R)	TAA CAT AAC ATA ACA TAA AAT AAA G		
29(F)	CAG ACA TCC AAA CCA TAT CAG	213	1.5
29(R)	GAA CCT ACT GAG AGA TTC AAC		
30(F)	AAT AGT CTT ATG CTA GTA GGC	292	3.5
30(R)	ACA GTG CTT ATG ACT AAC AAG		
31(F)	GTA CTC AAT GAT ATC AAA TAG C	230	3.0
31(R)	ACC AAT CTC TTA ACT ACT TAA TA		
32(F)	CCA AAA GAC ATT TGT GCT GAG	226	3.0
32(R)	GTG TAA TCT ATG CAG TCC TTG		
59(F)	GCG TGT ACA CAT CAT TTT TAG	222	3.0
59(R)	ATG TGT CAG GAG CTA GGT G		
63(F)	GGC TGC TGC CAC ACA TGC CGC	258	2.5
63(R)	GGT CAT GGT TGG AGA TTC TTT CA		
64(F)	CCT ACC TTG TCT TCC CAT TCT AAT G	268	2.5
64(R)	ACA GGA GAC ATC AGG AGA AAC		

(F)forward primer; (R)reverse primer

Table 2.2. cDNA screening: Oligonucleotide primer names and sequences (5' to 3'), the corresponding FBN1 exonic regions (CER), and size of generated PCR fragments (bp)

Name	CER	Primer Sequence	Size
#UF348(F)*	25-28	GAT ATC AAT GAG TGC AAG ATG	501
UF349(R)		GGC ACA CTG ATA CTT CCC T	
UF497(F)	28-32	GCC GTT GCG TGA ACC TCA T	530
UF390(R)		GTT GTG TGC TCC AAT TTC A	
UF428(F)	37-38	GTG ACT GCC CAC CTG ATT	346
UF495(R)		CTT GGC ACA GCC CTG GTA	
UF432(F)	38-41	TCC GAC CAA ATC CTA TCA	421
UF433(R)		TGT TCA CAG GGC TTG TTC	
UF833(F)	46-50	AAT GGA ACT TGC CGG AAC	446
UF896(R)		CCA CTG GAG GAC AAG GAA	
UF499(F)	49-53	CTT CAA ATG TCT GTG TCC A	463
UF396(R)		CAA GGA TTG CCA ACA GAA C	
Frag. "B"(F)	25-29	CAT TGG CAG CTT TAA GTG CAG G	480
Frag. "B"(R)		ACC ACC ATT CAT TAT GCT GCA	
Frag. "C"(F)	28-31	CAA TTC AAC TCC CGA TAG GCT	335
Frag. "C"(R)		TTT TCA CAG GTC CCA CTT AGG C	
Frag. "D"(F)	31-33	TTG TGT TAT GAT GGA TTC ATG G	392
Frag. "D"(R)		TGG TTC CTT GCA CAG ACA GCG G	

*# UF annotation, after Uta Francke (see text); *(F)forward primer; (R)reverse primer*

23, 24, 27, 29, 30, 31 and 32 were generously provided by the National Marfan Foundation and Dr HC Dietz, and for exons 25 and 26 by Dr DM Milewicz. The oligonucleotide primer sequences for exons 63 and 64 were designed by Jami Grossfield (Houston) and generously made available for my use. All the research patients underwent genomic-based screening of each exon shown in Table 2.1. Sequences of oligonucleotide primers for the genomic-based screening, the size of their respective PCR fragments, and the optimised $MgCl_2$ concentration are shown in Table 2.1. The 12 oligonucleotide primer sequences for 6 cDNA based fragments were made available from Uta Francke (Howard Hughes Medical Institute, Stanford CA). Primer sequences for the regions encoding cDNA fragments "B", "C" and "D" were designed and synthesised in Dr Milewicz's laboratory (Houston) and made available for my use. The primer names and sequences, corresponding exon regions of FBN1, and size of the PCR product are shown in Table 2.2. There is some overlap evident between the cDNA fragments screened. While initially learning the RT-PCR methods in Houston, I used the primers that were available in that laboratory to learn this technique to analyse 7 selected research patients. When I returned to Adelaide to continue the research using the RT-PCR approach, the UF series of primers were synthesised (Table 2.2), and these were applied in the analysis of all patients in the research. Using the combined approaches of cDNA and genomic-based screening, a total of 30 of the 65 FBN1 gene exons (46% of exons, or 3,750 of 8,613 bases, equivalent to 44% of coding sequence) were screened in all patients.

2.5.2 Oligonucleotide Cleavage and Deprotection

Reagents and apparatus used for oligonucleotide cleavage and deprotection were as follows: 1 ml disposable syringe, screw cap eppendorf tube, and concentrated ammonium hydroxide (> 30%). A 1 ml aliquont part of ammonium hydroxide was placed in the tube and the column containing the oligonucleotide was then screwed onto the tube. The syringe was inserted into the other end of the column. The tube was inverted and the plunger pumped until all the air had been expelled from the column. The tube was left inverted for 1-2 hours at room temperature, ensuring the ammonium hydroxide was in contact with the column. The tube was then turned upright, and the pumper plunged until all ammonium hydroxide was in the tube. This was followed by removal of the column and syringe, and capping of the tube. The tube was heated at 55°C overnight in a waterbath.

2.5.3 Oligonucleotide Purification

After cleavage and deprotection, oligonucleotide purification was carried out by the n-butanol method (Sawadogo and Van Dyke, 1991). The oligonucleotide was added to 10-15 ml of n-butanol (Ajax) in a 50 ml Falconer tube, ensuring an oligonucleotide:n-butanol ratio of at least 100 µl:1 ml. This was followed by vortexing for 15 seconds, and then centrifugation in a Jouan centrifuge at 2,900 rpm for 45 minutes. The supernatant was removed and discarded into a bottle in the fumehood, taking care not to disturb the pellet. The pellet was resuspended in 1 ml of distilled water to which 10 mls of n-butanol was added, the sample was vortexed for 15 seconds and then centrifuged as before for 20 minutes. The supernatant was removed and the pellet vacuum-dried and resuspended in water to give a final concentration of 1 mg/ml. The primers were stored at -20°C.

2.5.4 $MgCl_2$ Optimisation of Oligonucleotide Primers

The optimisation of $MgCl_2$ concentration for the oligonucleotide primer pairs shown in Table 2.1 was performed using PCR as described in Section 2.4.1, using double the stated volumes (total reaction volume of 20 µl), two separate "control" genomic DNA samples, and varying $MgCl_2$ concentrations (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mM) in each reaction. Twenty µl of product was then mixed with 2.2 µl of 10 x loading buffer (5% glycerol, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded into the wells of a 1% agarose (Promega) gel (made up to 100 mls with 0.5 x TBE (Tris base, boric acid, 0.5M EDTA) and heated to dissolution, with 7 µl ethidium bromide (Sigma) added per 100 mls prior to pouring). Electrophoresis was performed, using 0.5 x TBE buffer at 120 volts for 30-45 minutes, according to the expected size of the product. The products were visualised under ultraviolet transillumination in a dark room, and photographed. The optimal $MgCl_2$ concentration was deduced according to the most brightly fluorescent visible band.

2.5.5 Restriction Enzyme Digestion of PCR Products

Restriction enzyme digestion of PCR products with *Pst* I and *Bsm* I was conducted at 37°C and 65°C, respectively. Ten µl of PCR product were added to 2µl of the appropriate 10 x buffer, 2 units of restriction enzyme and 7 µl of water. Digestions were left for 3 hours.

2.6 PCR Product Detection

Twenty µl aliquot parts of PCR products were mixed with 2.2 µl of 10 x loading buffer. These were run on small 1% agarose gels as described in Section 2.5.4. A pUC 19/*Hpa* II marker 13 fragment ladder (range 501 to 26 bp) (Progen) was always included in lane 1 to estimate the size of the product. Visualisation of the bands was as described in Section 2.5.4.

2.7 Single Strand Conformational Polymorphism (SSCP) Analysis and Electrophoresis

Two different protocols were used for SSCP analysis and electrophoresis, and modifications were made to the second protocol. The first protocol was used exclusively in the analysis of cDNA fragments "B", "C" and "D" in 7 patients only, and exons 63 and 64 in all patients (see Tables 2.1 and 2.2). This work was undertaken in Houston, towards the beginning of the laboratory component of the research. At this time, the full genomic sequence of the fibrillin gene was not known and the majority of all work at that point was based on the extraction of total RNA from dermal fibroblasts, followed by RT-PCR. The first protocol, taught to me by collaborators in Houston, involved RT-PCR without the use of radionucleotide label, followed by SSCP analysis using MDE (Mutation Detection Enhancement) gel electrophoresis of amplified cDNA or genomic fragments, followed by the silver staining technique to visualise these fragments (see Sections 2.7.1-2.7.3).

The second protocol was an adaptation of various SSCP protocols used within the Department of Cytogenetics and Molecular Genetics and shown to be successful in mutation screening of the dystrophin and other genes (personal communication, K Friend). Once the full genomic sequence of the fibrillin gene was published, and the oligonucleotide sequences for intron-based exon-specific primers became available, the RT-PCR approach was abandoned in favour of the PCR amplification of genomic DNA (see Sections 2.7.4-2.7.6). This protocol was used in the analysis of PCR fragments generated by the UF primers shown in Table 2.2. A modification to the preparation of PCR fragments was subsequently used in conjunction with protocol II for the analysis of PCR products generated by the primers shown in Table 2.1, excepting those for exons 63

and 64.

In all circumstances, either cDNA or genomic DNA (in accordance with the samples being analysed by SSCP) from an unaffected individual was routinely included as a control, and loaded into the first well of each electrophoresed gel. Any band shift observed was not considered indicative of a potential mutation or polymorphism unless it was reproducible on at least two separate occasions. In such situations, two separate but identical PCR reactions were performed, and the two PCR products were run on separate gels for which the conditions were identical. Any reproducible band shift was further investigated, firstly by population screening (to eliminate silent polymorphisms) and then, where indicated, by direct DNA sequencing.

2.7.1 Preparation of Samples for SSCP Analysis: Protocol I

A polyacrylamide gel of the relevant RT-PCR products was used to visually quantify the amount of cDNA present, according to the band intensity. In general, 4 μl of PCR product was sufficient for band visualisation using the joint SSCP protocol I and silver staining method, but where the bands appeared notably less intense on the polyacrylamide gel, 8 or 12 μl of PCR product was used. For every 4 μl of sample, the following reagents were added: 25 mM EDTA, 0.1% SDS (sodium dodecyl sulphate) and 4 μl of denaturing solution. A mix of these reagents was made, and 4.6 μl of the mix was added to every 4 μl of PCR product. The denaturing solution was prepared in advance and stored at -20°C. The following reagents were used to make a total volume of 1 ml of denaturing solution: 20 mM EDTA, 86% of formamide (stock stored at -20°C) and 100 μl each of 0.1% bromophenol blue and 0.1% xylene cyanol. The samples were denatured by boiling at 100°C for 2.5 minutes. To avoid the reannealing of single strands while other samples were being loaded into wells, the denaturing and loading of samples was performed in sets of ten. Two markers, λ DNA digested with *Hind* III (Promega) for larger fragments, or ψX174 digested with *Hae* III (Promega) for smaller fragments were loaded into the first and final wells, respectively. The marker mix was made using 1 μl of the relevant marker, 2 μl of 6 x loading buffer and 9 μl of distilled water; 3 μl of the marker mix was loaded into the respective wells. The MDE gel was electrophoresed at a constant power of 40 watts until the xylene cyanol dye front had moved off the gel, usually some 4 hours later. The gel plates were separated, the gel was dismantled, and

immediately subjected to silver staining.

2.7.2 SSCP Analysis and Electrophoresis: Protocol I

An MDE gel was used for the SSCP analysis. The following reagents were used to make a 0.5 x MDE gel: 25 ml of MDE "Hydrolink" (stored at room temperature; Biochem), and 0.6 x TBE. When ready to pour, 400 µl of 10% APS and 40 µl of TEMED were added to the total 100 ml volume. After polymerisation for 1 hour, the gel was assembled in a cold room at 4°C, with 0.6 x TBE as buffer. This was allowed to equilibrate for 1 hour, the samples loaded, and then the gel was run at 40 watts, constant power.

2.7.3 Silver Staining Technique

The silver stain protocol was performed using the GELCODE silver staining kit (Pierce). The MDE gel was left attached to the front gel plate, placed (gel-side uppermost) in a plastic bath and fixed using a fixative solution of 50% ethanol and 5% acetic acid, made up to 1 litre with distilled deionised water. The gel was then washed three times for 5 minutes at a time with distilled water. The excess water was gently suctioned off after each wash, taking care not to tear the gel. The silver stain was made using 20 ml of the silver stain (kit) made up to 600 ml with distilled water. This solution was poured over the gel and left to stain for 20 minutes, while being gently rocked by hand every few minutes to ensure equal staining to all portions of the gel. After staining, the gel was washed quickly with distilled water. A reducing solution of 20 ml of reducer aldehyde (kit) and 20 ml of reducer base (kit) added to 560 ml of distilled water was prepared immediately prior to use. A small amount of the reducer solution was poured over the gel, ridding the gel of excess silver stain (which, if present, appeared as black grains and could render the gel uninterpretable). The remainder of the reducing solution was then poured over the gel, turning the gel an orange-brown colour, with visualisation of the DNA fragments as bands. After removal of the excess silver stain (usually approximately 10 minutes), the reducing solution was removed by gentle suction. The gel was then stabilised using 20 ml of stabiliser (kit) made up to 900 ml with distilled water. The results were read and recorded while the gel was wet, since there was a tendency for some dissipation of the bands after gel drying. The gel was then blotted onto filter paper and dried on a gel drier for 30-60 minutes.

2.7.4 Preparation of samples for SSCP analysis: Protocol II

Six µl of PCR product was diluted in 100 µl of a stock solution of SDS and EDTA. The stock solution contained the following reagents: 0.1% SDS and 10 mM EDTA. The samples were vortexed and then centrifuged at 14,000 rpm for 60 seconds. Five µl of formamide loading buffer (96% deionised formamide, 20 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) was then added to each sample. The samples were heat-denatured at 94°C for 3-5 minutes, placed immediately on ice and 4-6 µl of sample was loaded into the wells. The gel was not pre-electrophoresed.

2.7.5 Modification to the Preparation of Samples for SSCP Analysis: Protocol II

Practical experience of others working in the laboratory (K Friend, personal communication) had shown that it was possible to reduce the number of steps required in the preparation of samples for SSCP protocol II, without a loss in the final definition or resolution of visible bands on the developed gel. This involved the omission of the SDS/EDTA step altogether. An equal volume of 10 x loading buffer was added to each sample. The samples were vortexed and centrifuged as above, then heat-denatured at 94-96°C for 5 minutes, placed immediately onto ice to prevent re-annealing of the single strands, and loaded immediately into the wells. This modification was successfully adopted in the analysis of PCR fragments generated by the primers shown in Table 2.1 (exons 63 and 64 excluded, see Section 2.7.1-2.7.3)

2.7.6 SSCP Analysis and Electrophoresis: Protocol II

The PCR fragments were analysed under two different gel percentages to maximise the chance of detecting a band shift (Hayashi, 1991; Orita et al., 1989). Depending on the number of samples prepared, small or large gels were run. The dimension of the small gels was 50 cm x 21 cm x 4 mm and of the large gels, 50 cm x 37.5 cm x 4 mm.

Gel one: The samples were loaded onto a 4.5% nondenaturing gel (49:1 acrylamide:bis-acrylamide) containing 10% glycerol (Ajax), 1 x TBE, 100 µl each of 25% APS and TEMED per 100 ml of gel solution. The gel required polymerisation overnight before the samples were loaded. It was then run for a period of hours (range between 9-27 hours) that was determined by the size of the PCR fragment at a constant 600 volts, and at room temperature. The gel was dried on a vacuum slab dryer for 1-2 hours, before autoradiography for 1 to 5 days (see Section 2.11).

Gel two: The conditions were identical to those for gel one except that the gel percentage was 10% instead of 4.5%. All other constituents were identical. This gel only required 2 hours for polymerisation, after which the samples were loaded. These gels were run for a period of hours again (range between 9-34 hours) determined by the size of the PCR fragment. The exposure time for the auroradiograph varied from 1 hour to 5 days.

2.8 Population Screening and Polymorphism Detection

Population screening was done in all cases where a reproducible band shift was detected by SSCP analysis. In all such cases, genomic DNA from 70 unaffected individuals (140 chromosomes) and, wherever possible, from the affected individual's parents or an unaffected first degree relative, were included in the screening. A band shift was considered representative of a polymorphism if this screening detected similar band shifts in > 1% of the samples (Ford, 1953). Such band shifts were presumed to represent silent polymorphisms within the population and were not investigated further, or characterised by direct DNA sequencing.

2.9 Preparation for, and Direct DNA Sequencing of PCR Products

Three methods were used for the direct sequencing of PCR products. The first method, taught to me by collaborators in Houston, was used in sequencing the DNA of FBN1 exon 63 in the first of my research patients in whom a band shift was detected (patient 31), by SSCP analysis protocol I (see Sections 3.2.2, 4.1.1 and 4.3). The second and third methods were taught to me in Adelaide and were standard methods used in the laboratory, so that all the necessary reagents were readily available. These two methods was used in conjunction with the the SSCP protocol II (see Section 4.1.1) to analyse all the other patients in whom reproducible band shifts were detected on SSCP screening.

Method I: The DNA fragment used for direct sequencing were purified by excising the fragment from a low melting point agarose gel, followed by phenol-chloroform extraction (Sambrook et al., 1989). The purified DNA fragment was sequenced using an ABI Applied Biosystems Automated Sequencer Model 373A (University of Texas Medical School, Department of Microbiology Core Facility).

Method II: PCR products were purified using the reagents and protocols of the Qiaquick (Qiagen) PCR Purification Kit Protocol (Bresatec) in a 100 µl reaction volume. Cycle sequencing was used to produce fluorescently labelled double-stranded PCR products using the reagents and conditions specified in the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit protocol (Applied Biosystems). The purified labelled products were then separated and analysed by an Applied Biosystems Model 373A DNA Automated Sequencer, according to the methods in the 373A DNA Sequencing System User's Manual 1/1992. The DNA fragments were sequenced using both the forward and reverse amplification primers to confirm the mutation.

One hundred ng of PCR product was added to 9.5 µl of the reaction premix and 20 ng of primer. The final volume of 20 µl was overlayed with paraffin. This mix was then subjected to the following thermal cycles: (96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes) x 25. The reaction volume was then increased to 100 µl and the labelled products were extracted twice using 100 µl phenol:water:chloroform (68:18:14; ABI). The products were then precipitated using 15 µl of 2 M sodium acetate (pH 4.5) and 300 µl of 100% ethanol. Following centrifugation for 15 minutes at room temperature, the pellets were washed in 70% ethanol, vacuum-dried and then stored (in the dark) at -20°C.

Method III: One µl of PCR product was used in a ligation reaction to clone the relevant PCR product into pGEM-T vector (pGEM®-T Vector System I; Promega). Two separate ligation mixes (mix A and B) were used with each PCR product. Mix A and B both consisted of 1 µl of T4 DNA 10 x ligase buffer (50 ng/µl), 1 µl of T vector and 1 µl of T4 DNA ligase enzyme (3U/µl). In addition, mix A contained 1 µl of PCR product and 6 µl of water, whereas mix B contained 0.5 µl of PCR product and 6.5 µl of water. Ligation reactions were carried out with a vector:insert molar ratio of 1:1 to maximise intermolecular as opposed to intramolecular ligation. The reactions were allowed to proceed for 3 hours at 15°C and then overnight at 4°C.

Following this, transformation reactions were conducted in a laminar flow hood, using Luria broth(LB)/ampicillin (50 µg/ml) agar plates equilibrated to room temperature. Two µl from each ligation reaction were transferred to 0.5 ml microcentrifuge tubes. Frozen

competent cells (Eschericia coli, XL1-Blue strain)(Stratagene) were prepared according to the method of Chung et al. (1989). The cells were placed on ice until just thawed and 50 µl of competent cells were aliquoted carefully into 0.5 ml microcentrifuge tubes. The tubes were placed on ice for 20 minutes, heat-shocked at 42°C for 45-50 seconds, and returned to ice for a further 2 minutes. Four hundred µl of LB was added to 0.5 ml microcentrifuge tubes, the contents transferred to 10 ml tubes, and incubated at 37°C for 1 hour with shaking at 225 rpm. To each LB/ampicillin agar plate was added 100 µl of 100 mM IPTG and 30 µl of 6% X-Gal (50 mg/ml) which were spread and allowed to absorb over 30-45 minutes at 37°C. The entire volume from each transformation was then plated out onto respective agar plates, left at room temperature for 15 minutes, and incubated overnight at 37°C.

A number of white colonies (insert incorporated into vector) and blue colonies (insert not incorporated into vector) were obtained. Ten white colonies from each agar plate were picked individually with a tip, spotted onto a fresh ampicillin agar plate in an ordered numbered fashion, and each tip placed into a 0.5 ml microcentrifuge tube containing two drops of paraffin oil. The spotted colonies were allowed to grow at 37°C for 4 hours, and transferred to 4°C. The 0.5 ml microcentrifuge tubes were heated at 100°C for 10 minutes and 48 µl of PCR mix (comprising 5 µl 10 x PCR buffer (Boehringer Mannheim), 1 µl of 10 mM dNTP, 1 µl each of pUC forward and reverse primers (150 ng/µl) and 40 µl of distilled water were added to each tube. The forward and reverse primers are located at bases 173-200 and 2955-2980, respectively on the pGEM-3Zf(+) vector sequence, giving an expected PCR product size of 246 bp. The primer sequences are as follows: pUC forward primer 5' TGT GAG CGG ATA ACA ATT TCA CAC AGG A 3', and pUC reverse primer 5' CAC GAC GTT GTA AAA CGA CGG CCA GT 3'. After centrifugation at 13,200 rpm for 1 minute, heating at 96°C for 5 minutes and cooling to 80°C, 2 units of *Taq* DNA polymerase was added to each tube.

PCR incubations were performed in a Perkin Elmer Cetus thermal cycler for 30 cycles at 94°C for 30 seconds, at 60°C for 30 seconds and at 72°C for 2 minutes. Samples were stored at 4°C. Twenty µl of each amplified PCR product was mixed with 4 µl of 2 x loading buffer, and the products electrophoresed through a 2% agarose gel (0.5 x TBE and 7 µl ethidium bromide added /100ml gel) in 0.5 x TBE buffer at 120 volts. A pUC

19/*Hpa* II marker was also loaded, enabling sizing of PCR products and appropriate selection of colonies (in which the insert was clearly incorporated into the vector) for DNA purification. Selected colonies (six per patient) were aspirated from the plates, innoculated into respective 50 ml Falconer tubes containing 10 mls of LB and 10 µl of thawed ampicillin (50 mg/ml), and incubated overnight at 37°C with shaking at 225 rpm.

DNA templates were prepared according to the manufacturer's instructions using the plasmid purification protocol and Qiagen columns (Qiagen Plasmid Handbook for Plasmid Mini Kit, 1993). Overnight bacterial cultures were centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was removed and the tubes inverted for 5 minutes prior to returning right-side up. The bacterial pellet was resuspended in 300 µl of resuspension buffer (stored at 4°C), the pellet gently dislodged with a tip, and the contents added to 1.5 ml tubes containing 300 µl lysis buffer. The contents were mixed gently by inversion, and incubated at room temperature for 5 minutes. To this, 300 µl of neutralisation buffer (stored at 4°C) was added, the contents mixed gently by inversion, and incubated on ice for 5-10 minutes. The tubes were microcentrifuged at 13,200 rpm for 15 minutes. The supernatant was recovered into an eppendorf tube, and then microcentrifuged as before.

Qiagen columns were equilibrated with 1 ml of equilibration buffer, and each supernatant loaded onto a Qiagen column. The columns were washed twice with 1 ml wash buffer. The DNA was eluted with 700 µl elution buffer and collected into eppendorf tubes placed beneath the columns. The DNA was precipitated with 600 µl isopropanol, mixed well and centrifuged at 13,200 rpm for 15 minutes. The pellet was washed with 1 ml 70% ethanol and centrifuged as before for 10 minutes. The supernatant was discarded, the pellet air-dried, and resuspended in 20 µl water. The DNA was quantitated (see Section 2.2.4) and dilutions of 210-250 ng/µl made in preparation for sequencing PCR (dye primer). This was carried out according to the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit protocol (Applied Biosystems). In the first instance, cycle sequencing was undertaken using primer in either the forward (-21M13) or reverse (M13) direction (see Kit protocol). Once a sequence alteration was found, cycle sequencing using the alternate primer was performed in order to confirm the mutation.

2.10 Sequencing Gel Electrophoresis

Denaturing acrylamide gels, used for resolving fluorescently labelled dideoxy-terminated or dye-primer PCR fragments in conjunction with the software of the ABI 373A DNA sequencer, were made, according to the protocol supplied. The sequencing gel mix contained the following reagents: 7.5 M urea (Biorad), 6% acrylamide:bisacrylamide solution (19:1; Biorad), and 1 x TBE (pH 8.3). The contents were placed in a clean parafilm-sealed conical flask under hot water, then made up to an 80 ml volume with distilled water, vacuum-filtered through a Nalgene 0.2 µm filter unit, and de-gassed. To this solution, 350 µl of freshly made 10 % potassium persulphate was added and mixed, followed by 45 µl of TEMED. This solution was syringe-poured between the two pre-prepared gel plates which had been cleaned with a non-fluorescent detergent, rinsed with water, separated by 0.4 mm spacers, taped around the edges, and clamped. After pouring, a plastic strip was applied to the top edge of the plates (where the shark's teeth were to be placed), and the gel left to polymerise over at least 2 hours. Then the clamps and tape were removed, the plates cleaned again, and the teeth inserted.

The gel was pre-electrophoresed at 35 watts (constant) and 45°C for 1 hour prior to loading of the samples. The PCR product to be sequenced was resuspended in 2.5 µl of a 5:1 solution of formamide:50 mM EDTA (pH 8.0), heated at 90°C for 2 minutes, and then loaded onto the gel. The gel was run for 12 hours under the conditions stated above, using 1 x TBE, diluted from a 3 mm Whatman-filtered 10 x TBE stock. The sequence data was visualised as a 4-colour chromatogram, and then compared to normal FBN1 gene sequence obtained from Genbank database.

2.11 Autoradiography

All gels were blotted onto filter paper (Whatman 3mm), dried on a slab vacuum dryer (Model 583 Bio-Rad), and placed in a cassette with radiographic film (Cronex) at -70°C overnight prior to developing. The bands were then visualised. Some gels required either a shorter or longer radiographic exposure time, and this was adjusted accordingly (range 1 hour to 5 days) after viewing the first autoradiograph.

2.12 The Subjects

2.12.1 Patient Ascertainment and Research

Ethics committee approval was obtained from the Women's and Children's Hospital prior to commencement of the clinical research. A medical record data collection protocol was designed and this was used for all participating patients (see Appendix 2A). This data was also entered into the Marfan Syndrome International Consortium Database.

Individuals with Marfan syndrome (Group 1) or an unclassified severe connective tissue dysplasia (Group 2) were ascertained through the records of the Department of Medical Genetics and by referral from cardiologists, ophthalmologists or geneticists. Initially, 58 patients were eligible to participate in the research and of these, 48 patients (26 Group 1; 22 Group 2, one of whom had a proven diagnosis of homocystinuria) or 81%, chose to participate. This represented 35 families. There were no patient withdrawals during the study period.

Patients were contacted by mail and invited to participate in the research study (see Appendix 2B). Those who expressed an interest were offerred a 2-3 hour genetic consultation. At this time, the details of the research, current knowledge of Marfan syndrome, and any genetic counselling issues were addressed. Confidentiality was ensured. Patients were asked to sign consent forms for the collection of DNA, skin biopsy and photographs (see Appendix 2C). All participants underwent clinical re-evaluation of their diagnosis, according to the internationally accepted diagnostic criteria (Beighton et al., 1988). In some cases, this required echocardiography, slit-lamp examination of the eye and orthopaedic consultation. Detailed medical histories and family pedigrees were obtained, and clinical features were documented with photographs of the individual, lateral and frontal views of the face, hands and feet, and views of the back and skin where indicated. Twenty mls of blood was collected from at least one affected individual from each family (83 samples in total). During the course of the research, DNA was received from one patient with neonatal Marfan syndrome, and from a kindred with autosomal dominant ectopia lentis, constituting a further 33 DNA samples (total 116 DNA samples). Where necessary, urine samples were obtained from patients to exclude a diagnosis of homocystinuria (44 samples in total; minimum of one sample per family). After the first visit, letters were sent to all participating families and to their

general practitioners and specialist physicians. At a second visit, a full thickness skin biopsy (see Section 2.3.1) was collected from one individual in all but one family (34 samples). During the course of the research, a further 2 skin biopsies were collected (36 in total). Those patients on anticoagulant medication required dosage adjustment and careful monitoring of their coagulation profile prior to the skin biopsy date. In some instances parental request led to their child's skin biopsy being collected whilst the child was under a general anaesthetic for another surgical procedure.

After commencement of the research, there were several referrals by geneticists, of interesting cases or families, including a large kindred with autosomal dominant ectopia lentis (Edwards et al., 1994), a baby with neonatal Marfan syndrome and a kindred with Shprintzen-Goldberg syndrome. These additional individuals were also studied.

A newsletter was sent to all participants annually, updating the knowledge given at the first visit (see Appendix 2D). All patients were contacted by mail at the end of the research with a general summary of the research findings in the group as a whole, at all times maintaining confidentiality. Individuals in whom mutations were found were contacted by mail and offered a further genetic visit.

CHAPTER THREE

CLINICAL RESULTS

3.1 Clinical Results

3.1.1 Patient Classification and General Characteristics

Forty-eight patients were clinically assessed during the course of the research. The patients were classified into one of two groups. Group 1 comprised those patients who fulfilled the diagnostic criteria for Marfan syndrome. Group 2 consisted of some patients with a severe undiagnosed connective tissue dysplasia, others with some clinical features overlapping those seen in Marfan syndrome, and one individual who was known to have pyridoxine-resistant homocystinuria. There were 26 patients (54%) in Group 1 and 22 (46%) in Group 2. The age of the patients at the time of their first research clinic visit, their sex, classification group (final not initial), and the results of urine screening for homocysteine are detailed in Table 3.1. Two patients underwent aortic surgery during the course of the research. The mortality in Group 1 over the course of the research period was 1/26 (3.9%),and in Group 2, the mortality was 1/22 (4.5%). No patients withdrew from the research project.

3.2 Clinical Features of Group 1 Patients

Anthropomorphic measurements on all patients included height, weight, head circumference, upper:lower segment ratio, arm span, hand length, mid-finger length, foot length and facial measurements, all of which were recorded as centiles, using standard charts for the normal population. Analysis of the clinical features of these patients clearly demonstrated that no two patients within the group were the same. In addition, text-book descriptions and photographs of classic Marfan syndrome clearly represent only a proportion of these patients. In those patients with familial Marfan syndrome, interfamilial phenotypic variability was often noted. There were 10/26 (39%) with sporadic Marfan syndrome, and 16/26 (61%) with familial Marfan syndrome. These relative frequencies are in keeping with those reported in the literature (see Section 1.1.1).

The clinical features of Group 1 patients are summarised in Table 3.2, and the craniofacial features are detailed separately in Table 3.3. A three-generation Aboriginal kindred with Marfan syndrome was ascertained through the research and two of the affected members (P13 and P14) were studied. One infant (P47) had neonatal Marfan

Table 3.1 Patient Classification

Patient Identification	Age (years)	Sex	Sporadic or Familial	Patient Group	Urine Screening for Homocystinuria
P1	16.3	F	familial	2	-
P2	10.2	M	sporadic	2	-
P3	20.2	F	familial	2	-
P4	19.3	M	familial	1	-
P5	26.3	M	sporadic	2	-
P6	40	F	familial	1	-
P7	19	M	familial	1	not done
P8	13.8	F	sporadic	1	-
P9	4.5	F	sporadic	2	-
P10	29.8	F	familial	1	-
P11	5	F	familial	1	-
P12	7.7	M	familial	1	-
P13	11.8	F	familial	1	-
P14	10.3	F	familial	1	not done
P15	9.7	F	familial	2#	-
P16	9.7	F	familial	2#	-
P17	14.1	F	familial	2	-
P18	46.9	M	familial	1	-
P19	16	M	familial	2	-
P20	14.9	M	sporadic	1	-
P21	2.6	M	familial	1*	not done
P22	14.4	M	familial	1	-
P23	19.6	M	sporadic	1	-
P24	40.9	M	sporadic	1	-
P25	26	F	familial	1	-
P26	18.1	M	sporadic	2	-
P27	9.8	F	sporadic	2	-
P28	3.6	M	sporadic	2	-
P29	12.4	F	sporadic**	1	-
P30	42.7	F	familial	1*	-
P31	29.8	M	sporadic	1	-
P32	28.2	F	sporadic	1	-
P33	53.8	F	sporadic	1	-
P34	26	M	familial	1	not done
P35	4.9	F	sporadic**	2	-
P36	37.8	M	familial	2#	-
P37	47.9	M	sporadic	2	-
P38	18.8	M	sporadic	2	-
P39	3.9	F	familial$^{\infty}$	2	-
P40	3.9	F	familial$^{\infty}$	2	-
P41	1.9	F	familial$^{\infty}$	2	-
P42	13	M	familial	1	-
P43	43.7	F	familial	1	-
P44	6.3	M	sporadic**	1	-
P45	16.5	M	sporadic	2	-
P46	11.8	M	sporadic	2	-
P47	0.2	F	sporadic	1	-
P48	27.3	M	sporadic(AR)	2	+

 # familial aortic aneurysms; * most likely group 1 but young age or other factor precludes definitive clinical diagnosis; ** adopted; $^{\infty}$ autosomal recessive disorder possible; AR known autosomal recessive condition; - homocystine not detected; + homocystine detected

Table 3.2 Clinical Features of Group 1 Patients

Patient	ARD/AR	AD	MVP	TS	SR	ARA	SC/PD	JH	EL	RD	MYA	PTX	STR
P4	+/+	-	+	+	.76	+	+/+	++/#	+	-	+	-	+
P6(m)}	+/+**	-	-	+	.97	+	-/-	+	+	-	+	-	+
P7(s)}	+/-	-	+	+	.81	+	+/+	+	+	-	-	-	+
P8	+/-	-	-	+	.93	++	-/-	++/#	+	-	+	-	++
{P10 (m)	-/-	-	-	+	.88	++	+/-	++	-	-	+	-	++
{P11 (d)	+/-	-	-	+	.96	++	-/-	++	+	-	+	-	-
{P12 (s)	+/-	-	-	+	.92	++	+/+	+	+	-	+	-	+
P13 (si)}	+/+	-	-	+	.76	+	+/+	+	+	-	+	-	++
P14 (si)}	+/-	-	+	+	.75	+	+/+	++	+	-	++	-	++
P18	+/-	-	+	+	.85	++	-/+	-	-	-	+	-	+
P20	+/-	-	+	+	.79	++	-/+	++	+	-	+	-	+
P21*	-	-	-	+	1.3	+	-/-	++	-	-	-	-	-
P22	+/-	-	-	+	.91	++	-/+	++	-	-	-	-	++
P23	+/-	-	+	+	.83	++	-/+	-	-	-	-	-	++
P24	+/-	-	-	+	.89	++	+/+	-	-	-	+	-	-
P25	+**/-	+	+	+	.75	++	+/+	++	+	-	+	+ (r*)	++
P29	+/+	-	-	+	.82	-	+/-	+	+	-	+	-	++
P30	+/+	-	-	-	.79	++	-/-	-	-	-	+	-	-
P31	+/+	-	-	+	.84	++	+/+	+	+	-	-	-	++
P32	+/+	-	-	+	.92	+	+/+	#	+	-	++	-	+
P33	+/-	-	-	-	1.0	-	+/-	#	+	-	?	-	++
P34	?**	+	-	+	.79	+	+/+	+	-	-	-	-	+
{P42 (s)	+/-	-	+	-	.75	-	+/+	+++	-	-	-	-	+
{P43 (m)	+/-	-	+	+	.87	++	+/+	+++	+	-	+	-	++
P44	+/-	-	+	+	.96	-	+/+	+++	-	-	-	-	-
P47	+/-	-	+	+	?	++	-/-	#	-	-	-	-	-

*ARD aortic root dilatation; AR aortic valve regurgitation; AD aortic dissection; MVP mitral valve prolapse; TS tall stature (> 90th percentile; most were > 97th centile); SR upper:lower segment ratio; ARA arachnodactyly; SC scoliosis; PD pectus deformity; JH joint hypermobility; EL ectopia lentis; RD retinal detatchment; MYA myopia; PTX pneumothorax; STR striae; - absent; + present; ++ moderate; +++ marked; * most likely Group I but young age precludes definitive clinical diagnosis; ** aortic graft surgery +/- valve replacement; (r*) surgery for recurrent pyo/pneumothoraces; # joint contractures; ? unknown; m mother; f father; d daughter; s son; si sister; { or } groupings of family together*

Table 3.3 Craniofacial Features of Group 1 Patients

Features	Number of Patients/total	Percentage (%)
dolichocephaly	8/26	31
deep set eyes	16/26	62
epicanthus	4/25	16
hypertelorism	6/25	24
ptosis	3/25	12
malar hypoplasia	14/26	54
malar prominence	1/26	4
high and narrow palate	12/24	50
high palate	8/24	33
dental occlusion-overbite	2/25	8
dental occlusion-underbite	1/25	4
dental overcrowding	13/25	52
orthodontics required	5/25	20
abnormal ears	9/26	34
mandibular prognathism	3/26	12
retro/micrognathia	5/26	19

syndrome and died at age 2 months. Of the Group 1 patients, a FBN1 gene mutation was defined in six patients (representing five families) (see Section 4.3). The clinical and molecular details of these patients are described in Sections 3.2.2 and 4.3, respectively.

3.2.1 Re-classification from Group 2 to Group 1.

Patient P33 was re-classified from Group 2 to Group 1 during the course of the research, based on echocardiographic data. Her features included bilateral lens subluxation, a cataract of the left lens, elbow joint contractures and a cervico-thoracic kyphosis. The family history was significant. Her son had a high arch palate, generalised joint hyperextensibility, recurrent knee dislocations, pectus carinatum and tall stature. An echocardiogram 4 years prior was reportedly normal, and he had no ocular abnormalities. Her sister died suddenly at the age of 26 years after a routine tonsillectomy, and a postmortem did not help to elucidate the cause of death. Several other family members had recurrent joint dislocations. Although clearly an interesting family, none of the family members fulfilled the diagnostic criteria for Marfan syndrome until 1995, when a routine echocardiogram on P33 demonstrated aortic root dilatation for the first time, at the age of 54 years.

3.2.2 Clinical Features in Group 1 Patients with a Defined FBN1 Gene Mutation

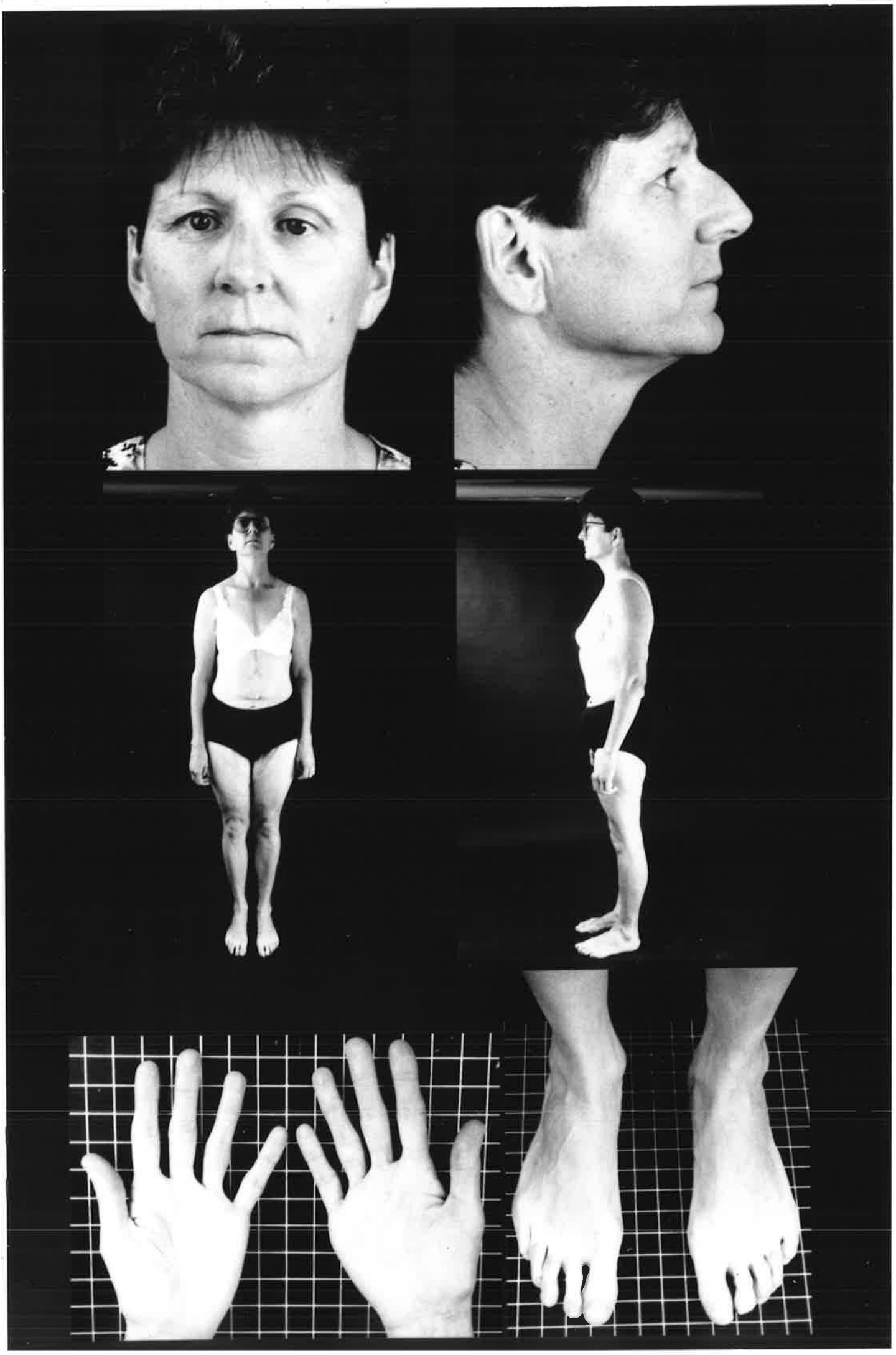
This patient subgroup consisted of one 2-generation Marfan kindred (P6 and P7), two sporadic cases in unrelated adult males (P20 and P31), one sporadic case in a 6 year-old boy (P44), and one sporadic case of neonatal Marfan syndrome in a 2 month old baby girl (P47). Clinical photographs of P6, P7, P20, P31, P44 and P47 are shown in Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6, respectively.

Patient 6

A diagnosis of Marfan syndrome was not suspected in this woman until the diagnosis had been made in her son, P7 at 10 years of age. As the family lived in the country, P7's paediatrician arranged an echocardiogram for P6 as a precautionary measure, although she was asymptomatic, and not considered to have Marfan syndrome on clinical grounds. The echocardiogram, done when P6 was 31 years old, confirmed aortic root dilatation. Bilateral lens dislocation was also confirmed on slit lamp examination. She was treated with propanolol and underwent regular echocardiographic surveillance. Six years later when the aortic root diameter exceeded 6 cm, she underwent a Bentall's procedure

Figure 3.1. Clinical Photographs of P6

(see overleaf)



(composite aortic graft repair and aortic valve replacement). She remains well.

Patient 7

The diagnosis of Marfan syndrome was first suspected in P7 at age 10 years, based on the paediatrician's findings of a high, narrow palate, tall stature, dolichostenomelia, arachnodactyly, scoliosis and a pectus defect. Echocardiogram confirmed aortic root dilatation, mild mitral valve prolapse and mild mitral incompetence. Slit lamp examination confirmed bilateral lens dislocation. He was noted to have progressive contracture of both elbow joints, hypermobility of the distal interphalangeal joints, and fixed flexion deformities of the proximal interphalangeal joints of both index fingers. He required orthodontic treatment, bilateral lens aspiration, and surgery to widen the palate. His clinical phenotype was more severe than that of his mother, P6. At age 21 years, aortic graft surgery is being planned because of progressive aortic root dilatation.

Patient 20

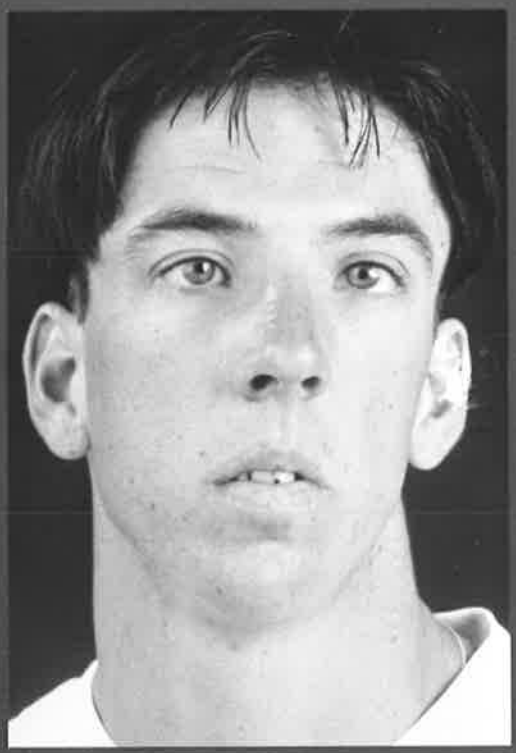
A diagnosis of Marfan syndrome was made at age 5 years, based on the clinical findings of tall stature, myopia, bilateral lens dislocation, a high arched palate, arachnodactyly and bilateral inguinal herniae. The patient moved abroad with his family and returned to Australia at the age of 13 years, at which time echocardiography demonstrated dilatation of the aortic root. He was treated with atenolol. Other clinical features included peripheral retinal degeneration of both eyes, hypermobility of the distal interphalangeal joints, fixed flexion contractures of the proximal interphalangeal joints of both little fingers and a combined pectus excavatum/carinatum defect. At the age of 17 years, there has been minimal progression of aortic root dilatation, and he remains well.

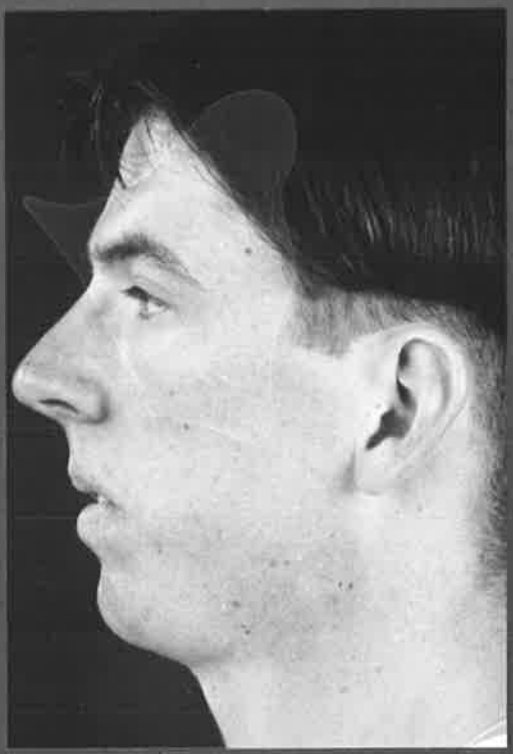
Patient 31

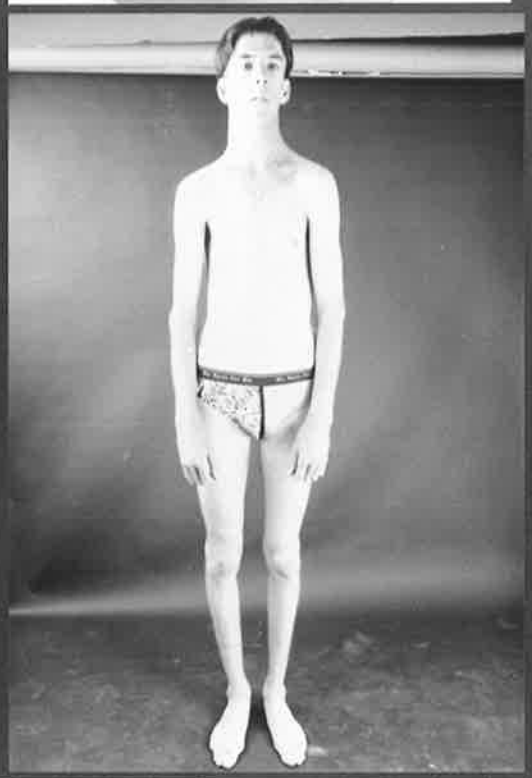
This patient was born to phenotypically normal parents. He underwent surgery to correct pyloric stenosis at the age of 3 weeks, hiatus hernia at 5 months, and bilateral calcaneovalgus foot deformities at the age of 7 years. He developed acute rheumatic fever at the age of 14 years, at which time echocardiography demonstrated dilatation of the aortic root. He had obvious facial asymmetry, myopia and bilateral lens dislocation. Skeletal manifestations included tall stature, dolichostenomelia, arachnodactyly, pes planus, scoliosis and pectus excavatum. At 20 years of age, he underwent aortic root and valve repair. He remains well at age 32 years.

Figure 3.2 Clinical Photographs of P7

(see overleaf)







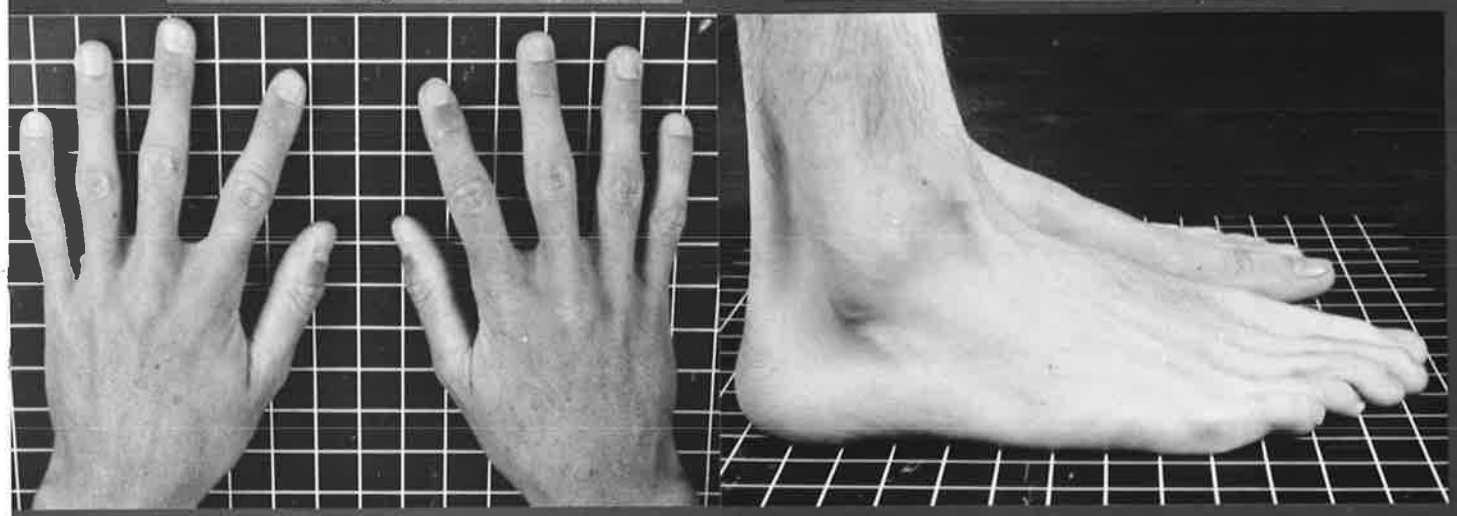
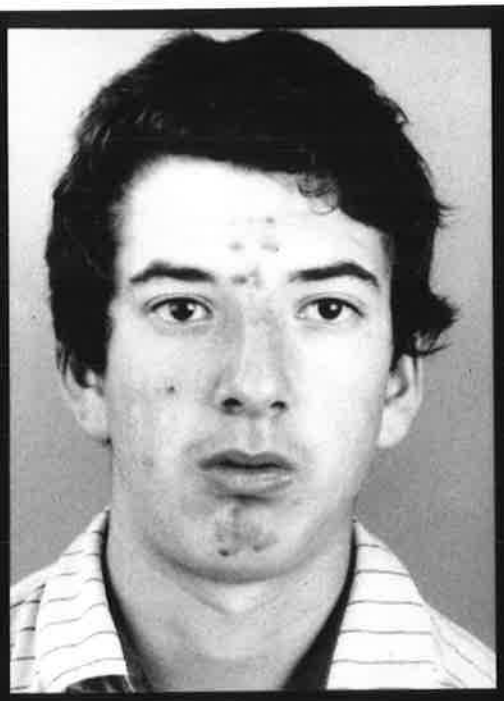
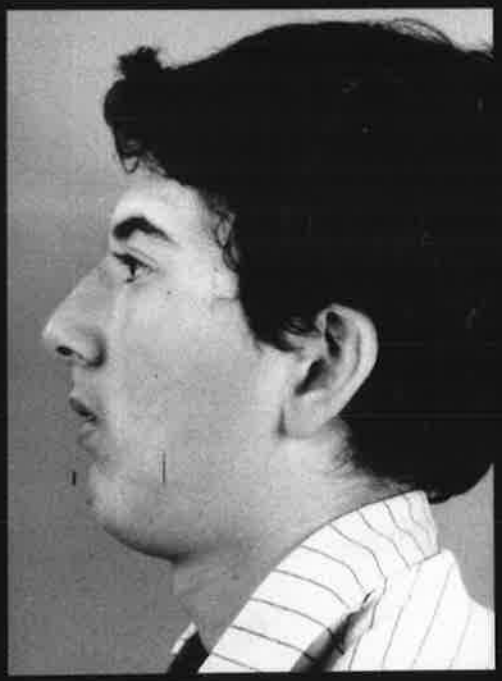
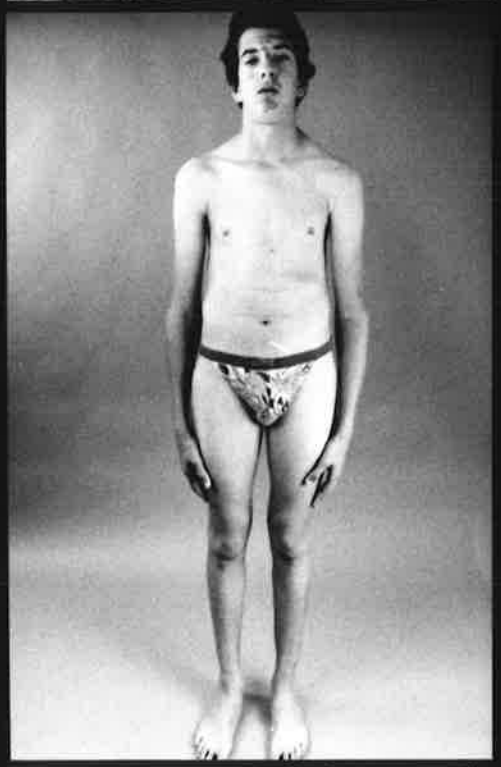


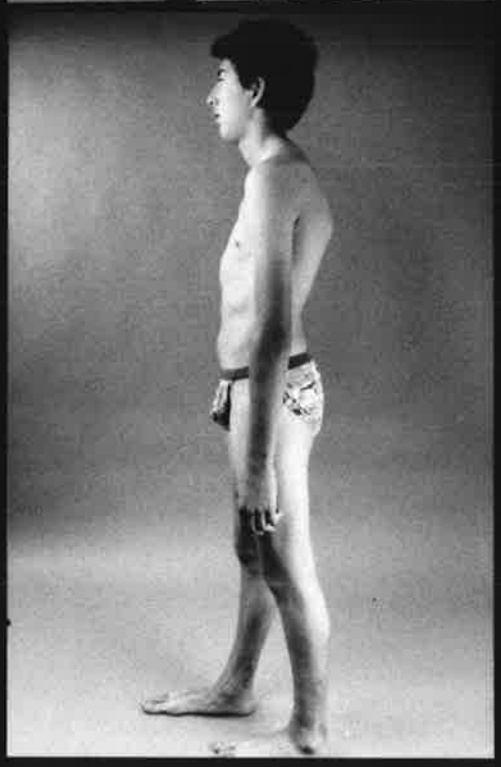
Figure 3.3 Clinical Photographs of P20

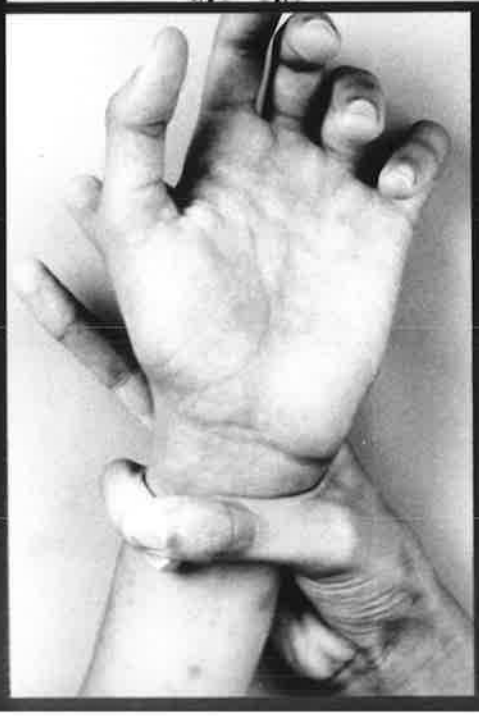
(see overleaf)











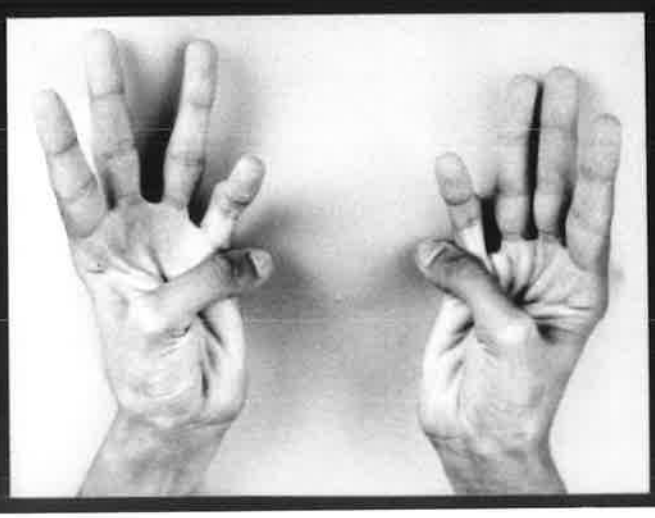
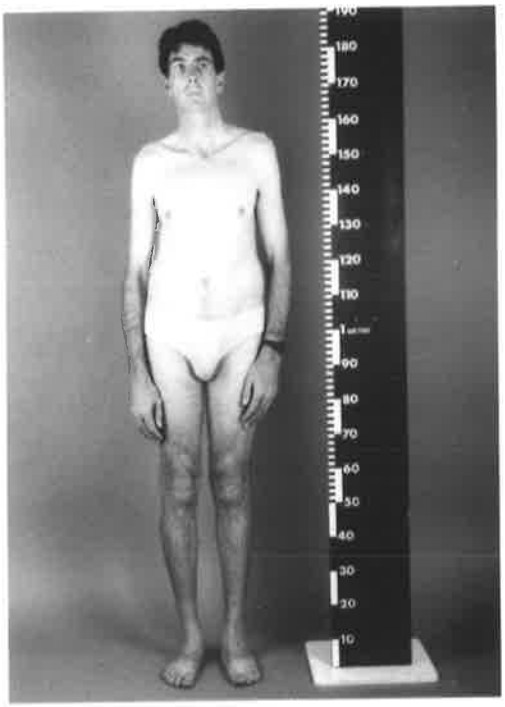


Figure 3.4 Clinical Photographs of P31

(see overleaf)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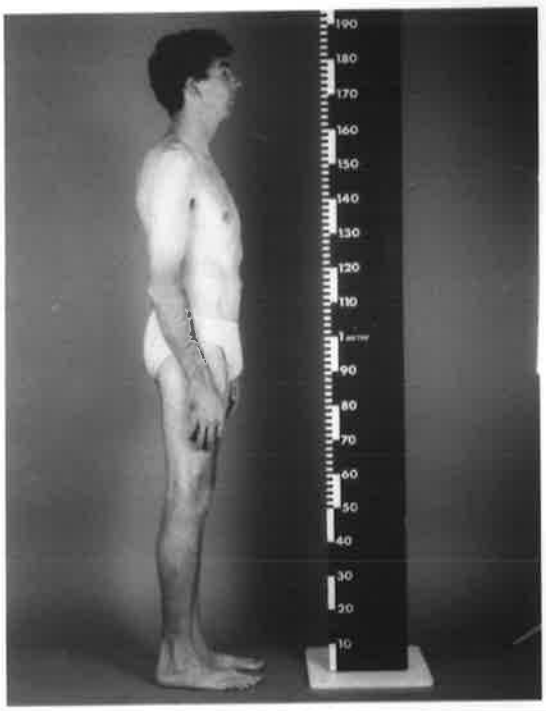
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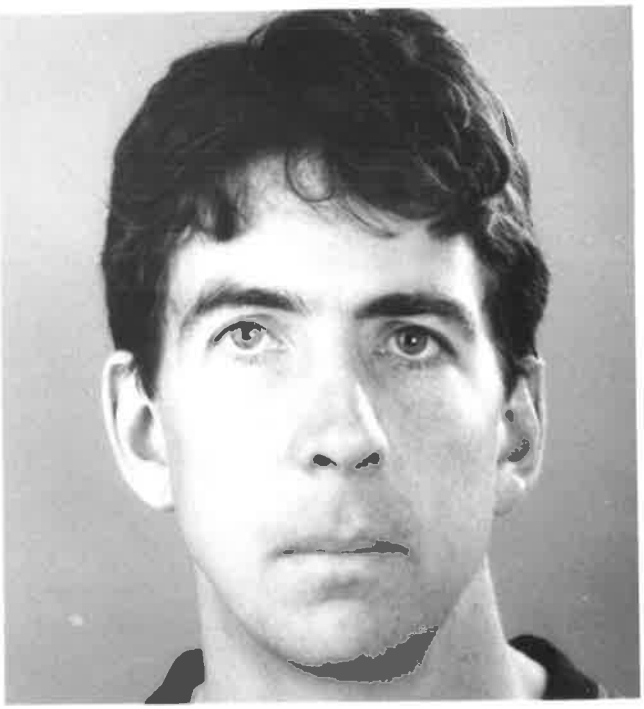
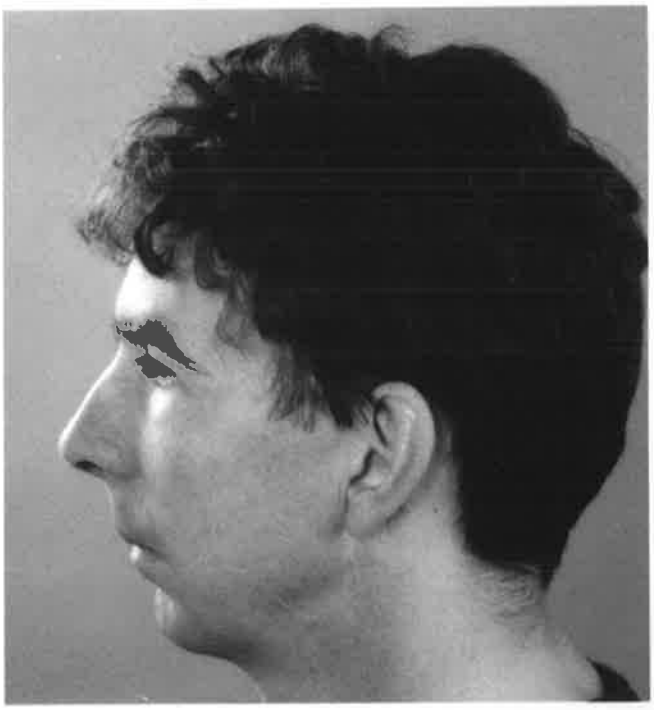
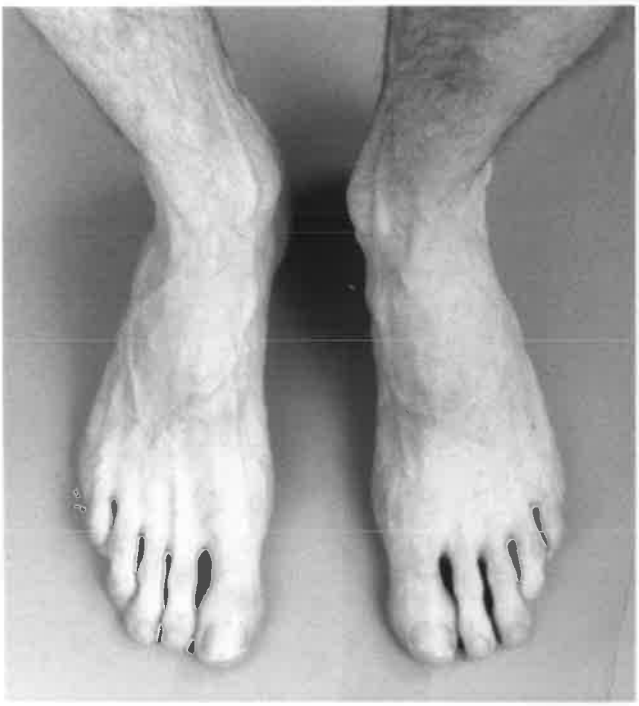






Patient 44

This patient, was born at term, with the following growth parameters: birth weight 3130 g (25th-50th percentile), birth length 47 cm (3rd percentile) and head circumference 35.5 cm (50th percentile). He was noted to have facial dysmorphism, scaphocephaly, pinpoint pupils, marked redundancy of facial skin, generalised hypotonia, and bilateral talipes equinovarus. He had congenital camptodactyly of the fingers which resolved with physiotherapy. There was a left undescended testis, bilateral inguinal herniae and an umbilical hernia, all of which were corrected surgically at age 11 months. Following the surgery, a cardiac murmur was heard, and echocardiography confirmed mitral valve prolapse. A cerebral CAT scan was performed at age 14 months because of macrocephaly in association with scaphocephaly. This showed early fusion of the metopic suture, and dilatation of the lateral and third ventricles suggesting mild communicating hydrocephalus. The carpal bone age was relatively advanced in relation to the remainder of the hand bones but overall, the bone age was within normal limits.

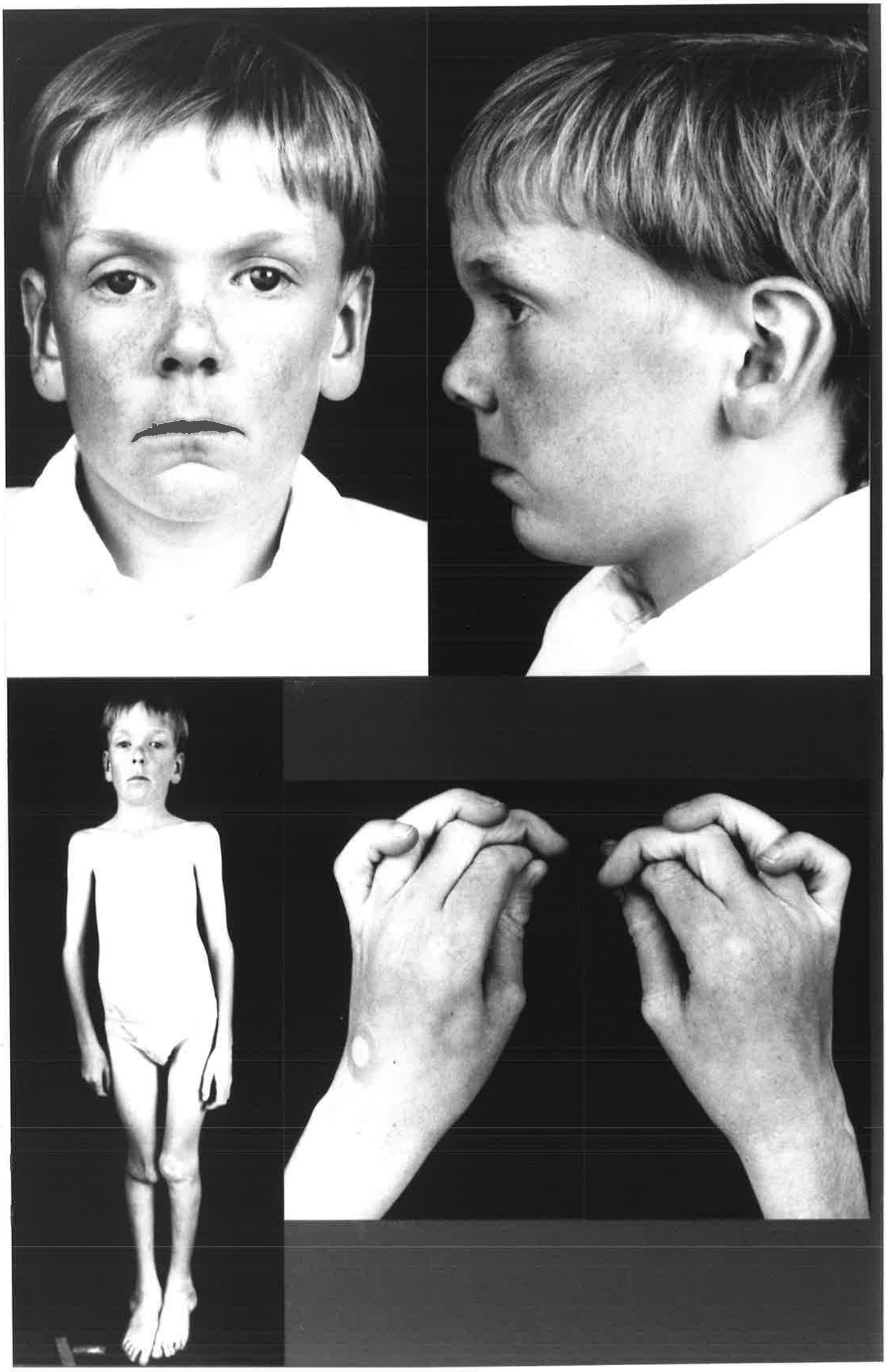
Since the age of 9 to 10 months, he was noted to have episodes of cyanosis, pallor and nausea with or without vomiting, headache and sleepiness. Sleep studies excluded the possibility of obstructive apnea, and the episodes were not considered to have a cardiac basis.

At age 15 months, he had a head circumference of 52.6 cm (> 97th percentile) and height of 86 cm (97th percentile). He had a ridged metopic suture, deep-set eyes, large ears (6 cm length, > 97th percentile), micrognathia and long, slender fingers. There was general redundancy of the skin, normal finger joint mobility, and limitated elbow extension and hip abduction. He had normal hearing, delayed speech and motor development, and was intellectually normal. A diagnosis of Weaver syndrome was made at this time, based on his craniofacial structure, rapid growth in the first year of life, camptodactyly at birth, and an advanced bone age.

At 3 years of age, echocardiography was performed. This showed aortic root dilatation and mitral valve prolapse, and led to a revised diagnosis of Marfan syndrome. Ophthalmologic examination at age 4 years confirmed myopic astigmatism and amblyopia of the right eye. The pupils could not be fully dilated because of iris dilator atrophy. Lens dislocation could not be fully excluded because of the inability to fully

Figure 3.5 Clinical Photographs of P44

(see overleaf)



dilate the pupils. He had a lean body build, generalised joint laxity, long narrow feet and slender fingers. There was an asymmetric pectus carinatum defect associated with prominence of the sternocostal junction. The accelerated growth had ceased. He was treated with verapamil since treatment with β-blockers was contraindicated because of asthma. At age 8 years of age, he continues to have the cyanotic episodes (aetiology unknown) but is otherwise well.

Patient 47

This baby girl was the second child of nonconsanguineous parents. The birth weight was 3200 g (50th centile), head circumference 36.8 cm (97th centile) and crown-heel length approximately 55 cm (97th centile), taking limb contractures into consideration. There was a relative lack of subcutaneous fat. The limbs were long. There was arachnodactyly, and the hand length (7.5 cm), mid-finger length (3.5 cm) and foot length (9.3 cm) were all greater than the 97th centile. There was ulnar deviation of the fingers, and flexion contractures of the shoulders, elbows, hips, knees and ankles. There was camptodactyly and flexion contractures of the toes. The skin was redundant and loose. The ears were long (4.5 cm) and soft with hypoplastic cartilage.

Ophthalmologic examination revealed poor dilator muscle of the pupils but no lens dislocation. A chest radiograph showed eventration of the right hemidiaphragm, slender long bones, and gracile ribs. Echocardiogram showed a mildly dilated aortic root, a normal aortic valve, prolapse of both mitral valve leaflets, mild mitral regurgitation, and moderate tricuspid valve prolapse with mild regurgitation. Cranial ultrasound was normal apart from an enlarged cisterna magna.

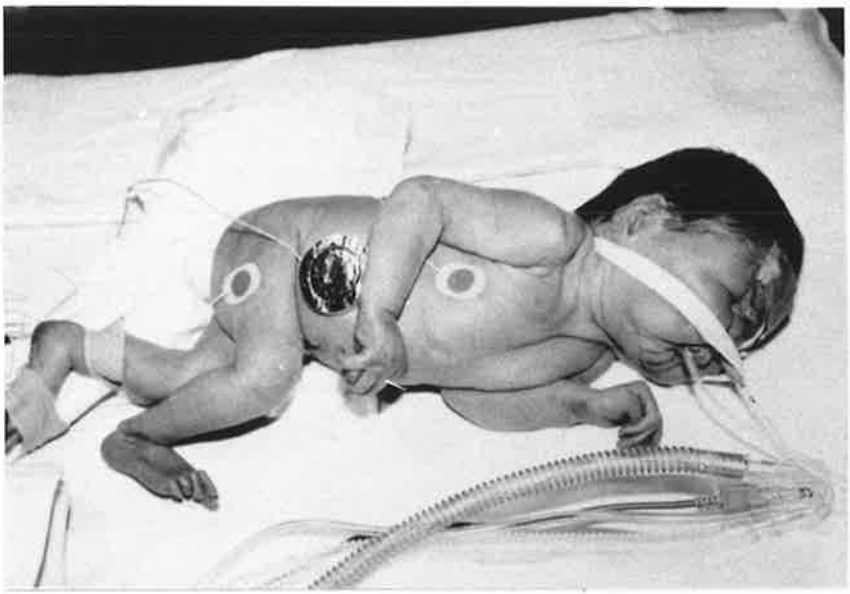
She died at home at the age of 2 months. The parents declined skin biopsy and post mortem examination.

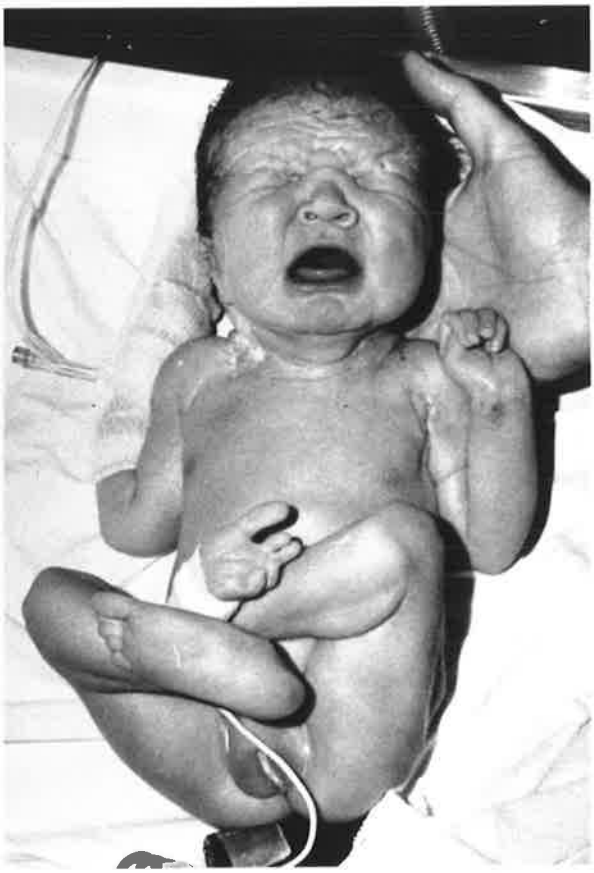
3.3 Clinical Features of Group 2 Patients

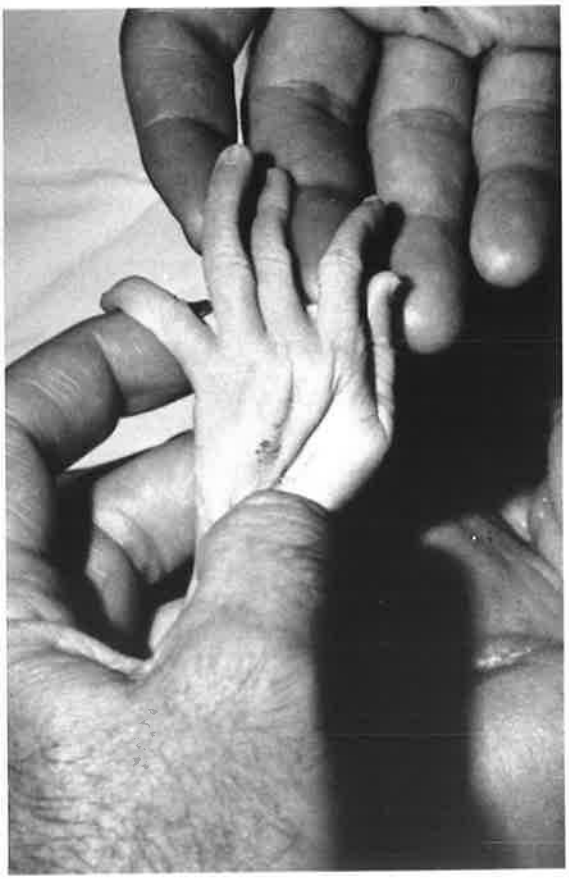
The clinical features of these patients are not described individually because of the heterogeneity of this patient group. All these patients had no known diagnosis at the commencement of the research. Subsequently, a diagnosis of Furlong syndrome was made in two patients (P2 and P46). These patients are described in Section 3.3.1. A

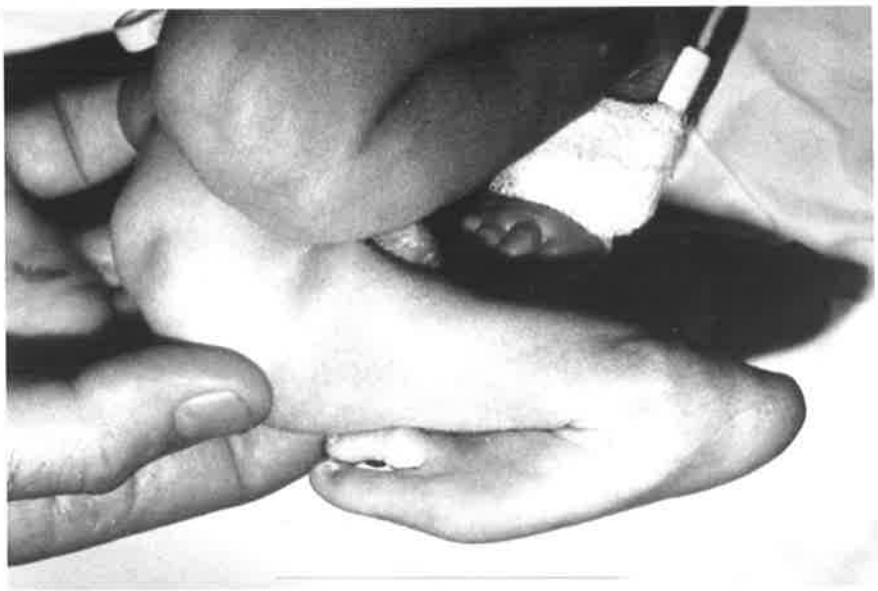
Figure 3.6 Clinical Photographs of P47

(see overleaf)









diagnosis of Shprintzen-Goldberg syndrome was made in one child (P27). The clinical and radiographic features of this child, and a second previously diagnosed family (P39, P40 and P41) are detailed in a publication (see Appendix 3A). In the manuscript, P27, P39, P40 and P41 are represented as Patients 1, 2 (twin 1), 3 (twin 2) and 4, respectively. In addition, two children (P28 and P35) with congenital aneurysms of the great vessels were ascertained, and details are provided below. Clinical photographs of P28 and P35 are shown in Figures 3.7 and 3.8, respectively.

Patient 28

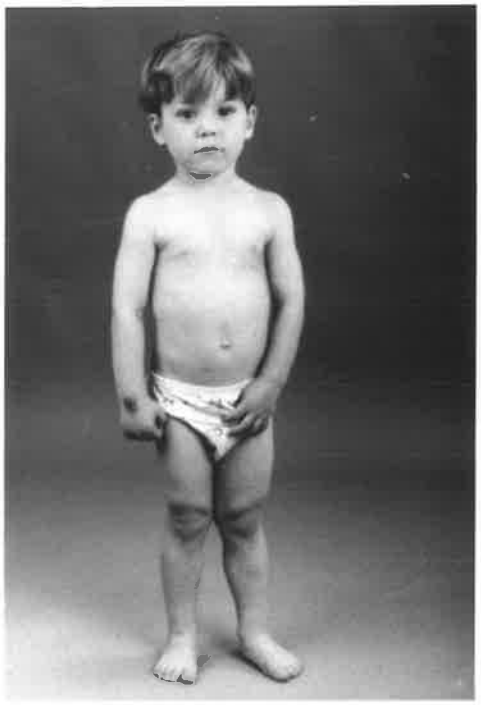
This male infant born at term was the second child of non-consanguineous parents. The BW was 3410 g (50th centile). A paternal niece had tetralogy of Fallot and a paternal female first cousin had aneurysmal dilatation of a cerebral vessel, confirmed on computerised axial tomography (CAT) scan.

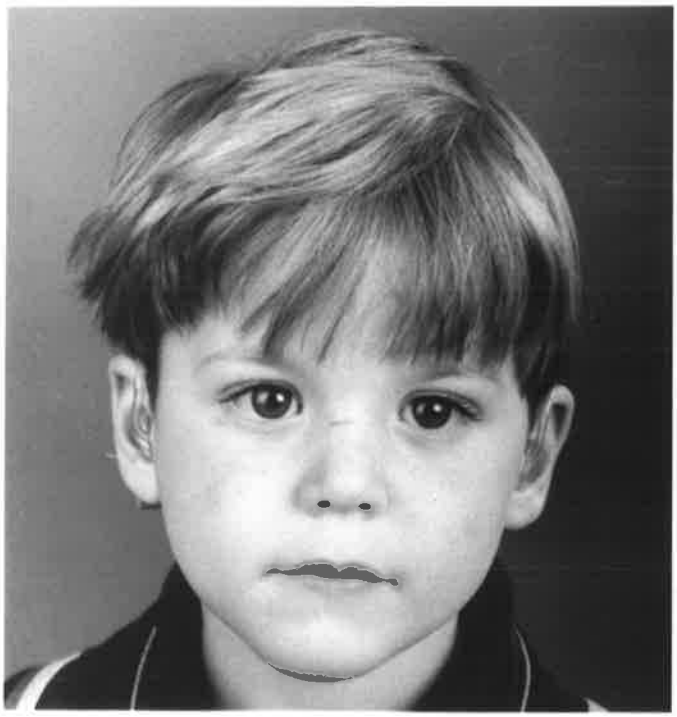
There was severe respiratory distress and cyanosis from birth. Echocardiography and cardiac catheterisation showed a dilated right atrium, a thickened tricuspid valve which was both regurgitant and stenotic, a dilated and misshapen right ventricle and a mildly thickened pulmonary valve. Pulmonary arteriography showed massive dilatation of the pulmonary trunk and severe pulmonary regurgitation. Despite the latter, pulmonary artery pressure was 55/30 (mean 40) mm Hg. There was a significant right to left shunt at atrial level. The left ventriculogram showed an intact venticular septum, a normal aortic valve, and marked dilatation of the ascending aorta and aortic arch. There was left to right shunting through the duct but due to the high pulmonary vascular resistance and pulmonary and tricuspid regurgitation, there was progressive retrograde opacification of the right ventricle, right atrium and then left atrium, with very little flow to the lungs. On echocardiography, the aortic root diameter (ARD), ascending aortic diameter, and main pulmonary and left pulmonary artery diameters were 2.1, 1.9, 2.2 and 1 cm, respectively, and tricuspid valve diameter 1.2 cm.

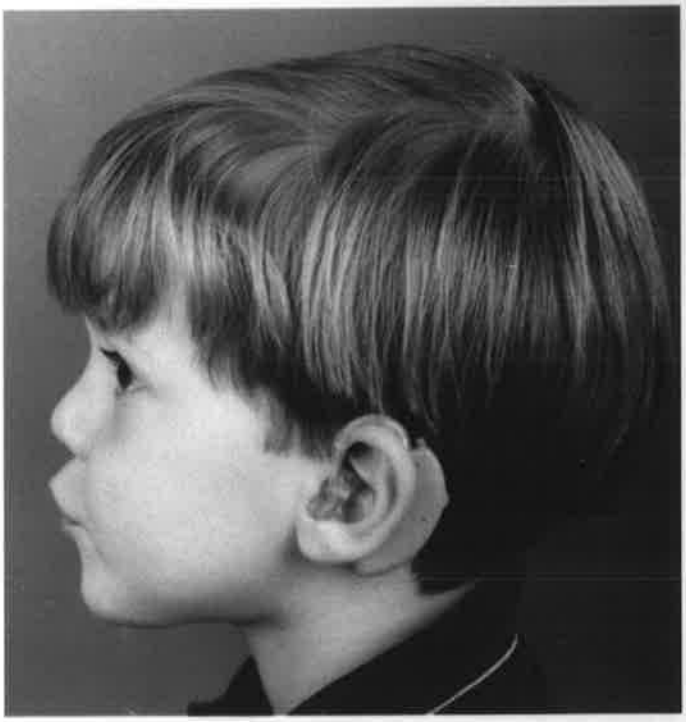
He was treated with prostaglandin E1 and dopamine and, at 3 weeks of age, underwent surgery because of unrelenting extrinsic airway compression by the aneurysmal great arteries. Operative findings included aneurysmal dilatation of the ascending aorta, aortic arch and upper half of the descending aorta; ectasia of the innominate, left subclavian and right coronary arteries and aneurysmal dilatation of the main, proximal right and left

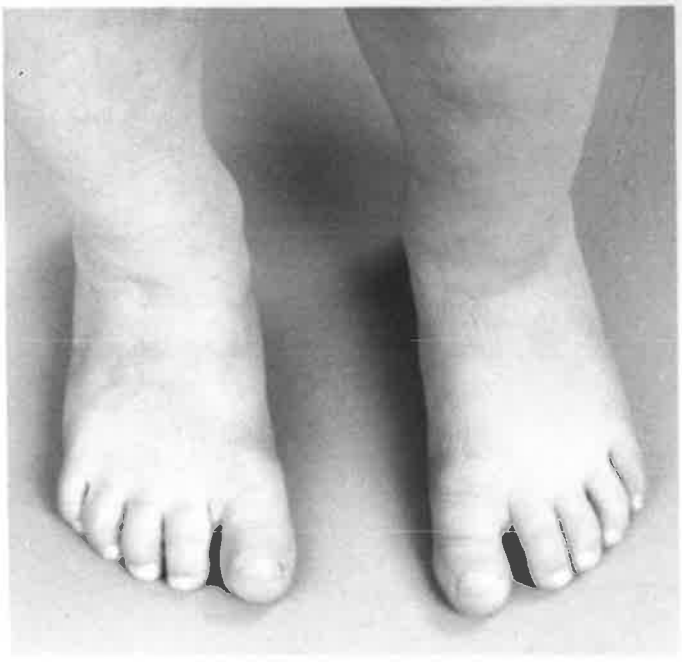
Figure 3.7 Clinical Photographs of P28

(see overleaf)









pulmonary arteries. The pulmonary and tricuspid valves were dysplastic, mildly stenosed and incompetent. There was a secundum atrial septal defect. The trachea was severely compressed. The entire ascending aorta and upper half of the descending aorta were replaced with a 12 mm dacron hemishield tube graft. The patent ductus arteriosus was ligated, the pulmonary valve and trunk replaced with a 13 mm aortic homograft, the atrial septal defect sutured and tricuspid valve commisurotomy was performed.

Histologic examination of the resected aorta showed normally ordered elastin fibres with a minimal increase in interstitial glycosaminoglycan deposition. There was mild fibrointimal hyperplasia.

At age 2 years, echocardiogram and cardiac catheterisation showed a significantly enlarged right atrium and ventricle, severe tricuspid regurgitation and moderate pulmonary homograft valve regurgitation. The right pulmonary artery diameter distal to the homograft was dilated, measuring 1.8 cm. The aortic root was slightly enlarged (1.8-2.0 cm) proximal to the graft. There was no aortic regurgitation. Distal to the graft, there was mild dilatation and tortuosity of the proximal descending thoracic aorta. The ARD showed progressive dilatation (1.95 and 2.1 cm, at 3 years 8 months and 5 years, respectively). Treatment consisted of daily aspirin. The use of beta-blockers was contraindicated because of severe asthma.

At the age of 3.5 years, his height was on the 3rd centile and weight was on the 10th centile. There was no arachnodactyly, joint hyperextensibility, dolichostenomelia, scoliosis, lens dislocation or skin abnormality. He had pes planus and severe high frequency sensorineural deafness (most likely attributable to neonatal hypoxia), with associated speech delay. Chromosome studies were normal, 46, XY. He remains well, at age 5 years and 10 months.

Patient 35

Details of the pregnancy, delivery, early neonatal period and family history were unavailable for P35, an adopted female infant who presented with symptoms of airway compression in the first 1-2 months of life. Cardiac catheterisation and angiography at age 6 weeks showed marked aneurysmal dilatation of the ascending aorta, and dilatation of the proximal aortic arch and innominate artery. There was massive dilatation of the

pulmonary trunk beyond the pulmonary valve, left pulmonary artery origin stenosis, stenosis of the right pulmonary artery beyond its origin, a huge ductal diverticulum on the pulmonary artery and moderate pulmonary regurgitation. There was no pulmonary stenosis. The remaining valves were normal. She was treated with propanolol.

At 2.5 months of age, she underwent shortening and extensive aneurysmorrhaphy of both the ascending aorta and pulmonary trunk, with pericardial patch repair of the stenotic left pulmonary artery and dilatation of the right pulmonary artery. The aorta and pulmonary trunk were thick-walled (the pulmonary trunk was thicker than the aorta), and the aortic layers came apart easily.

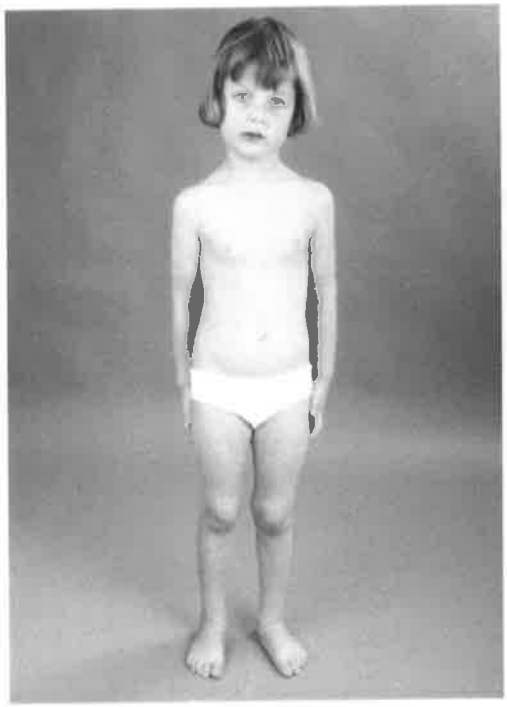
Life-threatening episodes of respiratory obstruction prompted cardiac re-catheterisation at 7 months of age. Aortography showed an even larger aneurysm of the ascending aorta and innominate artery. The ascending aorta was massive, pushing the bifurcation of the innominate artery superiorly, and the aortic valve inferiorly to the level of the xiphisternal junction. There was significant residual tracheal narrowing and obstruction from compression by the aorta. From the mid-arch, the aorta was normal.

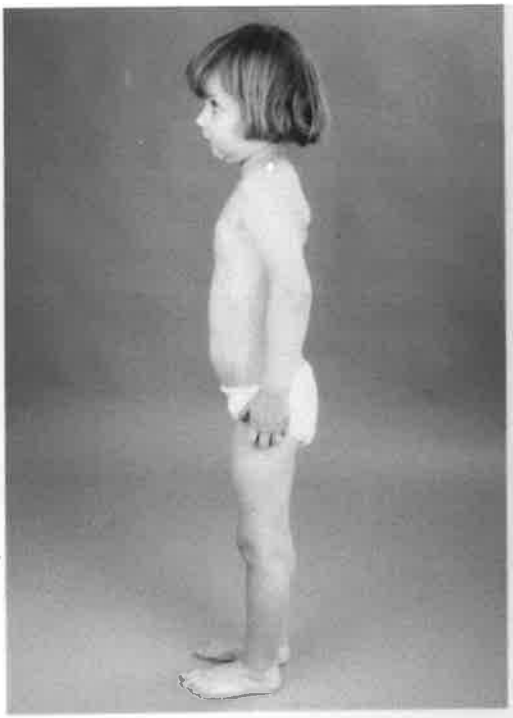
At reoperation at 8 months of age, findings included massive recurrent aneurysm of the ascending aorta, extending into the innominate artery. There was restenosis of the left pulmonary artery and a prominent ductal diverticulum extending upwards and to the left. The right atrial appendage and lower abdominal aorta were cannulated for cardiopulmonary bypass. The ascending aorta, proximal innominate artery and proximal arch were excised and replaced by a 14 mm collagen impregnated dacron graft. The innominate bifurcation was anastomosed end to side to the base of the left common carotid artery. The left main pulmonary artery origin was dilated. Post-operatively she developed left upper lobe collapse and consolidation which was considered to be due to compression by the aneurysmal ductal diverticulum.

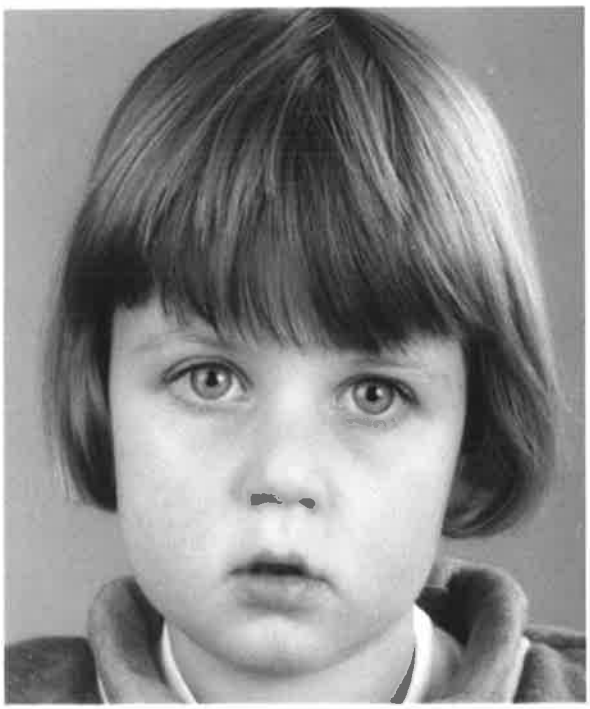
Histologic examination of the aorta and pulmonary artery from the initial surgery showed marked disruption of elastic fibres with an increase in deposition of interstitial glycosaminoglycans and marked thickening of the arterial intima. Large elastic fibres were markedly reduced in number and small elastic fibres were highly irregular in distribution.

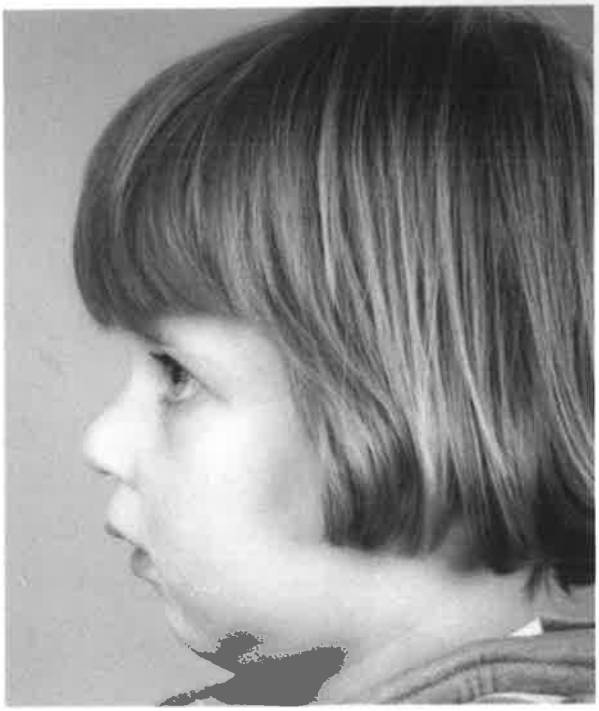
Figure 3.8 Clinical Photographs of P35

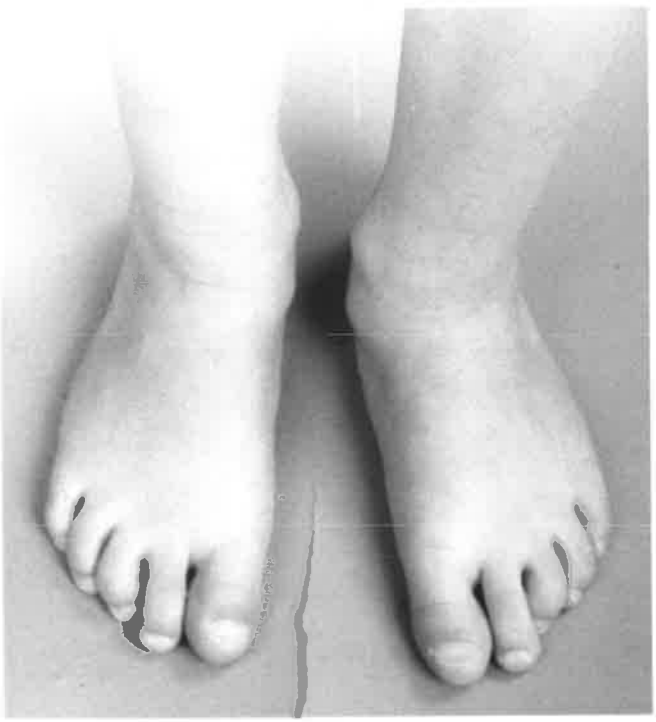
(see overleaf)











Echocardiogram at age 7, 20, 30, 46 and 76 months showed an ARD of 1.8, 2.6, 2.7, 2.9 and 3 cm, respectively. Cineangiography at age 39 months showed an unobstructed aortic graft and a redundant aortic arch. The pulmonary trunk (diameter 2.2 cm) and proximal right and left branches were significantly dilated. The abdominal aorta was slightly tortuous but not dilated. The major abdominal arteries were tortuous and dilated.

Ultrasound of the right common and internal carotid arteries at age 4.5 years of age showed marked tortuosity and mild fusiform dilatation of the right common carotid artery. The left common carotid artery showed less marked tortuosity. A cerebral angiogram at age 5.5 years showed extreme tortuosity and dilatation of intra- and extra-cranial arteries (Figure 3.9).

At 5 years of age, her growth parameters were normal. She had prominent eyes, a bulbous nasal tip, a prominent premaxilla, a highly arched palate, micrognathia, generalised mild joint hypermobility and smooth velvety skin with normal scarring. She was maintained on atenolol and at age 7 years and 2 months, remains well.

None of the patients in Group 2 were found to have a FBN1 gene mutation. Type IV Ehlers Danlos syndrome was excluded in several patients.

3.3.1 Furlong syndrome

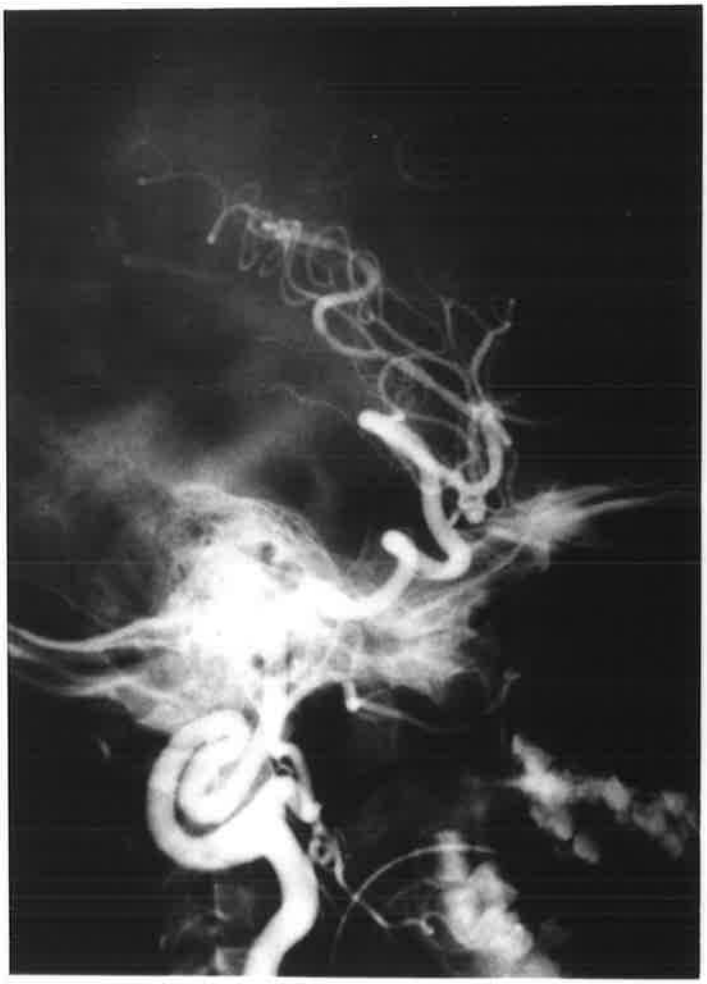
This Marfan-like disorder was first described by Furlong et al. (1987) who reported a male patient with dolichocephaly, dolichostenomelia, scoliosis, pectus carinatum, mitral valve prolapse, aortic root dilatation, myopia, recurrent inguinal herniae, and sudden death from aortic dissection. Craniosynostosis was also present, and intelligence was normal. The added findings of ptosis, hypospadias, spondylolisthesis, and absence of ectopia lentis suggested a possibly distinct connective tissue disorder separate from Marfan syndrome.

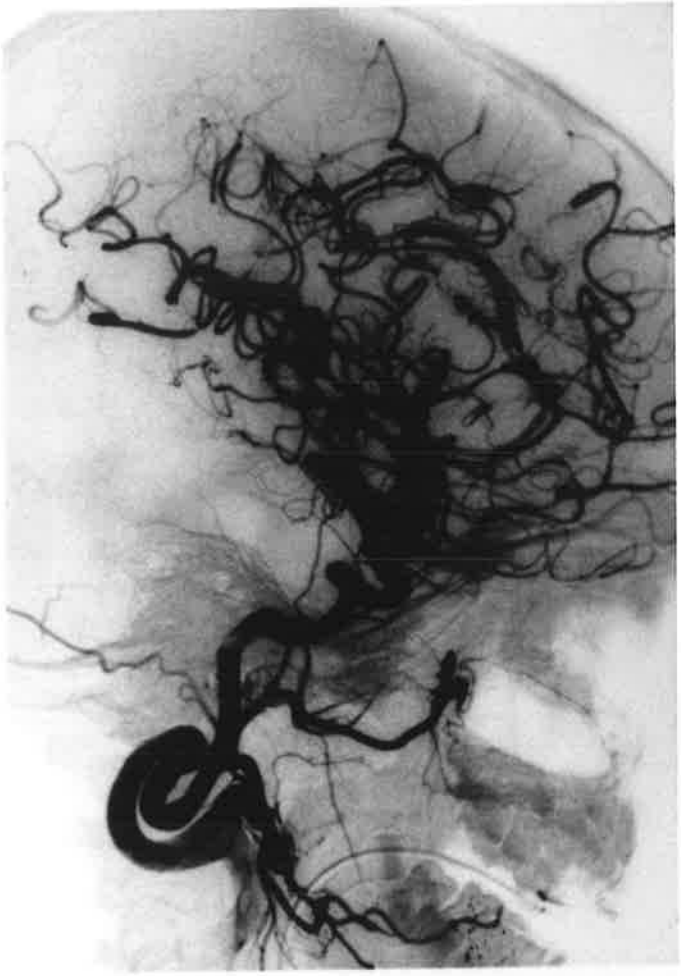
Patient 2

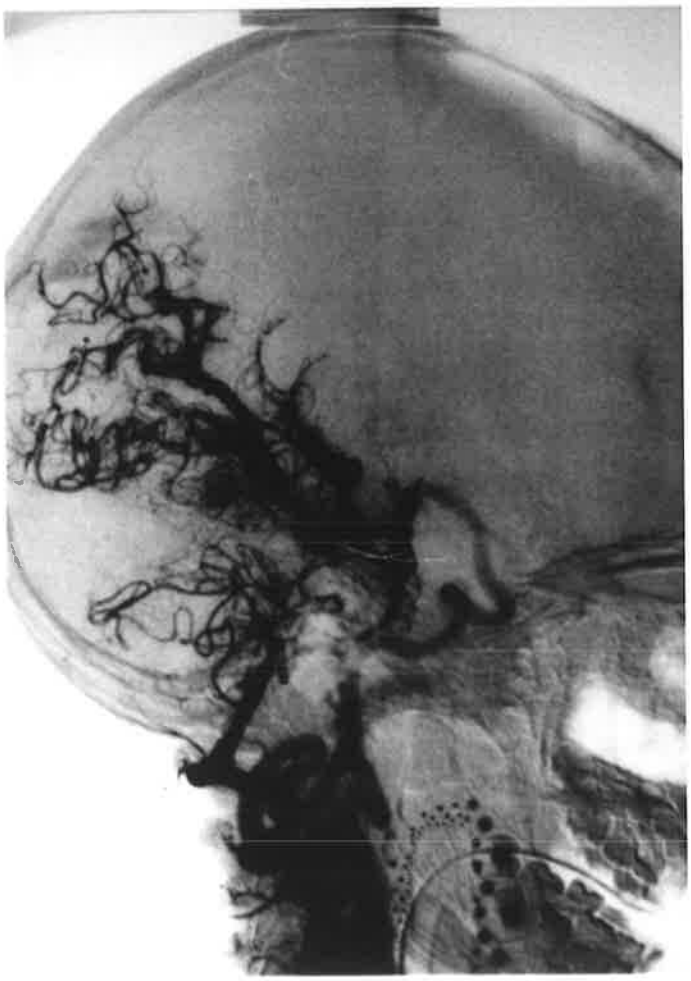
Patient 2 was born at term with a birth weight of 3120 g, birth length 52 cm and head circumference 35 cm. He had bilateral recurrent inguinal and femoral herniae which were corrected surgically. He had generalised hypotonia and at age 22 months, required surgical fusion of the second and third cervical vertebrae for spondylolisthesis. He also

Figure 3.9 Cerebral Angiogram of Abnormal Intra- and Extracranial Vessels in P35

(see overleaf)







had obstructive sleep apnoea, joint instability, delayed wound healing and normal scar formation. His intelligence was normal. Craniofacial features included scaphocephaly, frontal bossing, bilateral congenital ptosis, epicanthic folds, deep set eyes, down-slanting palpebral fissures, maxillary and mandibular hypoplasia, a high arched palate, bifid uvula, dental malocclusion and micrognathia. He had a slender trunk and limbs, normal body proportions, arachnodactyly, hypermobility of small and large joints, pectus carinatum and pes planus. There was reduced muscle bulk, lumbar striae and velvety hyperelastic skin. Echocardiogram confirmed the presence of dilatation of the aortic root and the main pulmonary artery. Slit lamp examination of the eyes showed that the lenses were normal. Refraction was also normal. A CAT scan showed lumbosacral dural ectasia. Clinical photographs of P2 are shown in Figure 3.10.

Patient 46

Patient 46 was referred to the Genetics Department by the Craniofacial Unit for diagnosis at age 12 years. He was born with a cleft palate and unilateral inguinal hernia. Craniofacial features included sagittal craniosynostosis with facial asymmetry, blue sclerae, long palpebral fissures, proptosis, malar hypoplasia, a high narrow palate and vestigial uvula, dental malocclusion, protuberant cup-shaped ears and micrognathia. He had normal body proportions and height, arachnodactyly, pectus carinatum and a thoracic scoliosis, and hypermobility of large and small joints. Radiographs of the spine showed non-fusion of the atlas. Echocardiogram demonstrated aortic root dilatation and dilatation of the right atrium and ventricle. Ophthalmologic examination detected mild astigmatism, a right divergent squint, normal lenses and normal visual acuity. Clinical photographs of P46 are shown in Figure 3.11.

3.3.2 Three Unusual Group 2 Patients

Three further patients from Group 2 are described here to demonstrate the diversity of the clinical phenotypes assessed.

Patient 26

Patient 26 was referred to the Genetics Department from a paediatric rheumatologist for diagnosis at age 18 years. Craniofacial features included dolichocephaly, mild synophrys, deep set eyes, upslanting palpebral fissures, malar hypoplasia, prominent ears with soft helices, and a broad, high palate. He had a Marfanoid body habitus, a pectus

Figure 3.10 Clinical Photographs of P2

(see overleaf)

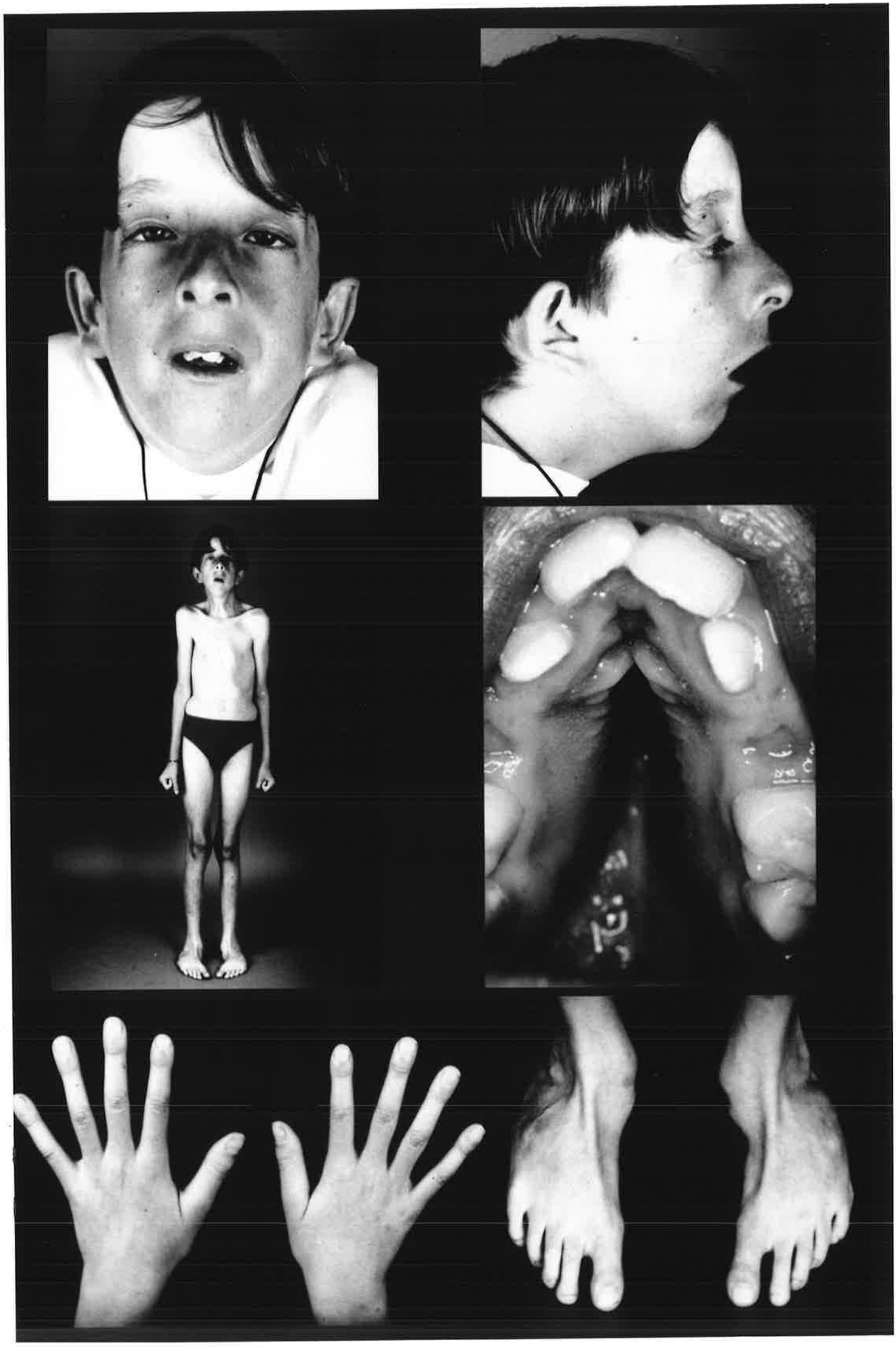
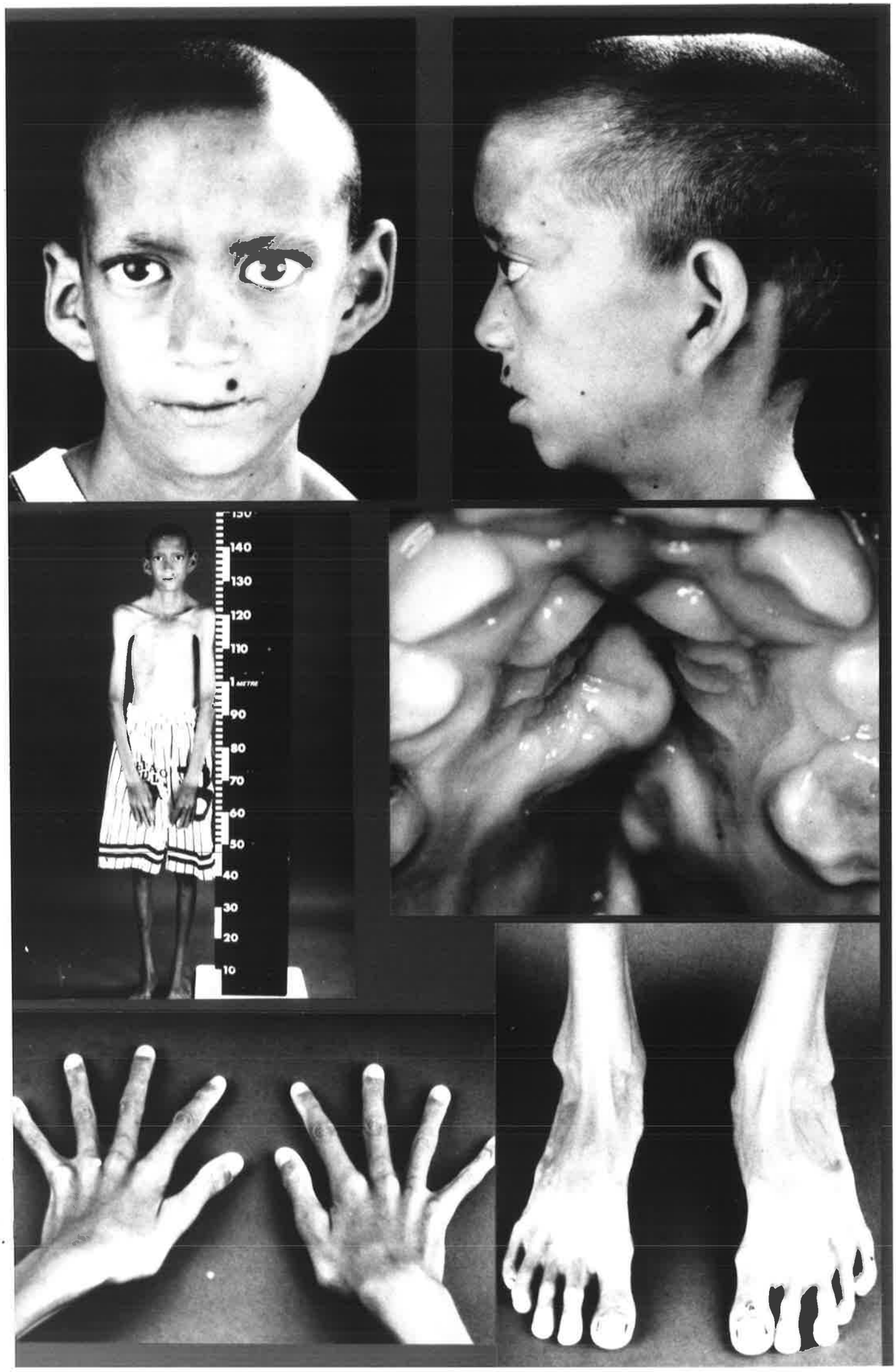


Figure 3.11 Clinical Photographs of P46

(see overleaf)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carinatum and thoracic scoliosis, fusiform swellings of the proximal and distal interphalangeal joints of the fingers, hypermobility of some large and small joints, fixed flexion deformity of both hips and the right knee and unilateral pes planus. Other features included velvety hyperelastic skin, sparse facial, body and axillary hair, bilateral undescended testes and subcoronal hypospadias. The skin around the lateral neck and both axillae had a "goose-flesh" texture reminiscent of pseudoxanthoma elasticum. These changes had developed in late adolescence. He had multiple naevi and café-au-lait spots over the trunk. Mitral valve prolapse was confirmed by echocardiography, and ophthalmologic review detected hypermetropia but no lens dislocation. He had mild mental retardation. Clinical photographs of P26 are shown in Figure 3.12.

Patient 37

Patient 37 had been well until the age of 45 years when he presented to the hospital's accident and emergency services with cardiac rhythm disturbances of supraventricular tachycardia and atrial fibrillation. Clinical features included a Marfanoid body habitus, marked hypermobility of the hip joints, and gross varicosities of the lower limb veins. Echocardiogram demonstrated aortic root dilatation, mild thickening of the aortic valve, and possible coronary artery dilatation. There was no myopia or lens dislocation. Clinical photographs of P37 are shown in Figure 3.13.

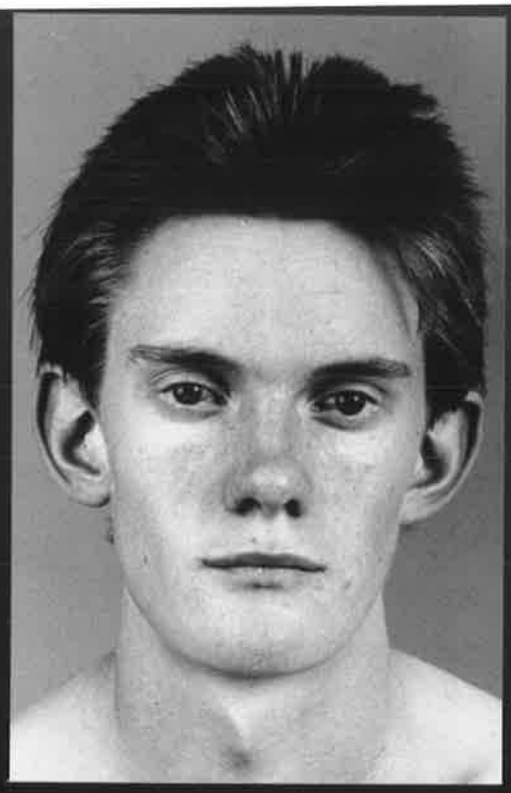
Patient 45

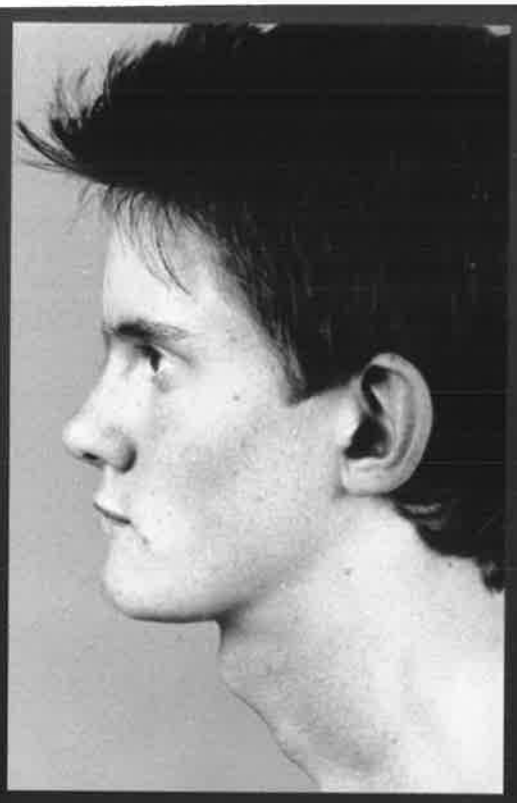
The proband was delivered at term following a normal pregnancy. There had been no exposure to a known teratogen. His parents were unrelated and there was no family history of hand or foot abnormalities, aortic or mitral valve abnormalities, nor of any structural ocular defects. At 6 months of age he underwent repair of bilateral inguinal herniae, at which time one testis was noted to be absent. A patent ductus arteriosus was surgically ligated at 3 months of age, and at that time, bilateral iris hypoplasia was detected. Psychomotor development was normal. He had mild asthma during childhood.

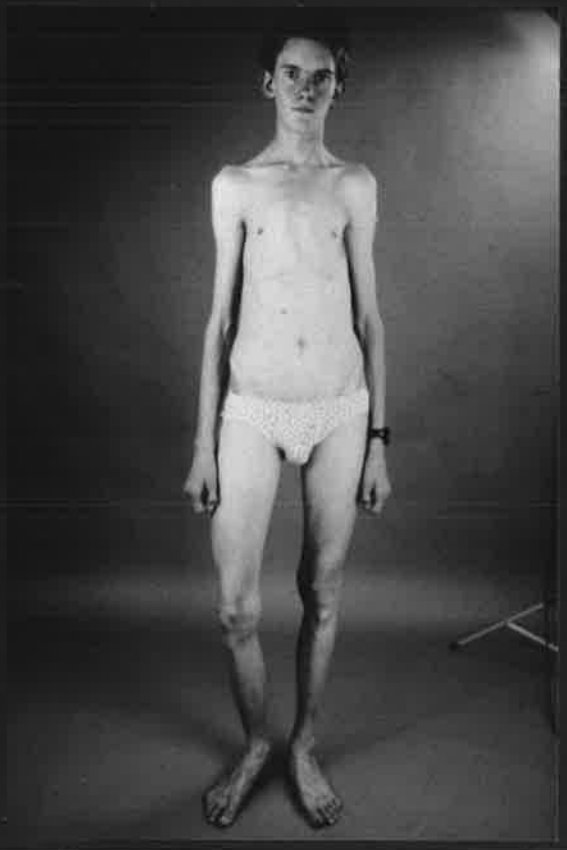
At 14 years of age he experienced acute pain in the lower abdomen and back during trunk bending exercises at school. this was followed immediately by weakness in both legs and numbness below the level of the umbilicus, associated with evolving oedema of the left leg. Sensation in the lower limbs returned within hours and he was able to walk five days later, but residual weakness and numbness remained in the distribution of the

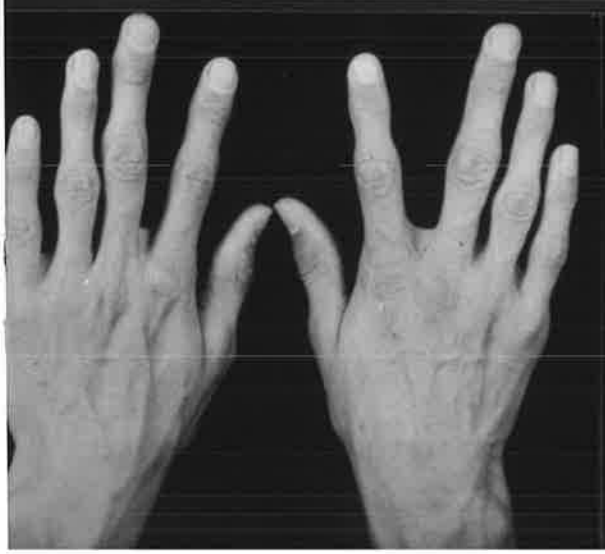
Figure 3.12 Clinical Photographs of P26

(see overleaf)









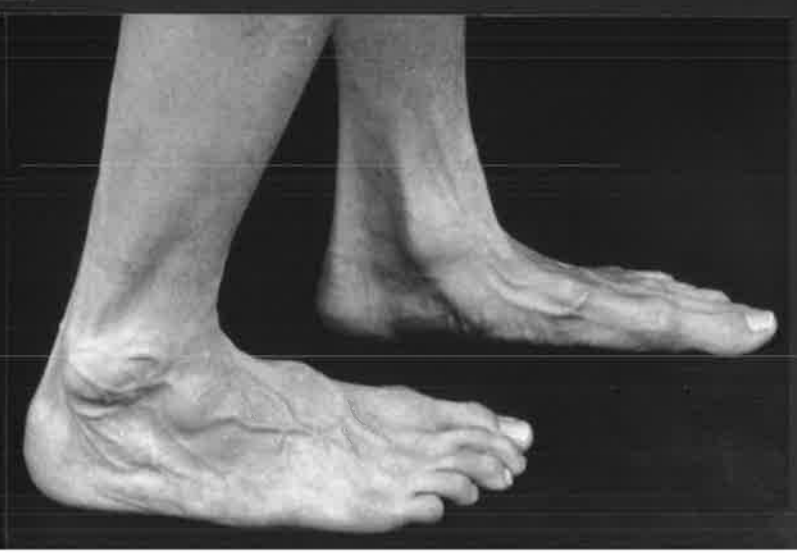
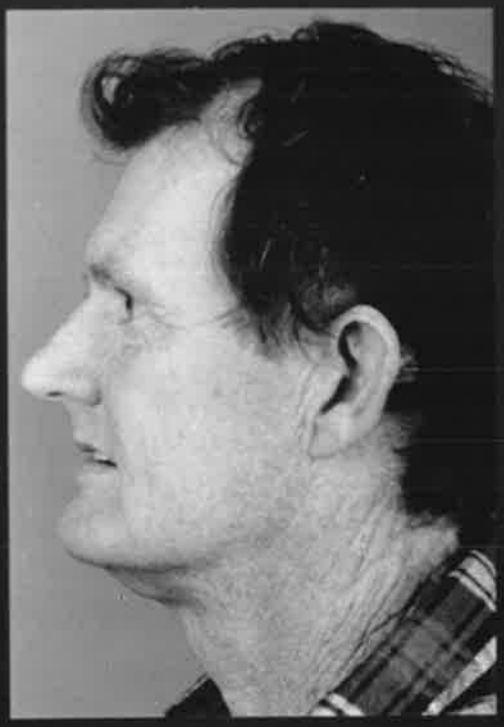
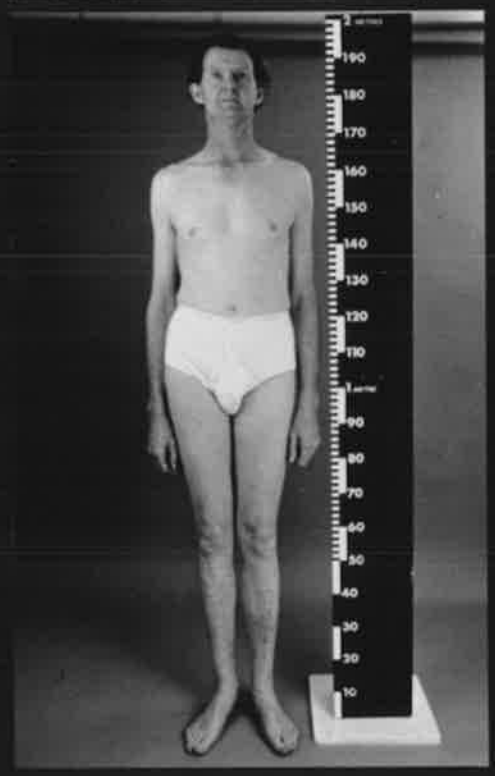


Figure 3.13 Clinical Photographs of P37

(see overleaf)

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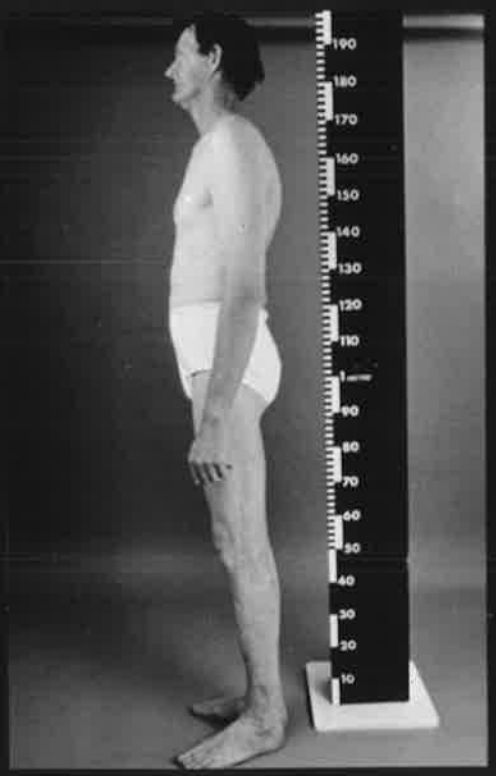
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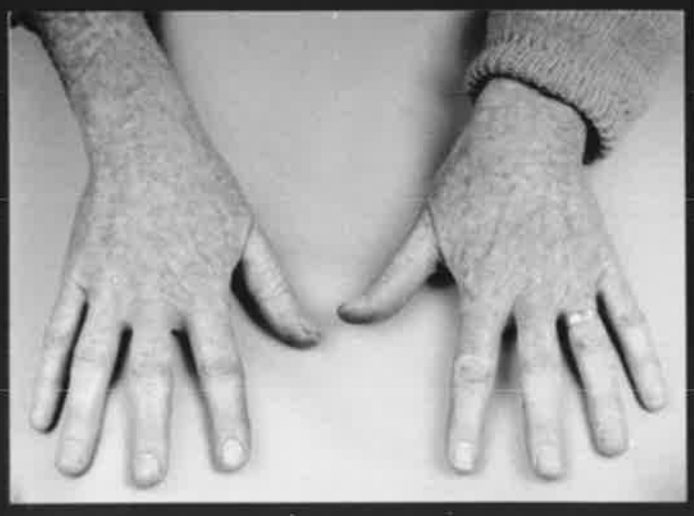
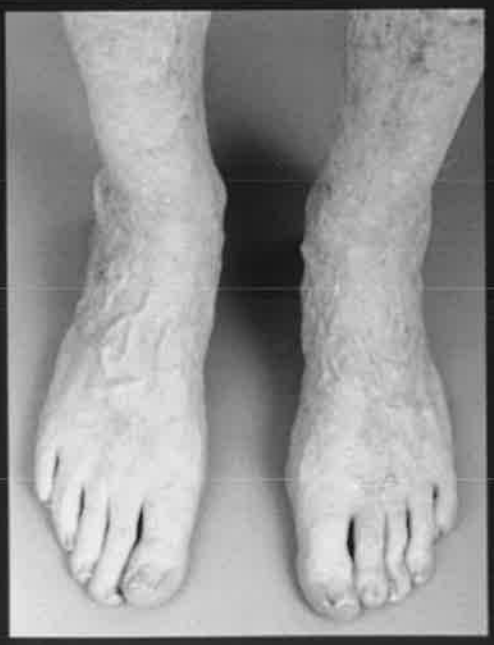




left common peroneal nerve.

When first examined by two geneticists (Dr L Adès, Dr E Haan) two weeks after this event he was noted to have an aesthenic build with a height of 176.7 cm (90th centile); the arm span: height (0.98) and upper segment: lower segment (0.94) ratios were normal. The presence of bilateral iris hypoplasia was confirmed but there was no other ocular abnormality. There was prominent hypoplasia of the terminal phalanges of both thumbs with mild shortening of the distal phalanges of the fingers. The abdominal aorta was readily palpable and lower limb pulses were slightly reduced, while the blood pressure was normal. The palate was normal, there was no joint laxity, chest deformity or scoliosis. The skin had normal thickness, texture and extensibility and was not translucent, but striae were present over the upper lateral thighs.

Radiographs of the hands showed short distal phalanges, the shortening being greater in the thumb (-6SD) than in the fingers (-1SD). In the feet the distal phalanges were also short with the hypoplasia being most marked in the hallux.

Initial investigation of abdominal pain by ultrasound revealed an abdominal "aneurysm". Thoracic and abdominal aortography demonstrated an extensive dissection of the descending thoracic and abdominal aorta, commencing 3 cm below the left subclavian origin and extending to involve the right renal artery and right common iliac origin. The true lumen showed moderate ectasia of the entire thoracic aorta, including the arch, extending to just below the left renal artery. Computed tomography confirmed the extent of dissection with the false lumen situated anteriorly in the thorax, and some clot within the adventitial layer of the aorta. Echocardiography also demonstrated mild aortic regurgitation. The mitral valve was normal.

Other investigations including urine copper, amino and organic acids, blood picture, ESR, plasma copper and caeruloplasmin, chromosomes and antinuclear antibodies gave normal results. Light microscopy of a skin biopsy showed normal collagen fibres; no vascular tissue was biopsied.

He was commenced on β-blocker medication and remained in a stable condition under observation in hospital for one month, prior to returning to his home in the country. On

re-admission 3 months later, further fusiform dilatation of the descending thoracic and abdominal aorta had occurred. He died suddenly at home, at age 17 years and 4 months. Clinical photographs of P45 are shown in Figure 3.14.

3.3.3 Reclassification from Group 1 to Group 2

P2 was originally classified into Group 1, but a subsequent diagnosis of Furlong syndrome enabled reclassification into Group 2.

3.4 Response to Questionnaire

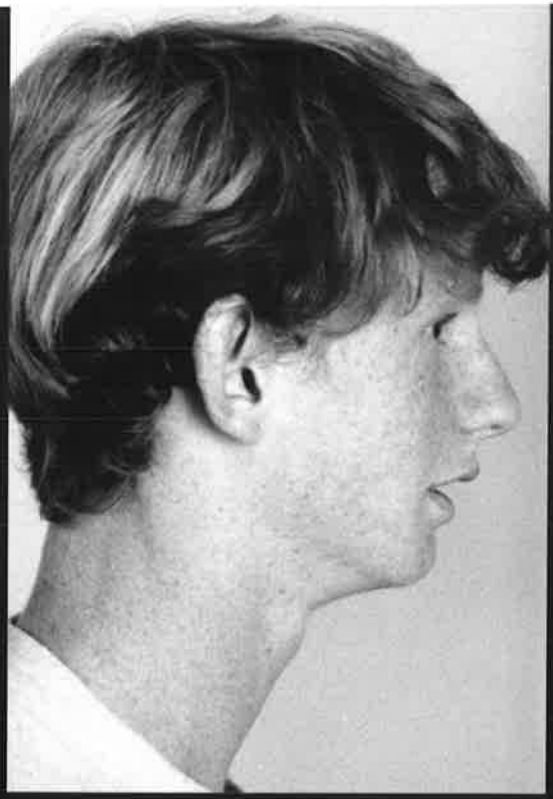
All the research patients were sent a questionnaire, as outlined in Appendix 2D. There were 19 responses (19/48 or 40%) in total. Of these, 18/19 (95%) were in favour of a Marfan syndrome support group. The one negative reply was from the mother of P9, (a Group 2 patient), who did not feel that a Marfan syndrome support group had any relevance to her child. Nine of the 48 patients (19%) had undergone aortic surgery; of these, 7/9 had an unequivocal diagnosis of Marfan syndrome. Of those patients responding to the questionnaire, 6/19 had undergone aortic surgery, 5 prior to the commencement of the research, and 1 during the course of the research. In other words, there was a high response rate (6 out of 7) Marfan syndrome patients who had had aortic surgery.

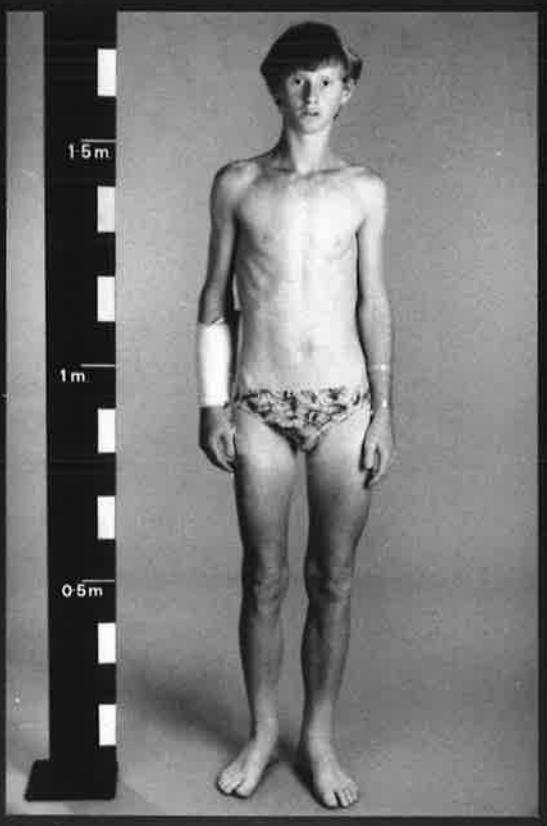
Eleven individuals of 18 indicated their desire for a fitness program tailored to meet their specific needs, 7/18 wanted more moral support regarding their condition, 6/18 requested further counselling, 5/18 were interested in post-operative information, 3/18 requested pre-operative information and 2/18 requested a post-operative rehabilitation program. Sixteen individuals of 18 were agreeable to being contacted by telephone by another individual with Marfan syndrome. The two patients who were not agreeable to this did not state their reasons.

Several patients had additional concerns or comments. Three patients commented that they did not believe the level of awareness of Marfan syndrome in the medical community, particularly of general practitioners, was adequate. One patient asked for information regarding the symptomatology associated with aortic aneurysms and was also interested in preventative aspects regarding the development of aortic aneurysms.

Figure 3.14 Clinical Photographs of P45

(see overleaf)

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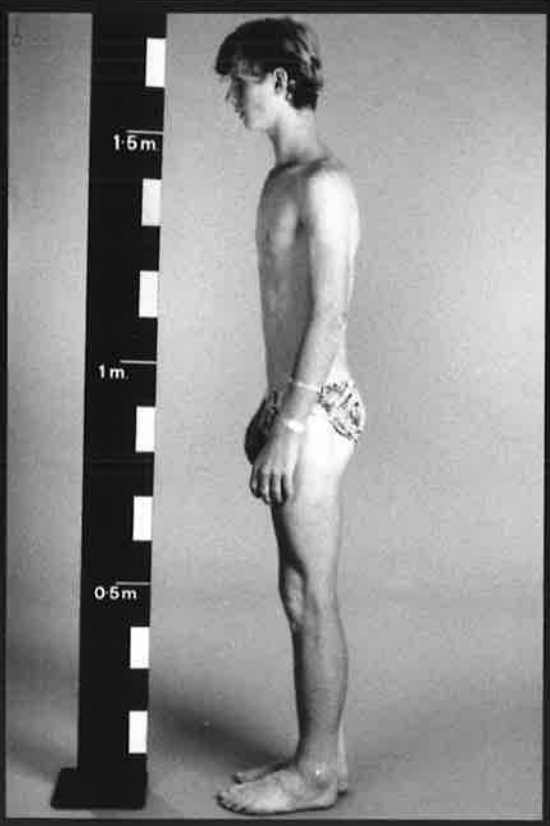
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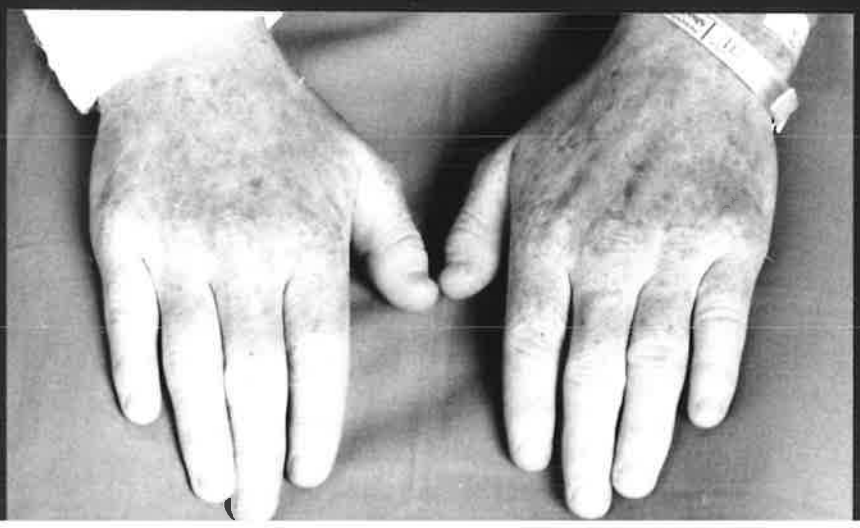


Another patient voiced a need for further education about pregnancy and Marfan syndrome.

Following the responses outlined above, a list of patients and telephone numbers were circulated to all those patients who had indicated that they were agreeable to this exchange. No formal Marfan syndrome support group has been established in South Australia as yet.

3.5 Discussion

Patients in Group 1 and Group 2 comprised two vastly heterogeneous patient populations. Many of the Group 2 patients were unique. A review of the Group 1 patients has led to some interesting observations, some of which differ from the standard text-book descriptions of classic Marfan syndrome (Harrison, 1994; Nelson, 1992). These patients are discussed below. Apart from those Group 2 patients already described, the remainder are not discussed here because of the diversity of their clinical phenotypes.

Many of the Group 1 patients shared similar facial features including deep-set eyes, malar hypoplasia, dental malocclusion/overcrowding, a high or high and narrow palate and ear abnormalities. While most of these facial features are well documented in the literature (Gorlin et al., 1990; Taybi and Lachman, 1990) they have not been used as part of the diagnostic criteria. Perhaps the inclusion of these clinical features for such a purpose warrants consideration.

Another interesting observation is that the so-called Marfanoid body habitus may not be evident in patients with Marfan syndrome.There were clearly patients within this group without tall stature, dolichostenomelia or a decreased upper:lower segment ratio. In addition, 3/26 patients were obese. While obviously helpful in making the diagnosis in some Marfan patients, an emphasis on the expected "typical" body habitus may be quite misleading. The absence of these expected skeletal findings, or the presence of normal stature and obesity may preclude the physician from considering a diagnosis of Marfan syndrome. This is important, especially in view of the fact that even today, Marfan syndrome remains undiagnosed in some patients. This is illustrated by the following case of P34.

Patient 34, a soldier, was referred to the research project by his cardiac surgeon, after undergoing emergency aortic graft and aortic valve replacement for acute aortic dissection. He was 26 years old at the time of his dissection and had been previously fit and well, apart from symptoms of constant paraesthesia in the fingers over the previous year. He complained of severe back pain after weight-lifting, but this was attributed to muscular strain by the attending army physician. Three weeks later, the pain had not subsided and review by a second army physician confirmed the presence of a cardiac murmur. Echocardiography demonstrated severe aortic incompetence, an aneurysm of the ascending aorta, and aortic dissection. The nature of P34's presentation is sobering. Apart from the failure of the physician to consider a diagnosis of Marfan syndrome in this tall male with all the skeletal features of Marfan syndrome and a past history of bilateral inguinal herniae, the physician omitted to enquire about a family history. In P34's case, his own father had died at age 29 years from presumed rupture of an aortic aneurysm. It has been well documented in the past that some undiagnosed Marfan syndrome athletes have died during sport because of aortic rupture (anonymous, 1993). Clearly, there are other groups potentially at high risk, such as those entering the police force or the armed forces. It is encumbent upon the physicians responsible for these individuals in particular to consider a diagnosis of Marfan syndrome where appropriate.

Patient 25 was one of two patients who underwent aortic graft and valve surgery during the research period. She was known to have dural ectasia (demonstrated previously on CAT scan). She had had unilateral sciatic nerve pain from childhood and intermittent paraesthesia in the legs which was never investigated. One year after her major aortic surgery, she presented with acute abdominal pain which was presumed ovarian in origin. The patient had alerted the attending gynaecologist to the presence of dural ectasia. At operation, no ovarian pathology could be demonstrated, but a clear, large, fluid-filled cystic structure was drained. Post-operatively, it was realised that the redundant ectatic dural sac had caused the abdominal symptoms, and that the dural sac had been inadvertently drained. The failure to recognise P25's symptoms as being due to dural ectasia placed P25's life at unnecessary risk. This example shows that even with the accurate documentation of dural ectasia in the setting of a major teaching hospital, the significance of dural ectasia in this patient was not appreciated. There is clearly scope for improving the knowledge base of many physicians with regard to the diagnosis and potential complications of Marfan syndrome.

The late re-assignment of P33 from Group 2 to Group 1 on the basis of aortic root dilatation occurring in her 50s is a salient reminder that aortic root dilatation may not occur until relatively late. Thus, any patient suspected to have Marfan syndrome should have regular echocardiographic surveillance.

The ascertainment of Marfan syndrome in an Aboriginal kindred highlights the fact that Marfan syndrome does occur in this population. The added factor of geographic isolation places this group at more risk of underdiagnosis than those individuals living in urban areas.

Some of the unusual features and unexpected findings in the Group 1 patients are listed in Table 3.4. Paraesthesia occurred in four patients and all these patients subsequently required aortic surgery. This finding suggests that perhaps more attention should be placed on the presence of this symptom. A larger study would be necessary to determine whether or not paraesthesia might be a predictor of those Marfan patients likely to need aortic surgery. Iris dilator hypoplasia was found in 3/26 patients. Two of these patients (P44 and P47) had a severe Marfan phenotype and congenital miosis. Specific iris abnormalities have been described in Marfan syndrome. These include iris transillumination, and a "velvety" appearance of the anterior iris leaf. There may also be aplasia or hypoplasia of the iris dilator muscle resulting in a congenital miosis resistant to the effects of pharmacologic dilators (Maumenee, 1981). A mother and daugther with classic Marfan syndrome have been reported, in whom the most striking manifestation on initial presentation was congenital miosis unresponsive to dilating agents (Summers et al., 1989). The presence of this unusual eye anomaly is important, since it may alert the physician to the possibility of Marfan syndrome. Recognition of congenital miosis in P44, and its association with Marfan syndrome may have led to an earlier correct diagnosis in this patient. Pyloric stenosis was present in 2/13 male Marfan patients. The frequency of this disorder in males in the general population is 1/200 (Sieber, 1990). Although the small patient numbers preclude any definitive conclusions, this finding is of interest, and raises the possibility that fibrillin may play a role in the formation and function of the pyloric muscle sphincter. Uterine prolapse occurred at a young age in one patient, P32. This has not been described as a complication of Marfan syndrome in standard texts (Harrison, 1994; Nelson, 1992). It is conceivable that the ligaments supporting the uterus may be abnormal in Marfan syndrome. This same patient had

several other interesting features (see Table 3.4) and was also shown to have a prolactinoma, a tumour of the pineal gland. The occurrence of prolactinoma in Marfan syndrome has not been previously described.

Twelve of 26 Group 1 patients had macrocephaly. In the majority (10/12), there was relative macrocephaly, and in the remaining 2/12 the macrocephaly was absolute. Relative macrocephaly is perhaps not unexpected, given that all the patients with this finding were tall. However, the finding of absolute macrocephaly is somewhat unexpected and this observation raises several questions. Is the macrocephaly due to an enlarged skull, and perhaps related to the process of cranial skull bone formation and/or cranial sutural closure? Alternatively, is the skull large because the underlying brain is large (megalencephaly). This is even more intriguing in view of the finding of communicating hydrocephalus in 2/12 patients, both of whom had relative macrocephaly (P23 and P44). Although based on a small number of patients, these observations suggest a possible role for fibrillin in cranial bone and/or cranial sutural development, and possibly also in the developing brain.

Although no FBN1 gene mutations were detected in the Group 2 patients, it was still worthwhile including these patients in the study, since a diagnosis was made in 3 of the 22 patients. In addition, the ascertainment of two children with rare congenital aneurysms of the great vessels has led to an international collaborative effort describing the clinicopathologic features of four children with these vessel abnormalities (see Appendix 3B).

The comparison of Marfan syndrome patients with the Marfanoid-craniosynostosis syndrome patients also proved fruitful. Patients with Shprintzen-Goldberg syndrome (four in Group 2) and Furlong syndrome (two in Group 2) fall into this second category. Several of the more common craniosynostoses syndromes (Crouzon, Jackson-Weiss, Pfeiffer and Apert syndromes) have recently been shown to be due to dominant mutations in one of the several fibroblast growth factor receptor (FGFR) genes, FGFR2 (Gorry et al., 1995). Findings from the research project suggested that the presence of craniosynostosis and radiological skeletal abnormalities in the Marfanoid-craniosynostosis patients might be features that could help to distinguish these patients from those with Marfan syndrome. This led to my hypothesis that Shprintzen-Goldberg syndrome might

Table 3.4 Unusual Features and Unexpected Findings in Group 1 Patients

Feature	Number of patients (/26)	Percentage (%)	Patient identification
macrocephaly	12	46	P4, P7, P10, P12, P14, P18, P22, P23, P25, P29, P31, P44
FFD DIP/PIP joints*	5	19	P4, P7, P8, P20, P33
limb paraesthesia	4	15	P25, P31, P32, P34
large joint contractures	3	12	P4, P7, P33
obesity (wt > 97th centile)	3	12	P11, P30, P33
iris dilator hypoplasia/aplasia	3	12	P8, P44, P47
communicating hydrocephalus	2	8	P23, P44
pyloric stenosis	2	8	P12, P31
recurrent knee dislocations	2	8	P4, P43
abnormal tooth wear	1	4	P44
congenital corneal leukoma	1	4	P29
epi/metaphyseal dysplasia	1	4	P44
keratoconus and coloboma	1	4	P8
massive dural ectasia	1	4	P25
partial absence of secondary dentition	1	4	P8
partial IgA deficiency	1	4	P44
peripheral retinal degeneration	1	4	P20
prolactinoma	1	4	P32
TMJ locking	1	4	P22
uterine prolapse	1	4	P32

**FFD fixed flexion deformity; DIP distal interphalangeal joint; PIP proximal interphalangeal joint; **TMJ temporomandibular joint*

be due to an FGFR2 gene mutation, rather than a FBN1 gene mutation. This concept led to a collaborative study with colleagues in the United Kingdom, and the identification of an abnormally migrating band on SSCP analysis at exon 1 of FGFR2 in the DNA from one Shprintzen-Goldberg syndrome patient, P27 (see Section 4.7). There has been a recent report of two separate FBN1 gene sequence alterations at exons 28 and 29 in two unrelated patients with the Shprintzen-Goldberg syndrome (Dietz et al., 1995b). At present, the significance of these findings is unclear, and both require further investigation. Depending on the outcome of molecular studies, these, together with the clinical and radiographic "handles" may help to differentiate those patients with a FBN1 gene mutation from those with a FGFR gene mutation, and facilitate correlation between the clinical phenotype and molecular defect in these patients. An extension of this hypothesis would include the analysis of FGFR genes in patients with Furlong syndrome.

CHAPTER FOUR

LABORATORY RESULTS

4.1 Laboratory Results

Using a variety of methods (see Section 2.7), several SSCP band shifts were identified in the Group 1 and Group 2 patients. Most but not all of these band shifts were reproducible. These results are given below in Sections 4.1.1, 4.1.2 and 4.1.3. A total of thirty exons (44% of total FBN1 coding sequence) were screened in all patients. All the FBN1 gene mutations identified were in Group 1 patients. In addition to screening the forty-eight research subjects, similar SSCP screening of genomic DNA was also undertaken in a large kindred with autosomal dominant ectopia lentis. The clinical details of this family have been previously described (Edwards et al., 1994). No FBN1 mutation was identified in this kindred.

4.1.1 Identification of SSCP Band Shifts in the FBN1 Gene in Group 1 Patients

Five SSCP band shifts were identified by genomic DNA screening of thirty FBN1 exons in six Group 1 patients, P6 and P7 (parent and child), P20, P31, P44, and P47. All band shifts were reproducible on at least two separate gels run under identical conditions, and using two separately prepared PCR reactions. In each case where a reproducible band shift was detected, population screening of seventy DNA samples (140 chromosomes) from unaffected individuals was performed. Population screening of the relevant exons failed to reveal any band shifts that were detected in the patient DNA samples. The corresponding clinical details of these patients are described in Section 3.2.2.

Patients 6 and 7 (Parent and child)

The SSCP band shifts in genomic DNA from P6 and P7 were detected in exon 17 of the FBN1 gene (Figure 4.1) under both the 4.5% and 10% gel conditions.

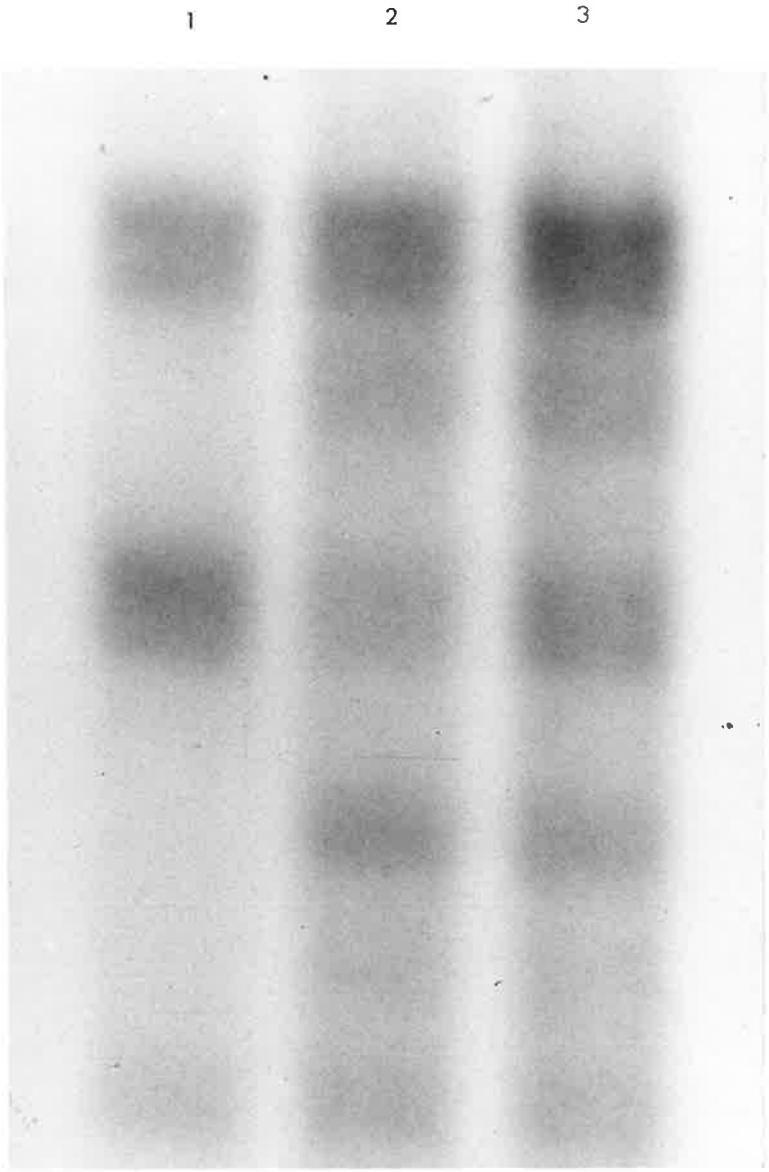
Patient 20

The SSCP band shift in genomic DNA from P20 was detected in exon 16 of the FBN1 gene (Figure 4.2) under the 10% gel condition only.

Patient 31

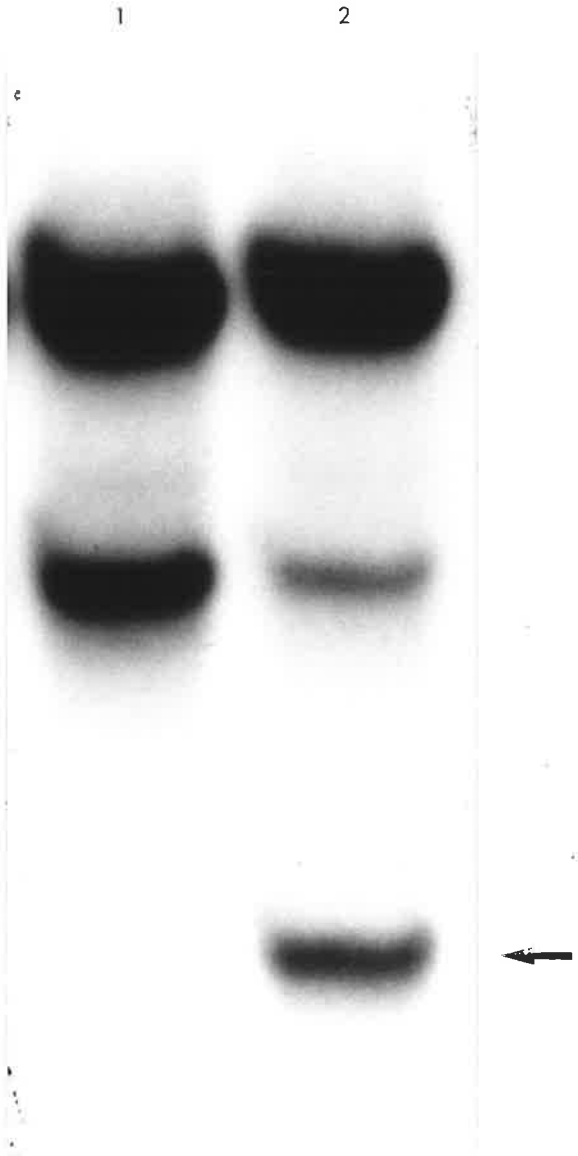
SSCP analysis of exon 63 using genomic DNA from P31 revealed an aberrantly migrating fragment on a non-denaturing 25% MDE gel.

Figure 4.1 10% SSCP of FBN1 Exon 17



Photograph of autoradiograph. Lane 1 represents bands obtained from amplified PCR product of genomic DNA from an unaffected individual; lanes 2 and 3 represent bands obtained from amplified PCR product of genomic DNA from P6 and P7, respectively. Aberrantly migrating bands are indicated by the arrows.

Figure 4.2 10% SSCP of FBN1 Exon 16



Photograph of autoradiograph. Lane 1 represents bands obtained from amplified PCR product of genomic DNA from an unaffected individual; lane 2 represents bands obtained from amplified PCR product of genomic DNA from P20. Aberrantly migrating band is indicated by the arrow.

Patient 44

The SSCP band shift in genomic DNA from P44 was detected in exon 27 of the FBN1 gene (Figure 4.3) under the 10% gel condition only. Since P44 was adopted, parental DNA samples were not available for screening.

Patient 47

The SSCP band shift in genomic DNA from P47 was detected in exon 25 of the FBN1 gene (Figure 4.4) under both the 4.5% and 10% gel conditions.

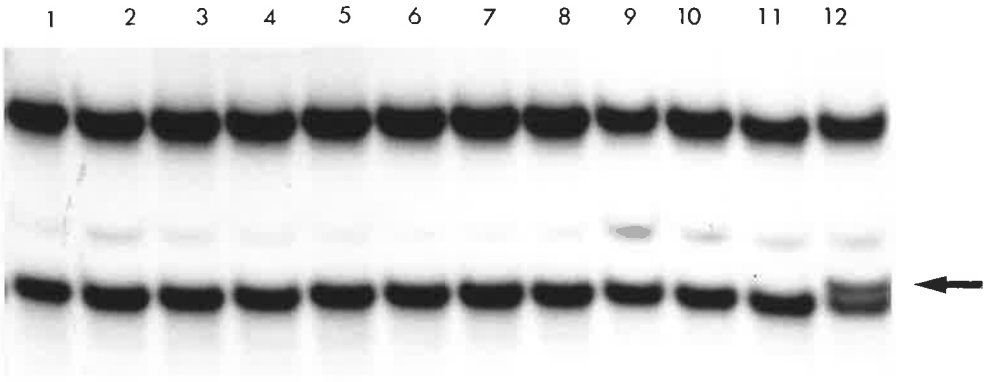
4.1.2 False Positive Results Obtained by RT-PCR and SSCP of the FBN1 Gene

Non-reproducible band shifts were detected in two Group 1 patients, P13 and P24. Both of these false positive results occurred using the RT-PCR approach. There were no false positive results obtained using the genomic DNA approach. The reason for the failure to reproduce the original SSCP band shift is unknown. Direct DNA sequencing was undertaken in one of these patients (P13) in an attempt to clarify the SSCP result. Direct sequencing was not undertaken in P24.

Patient 13

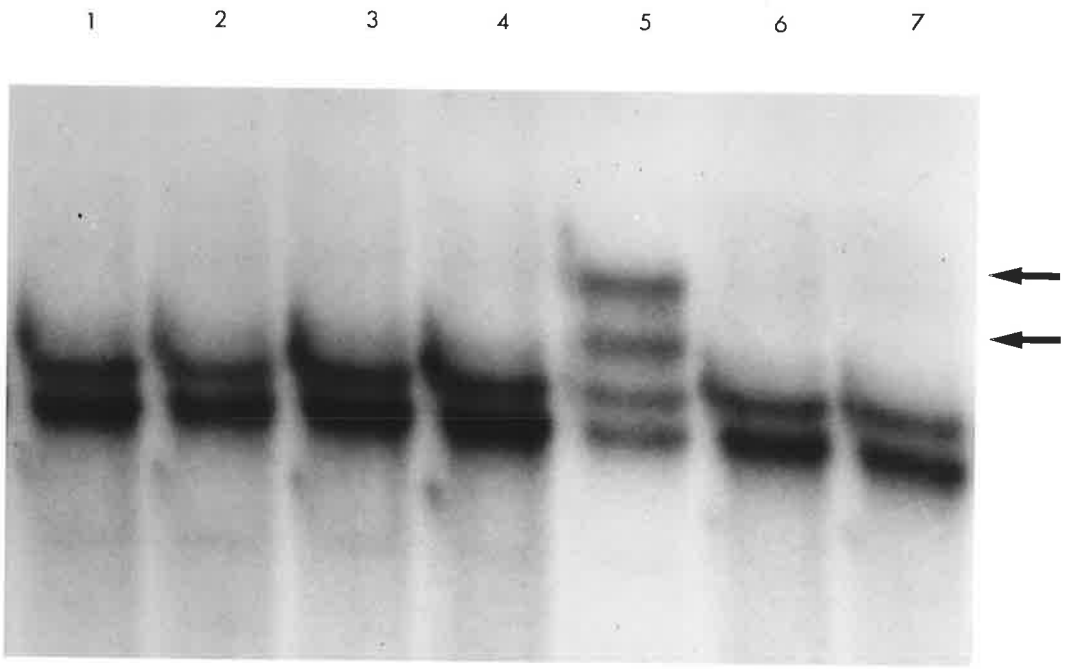
An SSCP band shift was detected in Fragment C (see Table 2.2) under the SSCP conditions outlined for MDE gel and silver staining (see Sections 2.7.1-2.7.3). The subsequent sequencing work was conducted by Dianna Milewicz (Houston). Exon 31 was amplified from genomic DNA, and sequenced in the forward and reverse directions. A heterozygous A to G point mutation at nucleotide 3949 was initially found. This was present in both directions on sequencing. The apparent putative mutation altered K1317E, thus changing the wild type lysine (basic) amino acid to a glutamine (acidic) amino acid. This change occurred within an EGF-like domain. However, because the sequence was immediately adjacent to a long poly-A stretch, a decision was made to clone the fragment and then sequence it. Four clones were sequenced and all were normal. Subsequent to these findings, genomic screening of exon 31 was undertaken, with DNA from P13 and her affected sister P14. This failed to detect an SSCP band shift under either the 4.5% or 10% gel conditions.

Figure 4.3 10% SSCP of FBN1 Exon 27



Photograph of autoradiograph. Lanes 1-10 represent bands obtained from amplified PCR product of genomic DNA from unaffected individuals (5 samples loaded per lane; total of 50 DNA samples or 100 chromosomes represented); lane 11 represents bands obtained from amplified PCR product of genomic DNA from a single unaffected individual; and lane 12 represents bands obtained from amplified PCR product of genomic DNA from P44. The aberrantly migrating band is indicated by the arrow.

Figure 4.4 10% SSCP of FBN1 Exon 25



Photograph of autoradiograph. Lanes 1-4 represent bands obtained from amplified PCR product of genomic DNA from unaffected individuals; lane 5 represents bands obtained from amplified PCR product of genomic DNA from P47; and lanes 6 and 7 represent bands obtained from amplified PCR product of genomic DNA from P47's mother and father, respectively. Aberrantly migrating bands are indicated by the arrows.

Patient 24

An SSCP band shift was detected in the PCR fragment amplified by the primers UF497 and UF390 (see Table 2.2) and corresponding to exons 28-32 of the FBN1 gene (Figure 4.5). This band shift was found under the 4.5% gel condition only, was not reproducible, and was not investigated further.

4.1.3 Identification of FBN1 Gene Silent Polymorphisms by SSCP and Genomic Screening

Several SSCP band shifts were detected in Group 1 and Group 2 patients and these are listed below.

Silent Polymorphism in Exon 15 of the FBN1 Gene

Band shifts were identified in DNA from the genomic screening of exon 15 in P3, P6, P29, P30, P32, P42, P43, P46 and P48. The band shifts were found in these nine patients under both the 4.5% and 10% gel conditions. After similar screening of seventy DNA samples (140 chromosomes) from unaffected individuals, identical band shifts were detected in at least nine DNA samples (9/140 chromosomes, or 6.4%) (Figure 4.6(a)).

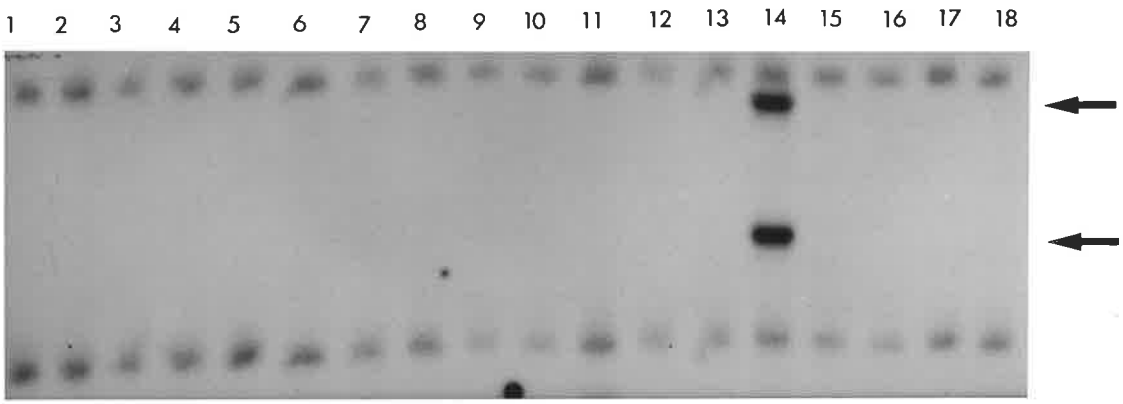
Silent Polymorphism in Exon 28 of the FBN1 Gene

Band shifts were identified from the genomic screening of exon 28 in the same nine patients for whom an exon 15 silent polymorphism was identified, again under both the 4.5% and 10% gel conditions (Figure 4.6(b)). Similar screening of seventy DNA samples (140 chromosomes) from unaffected individuals yielded identical band shifts in at least eight DNA samples (8/140 chromosomes, or 5.7%). Screening was undertaken using 4.5%, 10% SSCP PAGE/glycerol and 37.5% MDE gels in a subset of patients with Shprintzen-Goldberg syndrome, but no band shift was identified in these patients (see Section 4.8.5 for discussion).

4.2 Mutation Detection in Group 1 Patients

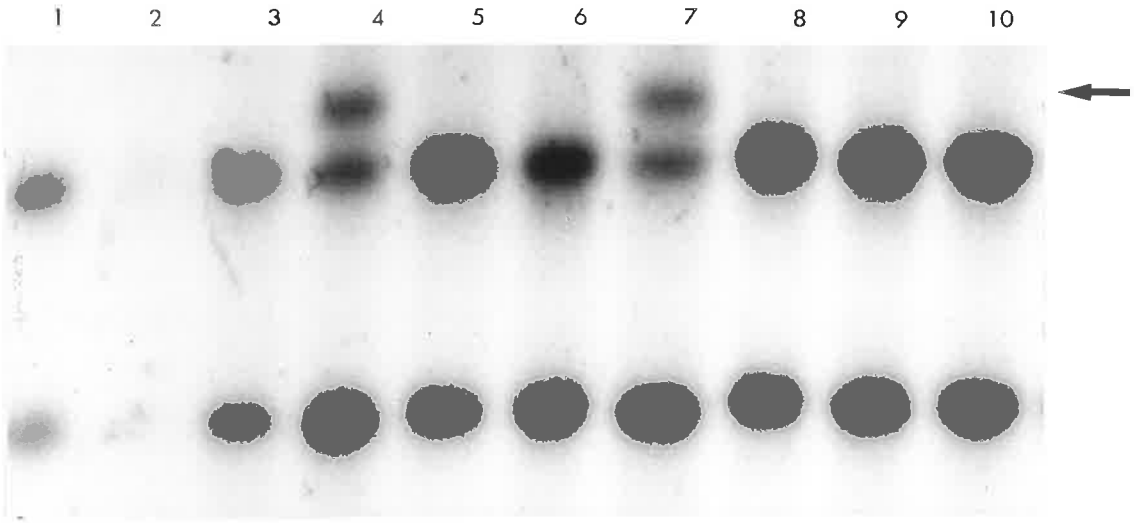
In this research project, a total of thirty-five families were studied by SSCP analysis. The twenty-six Marfan patients represented nineteen families, and the twenty-two Group 2 patients represented sixteen families. Five different mutations were defined in six of the Marfan patients. This represents five separate novel mutations in nineteen families,

Figure 4.5 4.5% SSCP of FBN1 Fragment Corresponding to Exons 28-32



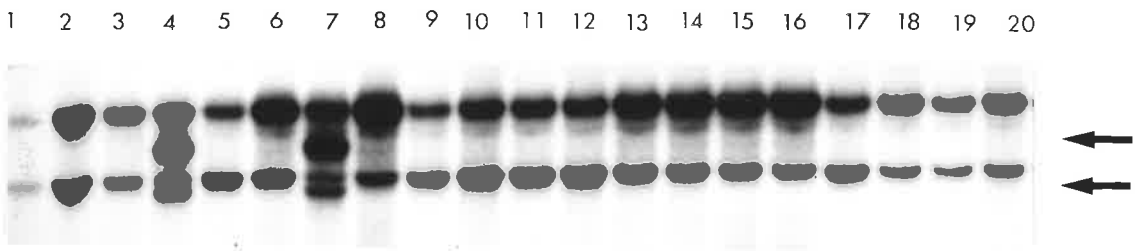
Photograph of autoradiograph. Lanes 1-13 represent bands obtained from amplified PCR product of cDNA (reverse-transcribed from mRNA extracted from dermal fibroblasts) from patients 1, 2, 3, 4, 5, 7, 8, 9, 12, 13, 20, 22 and 23, respectively; lane 14 represents bands obtained from amplified PCR product of cDNA from P24; and lanes 15-18 represents bands obtained from amplified PCR product of cDNA from patients 29, 27, 26, and 25, respectively. (Samples were prepared according to methods outlined in Sections 2.7.4 and 2.7.6; patient identification corresponds to those given in Table 3.1, Section 3.2). Aberrantly migrating bands are indicated by the arrows. These results were found only on 4.5% SSCP and were not reproducible.

Figure 4.6(a) 10% SSCP for FBN1 Exon 15 Silent Polymorphism



Photograph of autoradiograph. Lane 1 represents bands obtained from amplified PCR product of genomic DNA from an unaffected individual; lanes 2-10 represent bands obtained from amplified PCR product of genomic DNA from patients 1-9 (see Table 3.1, Section 3.2), respectively. Aberrantly migrating band is indicated by the arrow for patients 3 and 6.

Figure 4.6(b) 10% SSCP for FBN1 Exon 28 Silent Polymorphism



Photograph of autoradiograph. Lane 1 represents bands obtained from amplified PCR product of genomic DNA from an unaffected individual; lanes 2-20 represent bands obtained from amplified PCR product of genomic DNA from patients 1-19, respectively (see Table 3.1, Section 3.2). Aberrantly migrating bands are indicated by the arrows.

or 26.3%. Two patients (P6 and P7) from one family had an identical novel FBN1 mutation, and four patients (P20, P31, P44 and P47) had different novel FBN1 mutations. The SSCP band shift in P31 was detected by the MDE gel and silver staining methods. The SSCP band shifts identified in the remaining patients were all detected using the higher (10%) percentage PAGE/glycerol gel. Had only the 4.5% gel condition been utilised, band shifts would not have been demonstrated in two (P20 and P44) of these.

4.3 Sequencing of Putative Mutations in the FBN1 Gene in Group 1 Patients

The results of direct DNA sequencing of mutations in P6 and P7, P20, P31, P44 and P47 are described below individually, and summarised in Table 4.1. Chromatograms for mutations sequenced in P6 and P7, P20, P44 and P47 are shown in Figure 4.7 and schematic representation showing the location of the five characterised mutations is shown in Figure 4.8.

Familial Classic Marfan Syndrome

C711Y Missense Mutation in Patients 6 and 7

Direct sequencing of the PCR-amplified product of exon 17 was unsuccessful on two occasions and Method III (see Section 2.9) was utilised as an alternative approach. Single stranded plasmid DNA template was prepared from one colony of an unaffected individual (as control sequence) and six colonies each from P6 and P7. Cycle sequencing PCR was carried out using the forward primer of the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems)(see Section 2.9, Method III). Results of sequence analysis of this 133-bp fragment demonstrated a single base alteration in five of twelve DNA templates (four from P6 and one from P7) when compared to sequence from the unaffected individual and the FBN1 cDNA sequence (genbank database accession number L13923; complete coding sequence of HUMFIBRILLIN Homo sapiens fibrillin mRNA). Confirmation of the mutation was obtained by cycle sequencing PCR using the reverse primer and DNA template from the unaffected individual, and one DNA template each from P6 and P7 in which the mutant sequence was previously demonstrated.

The mutation identified was a heterozygous G to A substitution at nucleotide 2132 in

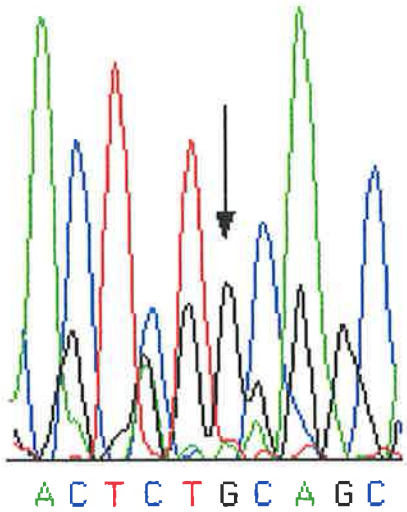
Table 4.1 FBN1 Gene Mutations in Group 1 Patients

Patient	Location of Mutation	Sequence Alteration	Amino Acid Alteration
Patients 6 and 7 (classic)	exon 17	T**G**C to T**A**C	C711Y (cysteine to tyrosine)
Patient 20 (classic)	exon 16	**G**CG to **A**CG	A705T (alanine to threonine)
Patient 31 (classic)	exon 63	C**A**C to C**C**C	H2629P (histidine to proline)
Patient 44 (infantile)	exon 27	T**G**T to T**A**T	C1152Y (cysteine to tyrosine)
Patient 47 (neonatal)	exon 25	**T**GT to **G**GT	C1055G (cysteine to glycine)

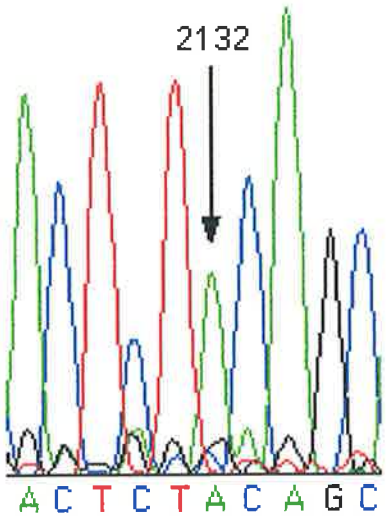
Figure 4.7 Chromatograms of Sequenced Mutations

C711Y in Patients 6 and 7

normal sequence

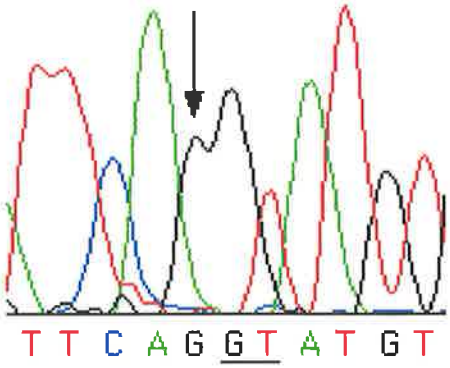


mutant sequence

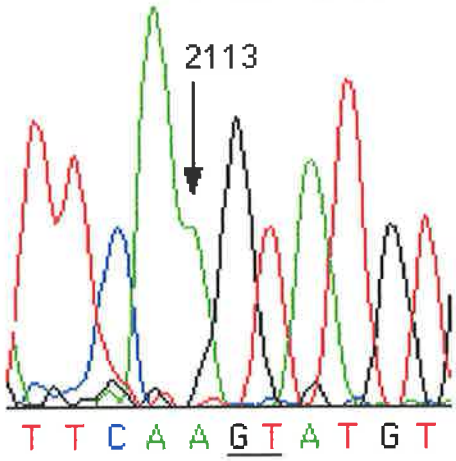


A705T in Patient 20

normal sequence

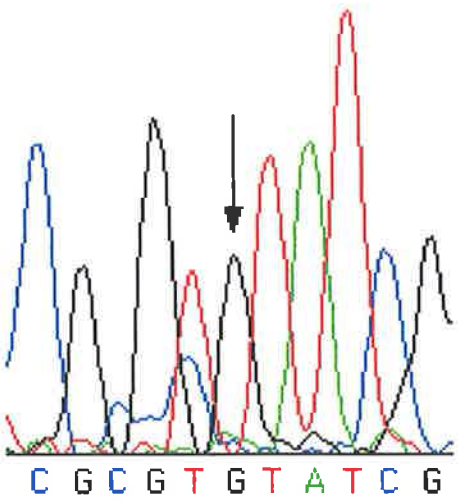


mutant sequence

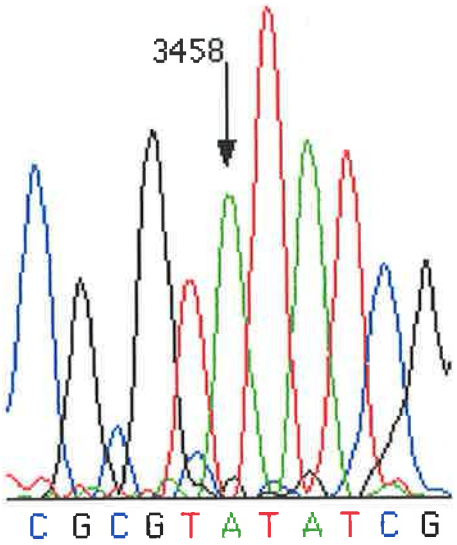


C1152Y in Patient 44

normal sequence



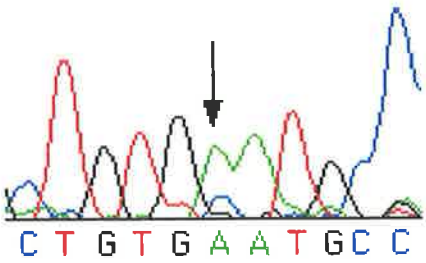
mutant sequence



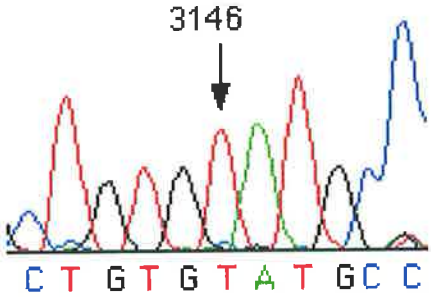
In the chromatogram representing A705T, the sequence alteration occurs adjacent to the splice site (underlined).

E1139V in Patient 44

normal sequence

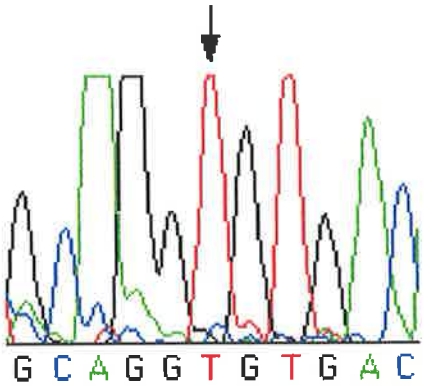


mutant sequence

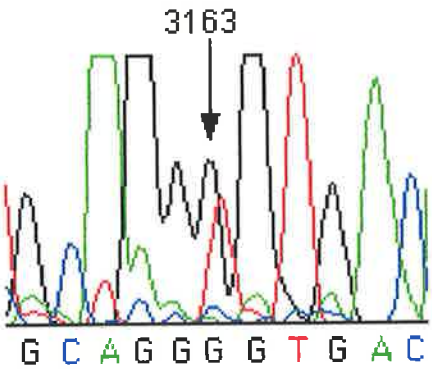


C1055G in Patient 47

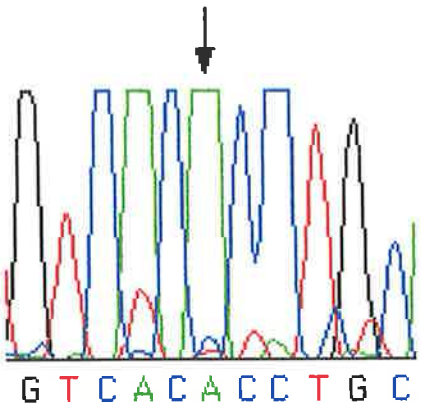
normal sequence (forward)



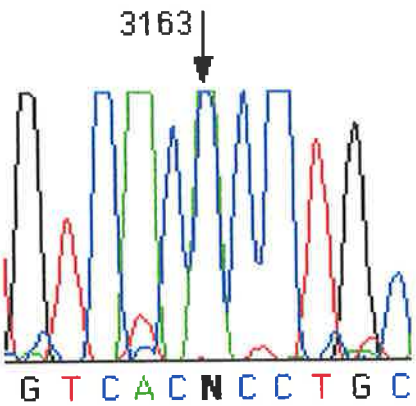
mutant sequence (forward)



normal sequence (reverse)

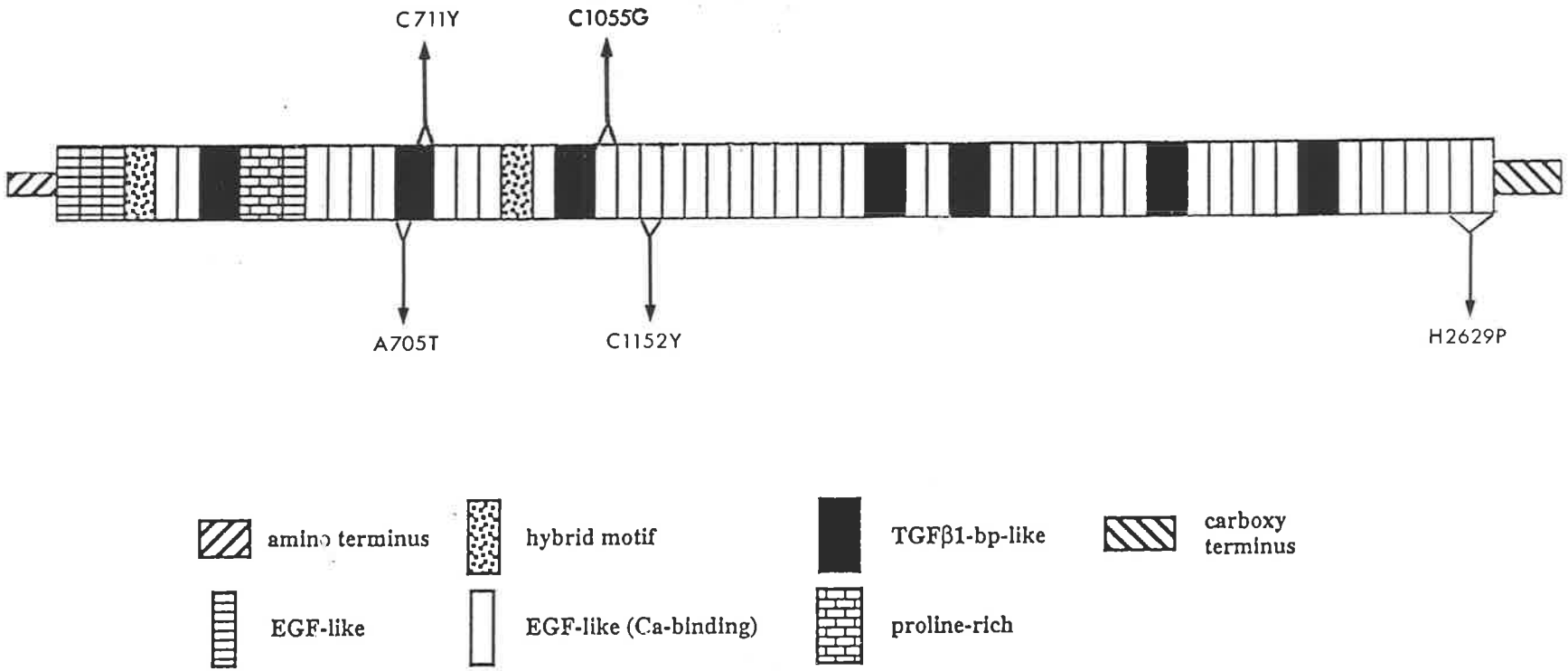


mutant sequence (reverse)



The sequence alterations shown above (arrow and number denote position of altered nucleotide in mutant allele) are presumed to be the disease-causing mutations in these Marfan syndrome patients, except for E1139V which most likely represents an artefact secondary to cloning (see Section 4.8.4 for discussion). Except for C1055G, all chromatograms represent sequence in the forward direction only.

Figure 4.8 Location of the Five Characterised Novel FBN1 Mutations



Novel FBN1 mutations in Marfan syndrome patients are superimposed on a diagrammatic representation of the complete domain organisation of fibrillin.

(modified from Dietz and Pyeritz, 1995)

exon 17 of the FBN1 gene (Figure 4.7). This substitution predicts a C711Y missense mutation at amino acid 711 caused by the alteration of the wild type cysteine, an uncharged polar amino acid, to a tyrosine amino acid in the second TGF-β1 BP-like motif of the fibrillin protein. The normal sequence in this region encodes a *Pst* I enzyme restriction site (recognition sequence 5'...CT<u>G</u>CA$^{\downarrow}$G...3'; arrow indicates site of enzyme cleavage) and the C711Y mutation was predicted to abolish this site (altered sequence in mutant allele: 5'...CT<u>C</u>CAG...3') in affected individuals P6 and P7.

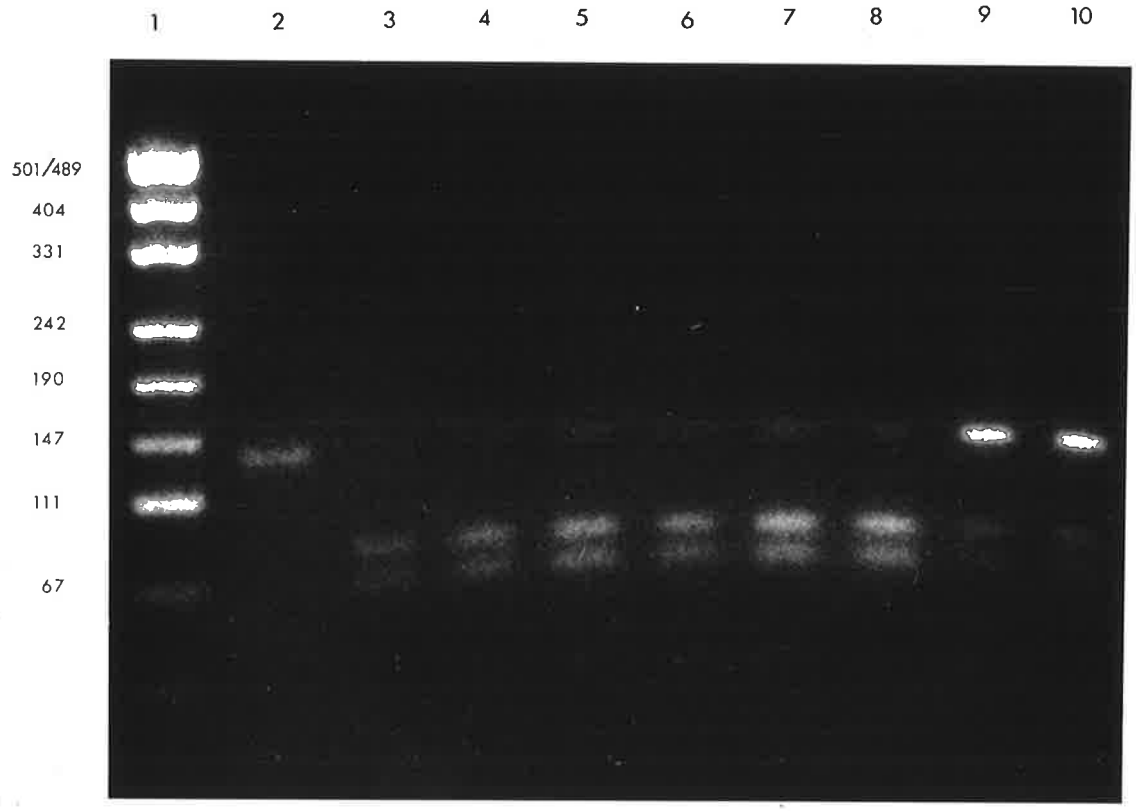
Exon 17-specific PCR amplification products of genomic DNA from six unaffected individuals, P6 and P7 were subjected to restriction digestion with *Pst* I. The products were electrophoresed through a 3% agarose gel stained with ethidium bromide and visualised by ultraviolet transillumination. In unaffected individuals with an intact *Pst* I site, one would expect *Pst* I digestion to yield two digested fragments whose sizes (57 and 76 bp) together add up to the size of the undigested PCR product (133 bp). In affected individuals, the normal allele would produce the same pattern as in unaffected individuals, while the mutant allele would not be subject to digestion by *Pst* I, resulting in the appearance of three bands in total: one fragment of 133 bp (undigested product;mutant allele) and two smaller fragments (digested products;normal allele). Precisely this pattern was obtained for *Pst* I digestion of P6 and P7 exon 17 PCR products, providing further verification of the putative mutation C711Y (Figure 4.9).

Sporadic Classic Marfan Syndrome

<u>A705T Missense Mutation in Patient 20</u>

Direct sequencing of exon 16-specific amplified genomic DNA proved unsuccessful on two occasions. Alternative methods were attempted in an effort to obtain sequence data for the mutant fibrillin allele. Twenty μl of PCR product was electrophoresed through a 1% low melting point agarose gel, and the relevant band was cut out of the gel under UV visualisation, melted at 65°C and then prepared for sequencing using the Qiaquick PCR Purification Kit Protocol followed by the Taq Dye Deoxy™ Terminator Cycle Sequencing Kit Protocol (see Section 2.9, Method II). This method gave poor sequence data. Sequencing of six separately cloned plasmid DNAs from P20 in the forward and reverse directions yielded apparently normal sequence, presumably due to representation of the normal allele only with no representation of the mutant allele in any of the clones.

Figure 4.9 *Pst* I Digests in Normal Individuals and Patients 6 and 7



Pst I digests of genomic DNA for exon 17-specific amplified PCR products. Lane 1 represents Hpa II digest of plasmid pUC19 DNA (13 fragment ladder), and numbers to the left indicate approximate fragment sizes (number of base pairs); lane 2 represents PCR fragment from unaffected individual not subjected to Pst I digestion; lanes 3-8 represent Pst I digested PCR fragments from six unaffected individuals; lanes 9 and 10 represent Pst I digested PCR fragments from patients 6 and 7, respectively demonstrating loss of Pst I restriction site.

Another approach involved electrophoresis of 6 µl of PCR product through a 10% PAGE/glycerol SSCP gel. DNA was recovered from the abnormally migrating SSCP fragment by cutting out the area of the gel that corresponded to the abnormally migrating band seen on autoradiography and soaking this gel fragment in 300 µl of distilled water at room temperature overnight. A 2 µl aliquot part of this sample was then used as a template for reamplification in a 50 µl total volume PCR reaction using primers specific to exon 16. Fifteen µl of this reaction was electrophoresed through a 2% agarose gel to allow quantitation of the amount of DNA required for sequencing. Direct sequencing of double stranded PCR product in forward and reverse directions as before was performed but again, there appeared to be no sequence alteration. Plasmid DNA templates were prepared from a further four clones from P20, revealing a sequence alteration which, on retrospective analysis of earlier sequence data, was present in the majority of clones.

The sequences showed a heterozygous G to A single base substitution at nucleotide 2113, adjacent to the GT splice site (Figure 4.7). This change predicts an A705T missense mutation at amino acid 705 resulting in alteration of the wild type alanine (nonpolar) to a threonine (uncharged polar) amino acid in the last codon of exon 16. Sequencing of plasmid DNA from P20's unaffected mother gave normal results.

H2629P Missense Mutation in Patient 31

Direct DNA sequencing was performed in the University of Texas Medical School, Department of Microbiology Core Facility, in collaboration with Dr Dianna Milewicz. Direct sequencing of the PCR-amplified product of exon 63 revealed a heterozygous A to C transversion at nucleotide 7868. This change predicts a missense mutation at amino acid 2629 caused by the alteration of the wild type histidine codon to a proline codon at a position immediately adjacent to one of the conserved cysteines in the second to last EGF-like domain of the fibrillin protein. Analysis of genomic DNA from P31's unaffected parents was normal.

Infantile Marfan Syndrome

E1139V Sequence Alteration and C1152Y Missense Mutation in Patient 44

Direct sequencing of exon 27-specific amplified genomic DNA was successfully performed in an unaffected (control) sample, but was unsuccessful on two occasions in

P44. Single stranded plasmid DNA template was prepared from one colony of an unaffected individual (as control sequence) and five colonies from P44. Cycle sequencing PCR was carried out using the forward primer of the PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems)(see Section 2.9, Method III). One sequence (from plasmid DNA preparation of a clone from P44) was of poor quality and excluded from analysis. Of P44's four remaining sequences, two clones showed an identical heterozygous A to T single base substitution at nucleotide 3146 (corresponding to amino acid 1139) (Figure 4.7). The E1139V sequence alteration in this allele predicts an alteration of the wild type glutamine (uncharged polar) amino acid to a valine (nonpolar) amino acid. The remaining two clones, representing the alternate allele, showed a G to A single base substitution at nucleotide 3458 (corresponding to amino acid 1152) (Figure 4.7). This C1152Y missense mutation predicts an alteration of the wild type cysteine amino acid to a tyrosine. Sequencing of these four plasmid DNAs using the reverse primer provided confirmation of these results. Sequencing from the unaffected control (from both double-stranded PCR product and single-stranded plasmid DNA) gave normal results.

The likelihood of two putative mutations within the same exon of the FBN1 gene was considered to be extraordinarily remote (see Section 4.8.6). Since P44 is adopted, there was no ready recourse to comparative sequence analysis of DNA from the biological parents. Given these restrictions, a further experiment was designed to determine whether the E1139V sequence alteration might represent a silent polymorphism, or reflect an artefactual change secondary to the sequencing of a cloned product.

The heterozygous A to T substitution at nucleotide 3146 occurs in a region that normally encodes a *Bsm* I enzyme restriction site (recognition sequence 5'...G<u>A</u>ATGCN$^{\downarrow}$...3'; arrow indicates site of enzyme cleavage) and was predicted to abolish this site (altered sequence in mutant allele: 5'...G<u>T</u>ATGCN...3') in the affected individual P44. Exon 27-specific amplification products of genomic DNA from 70 unaffected individuals (140 chromosomes) and from P44 were subjected to restriction digestion with *Bsm* I. The products were electrophoresed through a 3% agarose gel stained with ethidium bromide and visualised by ultraviolet transillumination.

In the unaffected individuals, analysis of exon-27 specific amplified PCR product restriction-digested with *Bsm* I yielded two digested fragments, whose sizes (117 and 64 bp) together added up to the size of the undigested product (181 bp). These findings are consistent with the control sequence data, confirm that the *Bsm* I site in exon 27 is intact in both FBN1 alleles of unaffected individuals, and suggest that the E1139V sequence alteration is unlikely to represent a silent polymorphism. Analysis of similarly prepared genomic DNA from P44 gave an identical pattern to that of unaffected individuals after *Bsm* I enzyme digestion. *Bsm* I restriction digestion was again performed on genomic DNA from several unaffected individuals, P44, and on plasmid DNA from P44's two separate alleles represented by the sequence alterations E1139V and C1152Y. Results of restriction enzyme digestion showed an identical pattern (two visible fragments) in unaffected individuals and in P44. Restriction digestion of the allele represented by C1152Y in P44 showed two fragments (digested products), while only a single fragment (undigested product) was seen in the allele represented by E1139V (Figure 4.10(a)).

The sequence data from P44's cloned products and the *Bsm* I digestion patterns obtained for P44's different plasmid DNAs gave consistent results which appear to indicate loss of the *Bsm* I site in two clones (represented by the sequence alteration E1139V), and preservation of this site in the other two clones (represented by the missense mutation C1152Y). However, these results are in direct conflict with the pattern obtained on *Bsm* I digestion of P44's chromosomal DNA, in which the anticipated loss of the *Bsm* I site in one allele could not be verified but instead, *Bsm* I restriction digestion clearly indicated the presence of two intact *Bsm* I sites (Figure 4.10(b)). From these data I concluded that the E1139V sequence alteration in two separate plasmid DNAs from P44 most likely represented cloning artefact (see Section 4.8.4 for discussion).

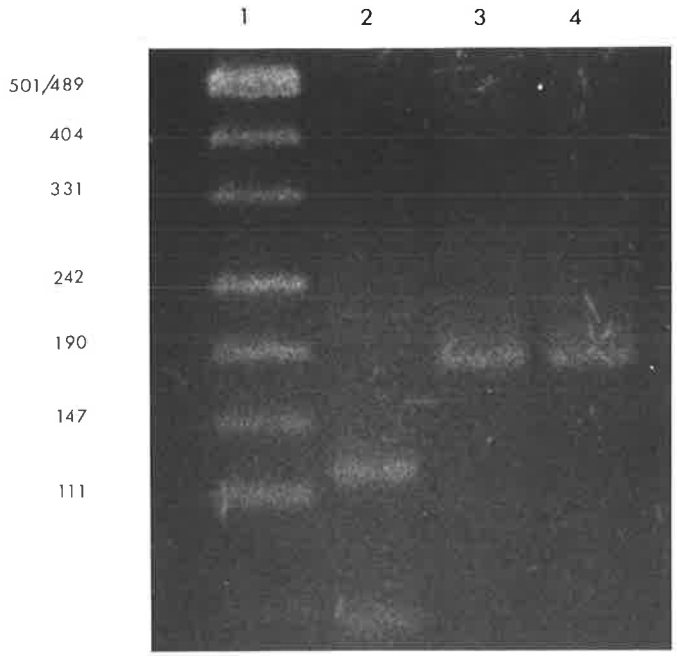
Neonatal Marfan Syndrome

C1055G Missense Mutation in Patient 47

Direct DNA sequencing and analysis of PCR-amplified fragments in both the forward and reverse direction of exon 25 revealed a heterozygous T to G single base substitution at nucleotide 3163 (corresponding to amino acid 1055) in one of the calcium-binding EGF-like domains constituting twelve such consecutive motifs in the central portion of the FBN1 gene (Figures 4.7 and 4.8). This C1055G substitution alters the wild type cysteine to a glycine amino acid.

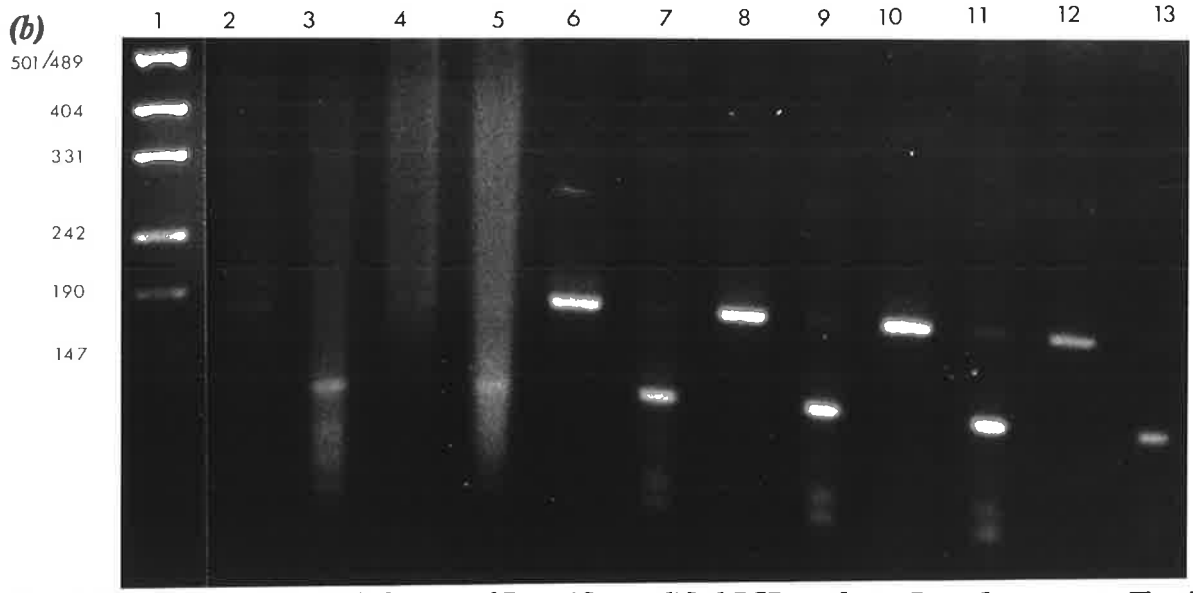
Figure 4.10 *Bsm* I Digests of Plasmid and Genomic DNA in P44

(a)



Bsm I digests of plasmid DNA for exon 27-specific amplified PCR products. Lane 1 represents Hpa II digest of plasmid pUC DNA (13 fragment ladder), and numbers to the left indicate approximate fragment sizes (number of base pairs); lane 2 represents Bsm I digested PCR fragments from plasmid DNA of the allele with the C1152Y mutation in P44; lanes 3 and 4 represent PCR fragments from plasmid DNA of the allele with the E1139V sequence alteration in P44 prior to and after Bsm I digestion, respectively, demonstrating apparent loss of the Bsm I site in this allele.

(b)



Bsm I digests of genomic DNA for exon 27-specific amplified PCR products. Lane 1 represents Hpa II digest of plasmid pUC19 DNA (13 fragment ladder), and numbers to the left indicate approximate fragment sizes (number of base pairs); lanes 2, 4 ,6, 8 and 10 represent PCR fragment from five unaffected individuals not subjected to Bsm I digestion; lanes 3, 5, 7, 9 and 11 represent Bsm I digested PCR fragments from the same five individuals; lane 12 represents PCR fragment from P44 not subjected to Bsm I digestion, and lane 13 represents Bsm I digested PCR fragments from P44, demonstrating preservation of the Bsm I site.

4.4 SSCP Screening of the FBN1 Gene in Group 2 Patients

SSCP screening of the FBN1 gene was conducted in all Group 2 patients for the same exonic regions of the gene as those screened in Group 1 patients. The phenotypes of Group 2 patients were heterogeneous, including one individual with isolated aortic root dilatation and clinical features suggestive of a mild form of type IV Ehlers-Danlos syndrome (P37), one patient with aortic dissection, patent ductus arteriosus, iris hypoplasia and distal digital hypoplasia (P45), two patients with Furlong syndrome (P2 and P46), two patients with congenital aneurysms of the great vessels (P28 and P35), and four patients with Shprintzen-Goldberg syndrome (P27, P39, P40, P41). Excluding silent polymorphisms, unique SSCP band shifts were not detected in any of the Group 2 patients.

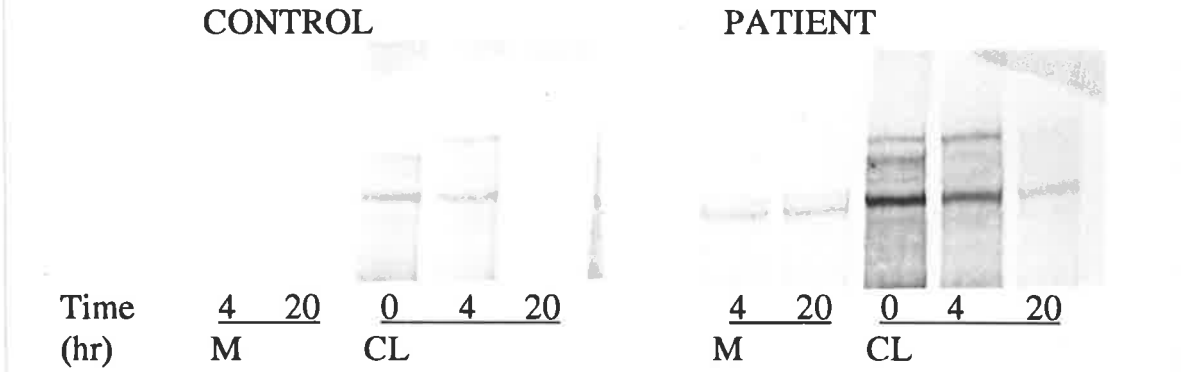
4.5 Fibrillin Pulse Chase Analysis

These studies were performed on dermal fibroblasts of five Group 2 patients (P26, P27, P28, P35 and P45) in Dr Milewicz's laboratory. Fibrillin processing abnormalities were detected in one patient (P26) selected for this analysis. These results are shown in Figure 4.12.

4.6 Collagen Screening

Screening of types I and III collagen was undertaken in eleven selected patients, two of whom belonged to Group 1 (classical Marfan syndrome, with unusual additional features). The remaining nine individuals were Group 2 patients, some of whom had clinical features suggestive of a possible collagen defect. Collagen screening was undertaken according to the methods outlined in Section 2.3.5, and the results are shown in Table 4.2. The normal results obtained in P28 and P35 enabled exclusion of the diagnosis of type IV Ehlers-Danlos syndrome in these two patients whose clinical features were suggestive of a collagen protein defect. Of the other patients screened, all had normal results, except for P37. There was markedly diminished secretion and abnormally slow migration of type III collagen in P37's dermal fibroblasts. These results were reproducible on at least two separate gels.

Figure 4.12 Fibrillin Pulse-Chase Studies in P26



Inefficient secretion of profibrillin by the proband's dermal fibroblasts. Cells from the proband and an age-matched control were incubated with [35S]cysteine for 30 minutes and then chased for up to 40 hours without label present. M, media and CL, cells were harvested separately at varying periods of time. Fibrillin is synthesised within the cells as profibrillin and then secreted from the cells. In the control fibroblasts, all profibrillin has been secreted by 20 hours. In contrast, in the proband's cells, a pool of profibrillin remains in the intracellular compartment up to 20 hours. Once outside the cell, profibrillin is processed to fibrillin.

(courtesy DM Milewicz)

Table 4.2 Types I and III Collagen Mutation Screening Results

Patient	Collagen synthesis[a]			α1/α2	Collagen secretion		% type III	Gel migration[b]		
	type III	type I	type V		type I	typeIII		α1(I)	α2(I)	α1(III)
P1	11993	128237	675	1.98	92.0	90.6	8.51	N	N	N
P2	25076	261739	236	2.04	94.8	93.8	8.67	N	N	N
P18	4538	32394	289	2.11	91.4	95.4	12.19	N	N	N
P26	6362	75556	1019	2.33	83.2	76.3	7.67	N	N	N
P28	5169	36375	280	2.08	94.4	93.3	12.36	N	N	N
P34	23874	169328	662	2.22	94.2	94.8	12.31	N	N	N
P35	13087	102738	1062	2.16	85.4	86.5	11.20	N	N	N
P36	24899	123550	546	2.21	89.7	92.2	16.71	N	N	N
P37	3360	96774	279	2.18	89.9	43.3	3.35	N	N	N
P38	8849	61412	133	2.01	82.7	84.7	12.57	N	N	N
P45	15291	83842	371	2.17	92.0	90.1	13.1	N	N	N
Controls										
S84	13403	138714	353	2.08	94	94.7	8.77	N	N	N
S87	14772	112380	573	1.85	91.9	94.1	11.57	N	N	N
S88	15331	111801	0	1.93	94.1	95.3	12.06	N	N	N
S89	10403	149510	600	1.93	93.7	94.6	6.48	N	N	N
S93	15435	129126	251	2.06	92.7	94.9	10.67	N	N	N
S101	14704	109784	1534	2.27	78.5	89.1	11.68	N	N	N
S103	12761	114226	659	2.07	91.5	92.3	9.97	N	N	N
S104	12439	78388	115	1.96	93.3	94.4	13.69	N	N	N
S113	8540	103165	1828	1.71	78.3	89.8	7.52	N	N	N
Mean	13088	116344	657	1.98	89.8	93.2	10.3			
SD (+/-)	2359	20803	626	0.16	6.5	2.3	2.3			

[a] Collagen production is expressed as [^{3}H]proline dpm/μg DNA. Control values are given as the mean +/- SD (standard deviation)

[b] The electrophoretic migration of α1(I), α2(I) and α1(III) were determined; α1(I), alpha-one chain of type I collagen; α2(I), alpha-two chain of type I collagen; α1(III), alpha-one chain of type III collagen; N normal α chain migration

(courtesy JF Bateman and D Chan)

4.7 Special Studies

Unusual clinical features prompted specialised investigations in four patients, P26, P27, P35 and P45. The clinical details of these patients can be found in Section 3.3.2, Appendix 3A (where P27 is represented as Case 1), Sections 3.3, and 3.3.2, respectively.

Patient 26

This patient was reviewed by an endocrinologist because of the absence of acne, the presence of poor muscle development, and the sparsity of facial, body and axillary hair. He also had mental retardation (cause unknown), a significant large joint arthropathy, and dermal changes of pseudoxanthoma elasticum. The clinical findings could not be ascribed to any recognisable syndrome. Investigations included a bone age, serum LH, FSH, oestradiol, DHEA-sulphate, testosterone and hGH. These results were normal, apart from a low serum hGH (< 1μU/ml), elevated oestradiol (360 pmol/l; normal reference range < 110) and elevated serum FSH (20.4 IU; normal range 10 +/- 4.8). The androgen receptor assay (performed on cultured dermal testicular fibroblasts) was normal (22.8 fmol ^{3}H-R1881 bound/mg protein; normal range 7.6-24.2). He had a normal karyotype, 46 XY.

Patient 27

Prior to the diagnosis of Shprintzen-Goldberg syndrome being made in this patient, collagen screening was undertaken, results of which were normal. The clinical findings of craniosynostosis and a generalised skeletal dysplasia led to a hypothesis invoking one of the fibroblast growth factor receptor genes, FGFR2, as a potential candidate gene in this patient with Shprintzen-Goldberg syndrome. Preliminary screening undertaken by collaborators in London (W Reardon, personal communication) uncovered an abnormally migrating band on 6% PAGE/glycerol gel SSCP analysis in exon 1-amplified PCR product of the FGFR2 gene in P27. Attempts to sequence the region of interest have been unsuccessful so far (W Reardon, personal communication) and this research is continuing.

Patient 35

Results of collagen screening and crosslinking were normal in P35 (Tables 4.2 and 4.3). Fluorescent in situ hybridisation of the elastin gene (WSCR, chromosome 7q) was performed in P35. The presence of a fluorescent signal on both chromatids of both chromosome 7 homologues confirmed non-deletion of this chromosomal region.

Patient 45

The clinical findings in P45 could not be matched to a recognised syndrome. A chromosomal karyotype and high resolution banding studies of chromosome 15 failed to demonstrate a microdeletion or chromosomal rearrangement. In view of the presence of iris hypoplasia, it was hypothesised that there might be a small microdeletion in the PAX6 (aniridia) gene on chromosome 11p13 (Ton et al., 1991; Hanson et al., 1995)). Analysis of the 11p13 locus using six microsatellite primer pairs for the 11p13 region was conducted by Michael Eccles (Cancer Genetics Laboratory, University of Otago, Dunedin) to examine this postulate. This research is in progress and results are pending.

4.8 Discussion

4.8.1 Current Methods of Mutation Detection

There are numerous techniques for the detection of naturally occurring mutations that disrupt genes. These methods for mutation detection can be divided into two categories. The first consists of techniques which efficiently identify known mutations, while the second consists of methods to scan DNA sequences for unknown mutations. The most appropriate screening technology is influenced by the expected nature of the mutation, size and structure of the particular locus, availability of mRNA, degree of sensitivity required and available resources. Single base alterations are the most common type of mutation at most loci (Grompe, 1993). A number of methods based on PCR amplification of DNA samples prior to analysis may be used for detecting these subtle changes.

Scanning methodologies based on the aberrant migration of mutant molecules during electrophoresis include denaturing gradient gel electrophoresis (DGGE) (Myers et al., 1987), heteroduplex analysis (HA) (White et al., 1992) and single-stranded conformation polymorphism (SSCP) analysis (Orita et al., 1989). Other scanning strategies rely on the

Table 4.3 Collagen Extraction from Fibroblast *In Vitro* Matrix in P35 and Control Subjects

	(a) Collagen matrix extraction				(b) Pulse-labelled collagen distribution			
	P35	**C1**	**C2**	**C3**	**P35**	**C1**	**C2**	**C3**
% collagen in acetic acid extract	57	63	37	32	18	29	20	9
% collagen in pepsin extract	43	37	63	68	79	66	64	85
% β-dimers	20	16	15	20	17	23	16	9

Fibroblasts were grown in the presence of ascorbic acid for 20 days and the in vitro matrix was (a) serially extracted and the proportions in acetic acid extract (newly crosslinked collagen), and pepsin extract (mature crosslinked collagen) was quantified and expressed as a proportion of the total collagen. The proportion of collagen crosslinked in β-dimers was also calculated. (b) After pulse labelling for 24 hrs and a 24 hr chase period the distribution of the newly synthesised labelled collagen in each extracted fraction, and in β-dimers, was quantified. C1, C2 and C3 represent age-matched controls.

(courtesy J Bateman and JAD Esquivel)

cleavage of RNA or DNA molecules prior to analysis using either ribonuclease A (Gibbs and Caskey, 1987) or chemical mismatch cleavage (CMC) (Cotton et al., 1988). All these methods are capable of detecting mutations with varying efficiencies, but none defines precisely the nature of the change. DNA sequencing achieves this and is, therefore, a necessary final step of any mutation detection method. Despite continued developments in the field of mutation detection in recent years, no one method detects all mutations.

While all these methods have been used successfully for identification of disease-causing alleles, SSCP (because of its simplicity and economy) and DDGE (because of its near 100% sensitivity) are the favoured techniques (Dean, 1995). Single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989) has become the most widely used scanning technique for identifying unknown mutations (Grompe, 1993). If performed with short PCR products (< 400 bp) and using two different conditions, SSCP analysis detects between 70-98% of mutations (Grompe, 1993; Sheffield et al., 1993; Ravnik-Glavac et al., 1994; Forrest et al., 1995). However, the sensitivity of the method is less than 50% when fragments of > 400 bp are analysed (Grompe, 1993). The FBN1 mutation screening in my research was performed by SSCP analysis because this was an established method of mutation detection in the laboratory.

4.8.2 Clinical Utility and Success of Mutation Detection

Currently, using RT-PCR of the whole FBN1 gene, the detection of mutations has been disappointingly low, in the order of 10% (Nijbroek et al., 1995). The recent development of the genomic approach appears to have a comparable degree of success in mutation detection, apart from one recent report claiming an ability to detect 78% of FBN1 mutations in unselected Marfan patients using genomic amplicons (Nijbroek et al., 1995). These results were drawn from a small number of patients only (7/9), and have yet to be reproduced by other groups. It is difficult to draw meaningful conclusions regarding the relative sensitivity of various techniques used in the detection of FBN1 gene mutations, largely because different approaches (RT-PCR and genomic amplicons) and a wide variety of different gel conditions have been applied. There is no published study comparing these differing methodologies and their relative sensitivity in FBN1 gene mutation detection.

In this research, thirty of sixty-five exons or 44% of the FBN1 gene cDNA were analysed in all patients by SSCP. Given that less than half of the coding sequence was screened in these patients, and that mutations are distributed randomly throughout the FBN1 gene, then by extrapolation, one could expect to detect mutations in some 60% of these Marfan patients if all exons were screened using the 10% SSCP gel system (see Section 2.7.4-2.7.6). While this level of sensitivity is clearly not high enough to consider using the SSCP screening approach in a diagnostic capacity, it is certainly more encouraging than the literature reports of an overall 10% mutation detection. In effect, this level of mutation detection (60%) differs little from that reported by Nijbroek et al. (1995), although in both cases, the total patient numbers remain small, limiting conclusions that might be drawn.

4.8.3 Technical Issues Regarding SSCP Gels and RT-PCR

Single-strand conformation polymorphism analysis has proved to be a simple and effective technique for the detection of single base substitutions (Sheffield et al., 1993). This technique is based on the principle that single-stranded DNA molecules take on specific sequence-based secondary structures under nondenaturing conditions. Molecules differing by as little as a single base substitution may form different conformations and migrate differently in a nondenaturing polyacrylamide gel. The sensitivity of SSCP is dependent on several factors and varies dramatically with the size of the DNA fragment being analysed. The optimal size fragment for sensitive base substitution detection by SSCP is approximately 150-200 bp (Savov et al., 1992; Sheffield et al., 1993). Ideally, the size of the PCR fragment should be kept small when performing SSCP to detect mutations. A second important factor is electrophoretic gel composition, and studies have shown that high percentage acrylamide gels improve resolution in SSCP analysis (Savov et al., 1992). This finding is supported by the results of the research described herein, where the detection of band shifts on 10% SSCP gels was superior to that of 4.5% SSCP gels. Other factors that may alter the sensitivity of mutation detection by SSCP relate to parameters expected to have a direct impact on DNA conformation, such as temperature, ionic strength and denaturant (Spinardi et al., 1991; Schoettlin et al., 1992). In some systems, excess PCR primers may interfere with the amplified sequence and inhibit the discriminative potential of SSCPs (Cai and Touitou, 1993).

There were several technical disadvantages to the 4.5% and 10% SSCP gels used in the

research. The 4.5% SSCP gels were slow to polymerise (6-8 hours), necessitating gel pouring the afternoon prior to gel electrophoresis. In addition, resolution of the bands obtained was not as crisp as those seen with 10% SSCP gels. Whilst the 10% SSCP gels required only 2 hours to polymerise (allowing pouring, loading and electrophoresing of the gel without an overnight delay), the single stranded DNA fragments migrated more slowly through the gel, necessitating electrophoresis for 24 hours in most cases. This placed serious limitations on the number of 10% gels that could be run, and the rate at which results could be obtained in any given time interval. The alternate MDE gel system has several advantages over the 10% SSCP gels, including relatively quick gel polymerisation (2 hours), a shorter gel electrophoresis time (8-12 hours overnight) and good resolution of bands. The MDE gel system is however, more expensive than the PAGE/glycerol gels used in the research.

There were two false positive SSCP results (P13 and P24). Both of these occurred in samples prepared by extraction of total RNA from cultured dermal fibroblasts and subsequent RT-PCR. Despite the precautions taken to minimise ribonuclease contamination, this may account for the spurious results obtained on these two occasions. The possibility of sample contamination is clearly greater using this multiple-step approach when compared to samples obtained from DNA extracted from whole blood.

4.8.4 Technical Issues Regarding Direct Sequencing of Genomic and Plasmid DNA

The use of the dideoxy chain termination procedure to sequence linear double-stranded PCR products may be hampered by fast renaturation of the template. Heat-denaturation of the template in the presence of the primer, and initiation of the reaction after the shortest possible annealing phase should minimise template renaturation (Casanova J-L et al., 1990). Other factors contributing to the success of this method include the recommended times and temperatures of incubation as well as DNA concentrations in the reaction mixture, factors that differ widely from author to author.

The fidelity of DNA synthesis, at least for single base substitutions, has been described for several DNA polymerases useful in molecular biology, including the DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus (Taq)* (Chein et al., 1976). The optimum temperature for DNA synthesis with the *Taq* DNA polymerase is 80°C (Chein et al., 1976). Polymerase chain reaction provides a method by which

specific DNA sequences can be amplified from genomic sequences in vitro with appropriately positioned primers, but it requires multiple rounds of heat denaturation, rehybridisation, and DNA synthesis. The thermal stability of *Taq* DNA polymerase and its ability to polymerise DNA at elevated temperatures simplify the procedure, and make it a particularly attractive polymerase for use in PCR amplification (Saiki et al., 1988).

The fidelity of *Taq* DNA polymerase at high temperature for both base substitution errors and frameshift errors has been examined (Tindall and Kunkel, 1988). A single round of synthesis at 70°C with the *Taq* DNA polymerase will yield one base substitution error per 9,000 nucleotides and one frameshift error per 41,000 nucleotides polymerised (Tindall and Kunkel, 1988). In addition, manipulations requiring multiple rounds of heat denaturation and extended time periods at 55-70°C are likely to increase the mutations occurring during PCR. Heat treatment of DNA is mutagenic (Drake and Baltz, 1976), generating both transitions (Baltz et al., 1976) and transversions (Bingham et al., 1976). These factors may significantly affect the mutation frequency of amplified sequences depending on experimental conditions. During PCR, the target sequence is amplified exponentially, so that individual sequences that contain errors may become a significant portion of the reaction product, depending upon how early in the amplification process the error occurs. In spite of this potential limitation, experimental conditions can be designed (Saiki et al., 1988) to limit the generation of mutations occurring during PCR, resulting in a mutation rate that is approximately twice that observed in a single round of DNA synthesis (Tindall and Kunkel, 1988).

The direct sequence analysis of PCR-amplified DNA generates a consensus sequence independent of errors that occur during amplification, allowing the detection of genomic mutations (McMahon et al., 1987; Engelke et al., 1988). However, the cloning of any single amplified sequence may yield unacceptable alteration/s within the isolated clone. Considering these factors, sequence of greatest fidelity is more likely to be generated by directly sequencing double-stranded PCR products rather than cloned DNA.

In this research, direct sequencing of cloned and purified single-stranded plasmid DNA preparations was more successful in generating clear sequence data with little background, than was direct sequencing of purified double-stranded PCR products. The reason for this is not known but may be due to several factors. Firstly, not all

oligonucleotide primers are ideal for sequencing work, even when purified. The success of various primers for sequencing may depend on the secondary structure of the template. Secondly, oligonucleotide primers are not rigorously purified so that although PCR may be successful, use of the same primers for sequencing may not be as successful (R Richards, personal communication). Only two mutations (C1055G and H2629P) were determined and sequenced successfully from purified double-stranded PCR-amplified product.

Isolated base-pair changes were identified in some sequences, and in most cases, these could be readily recognised as *Taq* DNA polymerase-induced errors, since they occurred randomly in one but not both directions on sequencing. One possible exception to this was demonstrated by the E1139V sequence alteration present in two of four cloned plasmid DNAs from P44. This sequence alteration was present in both directions on sequencing and did not appear to be random. Fortunately, use of the *Bsm* I restriction enzyme site provided an independent alternative method for determining whether loss of the *Bsm* I site (predicted by the E1139V sequence alteration, and demonstrated on *Bsm* I digestion of plasmid DNA from this allele) (Figure 4.10(a)) could be confirmed. *Bsm* I enzyme digestion of the patient's chromosomal DNA showed that the mutation had not occurred in the patient's chromosomal DNA, since the *Bsm* I site was intact (Figure 4.10(b)). The possibility of low level mosaicism resulting in the E1139V sequence alteration in two clones cannot be excluded in P44, although this is unlikely since one would expect the plasmid DNA sequence to reflect the genomic sequence. These findings illustrate the caution that should be exercised in interpretation of sequence data obtained from cloned products, since even reproducible base changes in separate clones may be misleading and may not accurately represent genomic sequence. This example also highlights the importance of seeking to verify sequencing results by several alternative approaches wherever possible. The presence of a restriction enzyme recognition site may be extremely helpful in confirming or throwing into question results obtained by sequencing. This was clearly demonstrated by the analysis of *Pst* I sites in P6 and P7, and of *Bsm* I sites in P44, respectively. The possibility of *Taq* DNA polymerase-induced errors should be borne in mind, although this is unlikely to be a major problem when sequencing products of relatively small size.

4.8.5 Detection of Band Shifts by SSCP

The SSCP band shifts (representing two polymorphisms and four mutations) found using genomic amplicons and 10% SSCP gels were located in exons 15, 16, 17, 25, 27 and 28. The size of the amplified PCR fragments for these exons is shown in Table 2.1, and ranged from 133 to 273 bp. Theoretically, it should be possible to further improve the sensitivity of the system employed by PCR fragment digestion with suitable restriction enzymes to obtain an optimal fragment size of 150-200 bp for the other exons in which band shifts were not found.

Two polymorphisms were identified (exons 15 and 28) by SSCP screening of Group 1 and 2 patients and an additional seventy unaffected individuals. The nature of these polymorphisms has not been analysed by direct DNA sequencing. It is notable that both polymorphisms were found in the same patients. At least one exon 15 polymorphism (N625), has been previously identified in exon 15 of the FBN1 gene (Hayward et al., 1994). This neutral polymorphism results from a substitution of T by C at nucleotide position 1875. At the time of writing of this thesis, there are no reported polymorphisms in exon 28 of the FBN1 gene. A sequence alteration (P1148A) has been recently described in exon 28, the significance of which remains unclear at present (Dietz and Pyeritz, 1995; Dietz et al., 1995b) (see below).

Sequence Alteration in Exon 28

Towards the conclusion of this study, new information became available regarding a sequence alteration in exon 28 of the FBN1 gene. A P1148A mutation has been observed in a variety of clinical settings including Marfan syndrome, isolated aortic aneurysm, Shprintzen-Goldberg syndrome, and apparently unaffected relatives of probands from these families (Dietz and Pyeritz, 1995; R Pyeritz, personal communication). The mutation does not always segregate with disease, but appears to be more commonly found in individuals with a connective tissue disorder than in the general population (D Milewicz, personal communication). Currently, significance of this sequence alteration for pathogenesis remains unclear. The authors intimate that since the P1148A sequence alteration is associated with a variety of phenotypic manifestations, then perhaps this mutation defines a predisposing allele that is subject to modification by epistatic, stochastic, or environmental modifiers. They also hypothesise that P1148A requires either another mutation in FBN1 or at another locus in order to achieve phenotypic

expression.

In this research, a polymorphism in exon 28 was detected in several patients. To date, this has not been investigated further, but may prove to be the P1148A sequence alteration described above, or alternatively, another as yet undescribed silent polymorphism. Of further interest is the absence of an abnormally migrating band on SSCP analysis of exon 28-amplified genomic DNA in any of the four patients with Shprintzen-Goldberg syndrome, despite the application of three different gel systems (4.5% and 10% PAGE/glycerol and 37.5% MDE gels).

Whilst it is possible that each patient with Shprintzen-Goldberg syndrome may have a unique mutation in the FBN1 gene, it would be reasonable to postulate that any expected FBN1 gene mutation in these patients should lie in the region between exons 24 to 32, since FBN1 gene mutations associated with severe phenotypes (severe neonatal and infantile Marfan syndrome) have occurred largely though not exclusively within this region. However, no FBN1 gene mutations were identified in these patients. It is difficult to explain how a FBN1 gene mutation could account for the craniosynostosis, generalised bone abnormalities and mental retardation in these patients. These clinical features more strongly support the involvement of FGFR2 gene as a candidate gene for the disorder. This is further supported by the finding of a reproducibly abnormal migrating band on SSCP analysis of exon 1 of FGFR2 in one of the four Shprintzen-Goldberg syndrome patients (P27), together with only normally migrating bands in P27's parents. Sequencing of this region is in progress (W Reardon, personal communication). It may be that a FBN1 gene polymorphism and a FGFR2 gene mutation (in the first immunoglobulin domain of FGFR2 gene, a domain of the gene previously not thought be of any functional significance) are both necessary for the phenotypic expression of Shprintzen-Goldberg syndrome. Determination of the precise sequence alteration in the exon 28 polymorphism detected as part of the research, and of the sequence alteration in the FGFR2 gene of P27 (in whom there was no demonstrable exon 28 polymorphism with SSCP screening) are both areas of research that warrant further investigation.

4.8.6 Genotype-Phenotype Correlation in Group I Patients

Five novel FBN1 mutations were detected in six Marfan syndrome patients. Considering that there are currently fifty-five reported FBN1 mutations in the world literature, this

research resulted in a significant contribution (8.3%, or 5/60) to definition of novel FBN1 mutations.

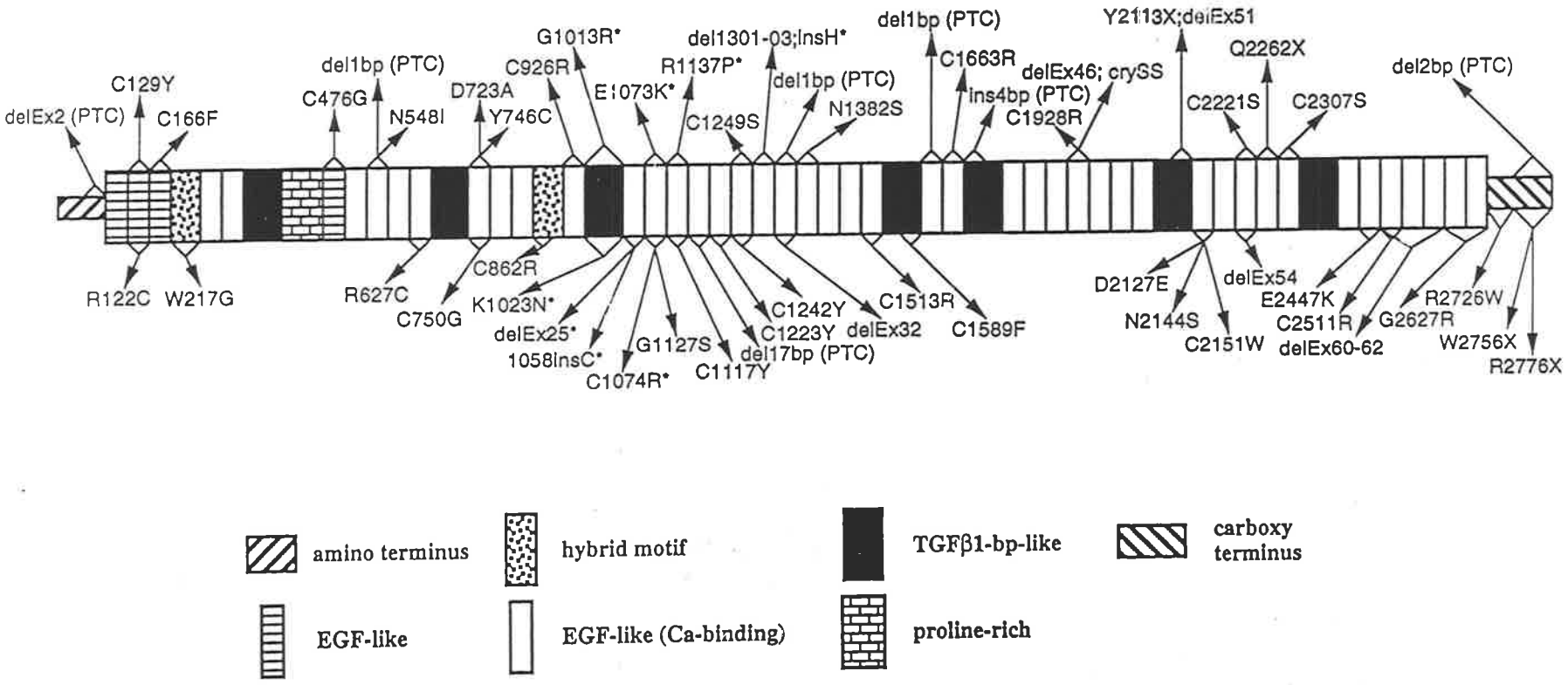
Classic Marfan Syndrome

Three novel heterozygous mutations A705T, C711Y and H2629P were identified in four patients with classic Marfan syndrome (P20; P6 and P7; P31)(Figure 4.8). The sites of these mutations are in exons 16, 17 and 63 of the FBN1 gene, respectively and in each case, the reading frame is maintained. No other FBN1 mutations have been reported in exon 16 or 17 in association with Marfan syndrome. Additionally, the A705T and C711Y mutations are the first reported in the second TGF-β1 BP motif. There are six other mutations in TGF-β1 BP motifs of FBN1. Of these, G1013R and K1023N both occur in the third TGF-β1 BP motif which corresponds to exon 24 (Figure 4.13). The third and fourth TGF-β1 BP motifs flank the twelve consecutive cb-EGF motifs in the central portion of the FBN1 gene. Both these mutations are associated with a severe neonatal phenotype. The C1589F mutation (exon 38) and a 4 bp insertion mutation (exon 41) that leads to a premature termination codon, occur in the fourth and fifth TGF-β1 BP motifs, respectively, and both are associated with a classic Marfan phenotype (Figure 4.13). Mutations Y2113X and del ex 51 both occur in the sixth TGF-β1 BP motif, which corresponds to exon 51 (Figure 4.13), and are associated with classic Marfan syndrome. Of the seven mutations identified so far in TGF-β1 BP motifs of FBN1, only one (C1589F) involves the alteration of a wild type cysteine residue. There are no reported mutations involving the first or last TGF-β1 BP motifs.

The A705T *de novo* mutation leads to the substitution of a nonpolar alanine for an uncharged polar threonine residue in the second TGF-β1 BP motif of the FBN1 gene. The wild type alanine is located precisely five codons up and downstream from two conserved cysteine residues. This cysteine-rich TGF-β1 BP domain may participate in protein-protein interactions, and the nature and location of the A705T mutation may be responsible for disrupted domain conformation. The affected patient (P20) has classic Marfan syndrome.

In keeping with the previously reported C1589F mutation, the familial C711Y mutation also leads to substitution of a conserved cysteine residue, but in this case, for a tyrosine

Figure 4.13 Mutations Causing Marfan Syndrome



Mutations causing Marfan syndrome and related disorders are superimposed on a diagrammatic representation of the complete domain organisation of fibrillin. The triangular brackets encompass single exons. Mutations associated with infantile presentation of severe, rapidly progressive disease are indicated by astericks , *. Mutations that create a premature termination codon are denoted "PTC".

(after Dietz and Pyeritz, 1995)

residue. The C1589F substitution occurred at the eighth cysteine of the fourth TGF-β1 BP-like domain of fibrillin and in the three original 8-cysteine domains described in TGF-β1 BP (Tynan et al., 1993). The affected patient had congenital contractures, no skeletal findings, and was only later diagnosed with Marfan syndrome when he developed aortic root dilatation. Fibrillin protein biosynthesis studies of dermal fibroblasts showed a normal rate of synthesis, delayed secretion, and fibrillin deposition into microfibrils of about 65% of normal (Aoyama et al., 1993). Although both mother (P6) and son (P7) have the C711Y mutation and a classic Marfan phenotype, P7 has significant pain and limitation of movement of the upper cervical spine, and is due for prophylactic aortic graft surgery in the near future because of progressive aortic root dilatation. He is clearly more severely affected than his mother. Patients P6 and P7 demonstrate the intrafamilial phenotypic variation known to occur in Marfan syndrome in differentially affected individuals within the same family in whom identical FBN1 mutations are found.

The *de novo* H2629P mutation was identified in the carboxy-terminal region of the FBN1 gene in a patient with classic Marfan syndrome (P31). This missense mutation alters the wild type basic histidine to a nonpolar proline amino acid, in the second to last EGF-like domain of the fibrillin protein. Early genotype-phenotype correlations suggested that individuals with heterozygous mutations in the carboxy-terminal region of FBN1 had disproportionately mild cardiovascular involvement, compared to the involvement of the ocular and skeletal systems (Grossfield et al., 1993). These findings were not borne out by subsequent assessment of additional affected individuals with carboxy-terminal mutations and a spectrum of phenotypes, nor by P31 who had significant cardiovascular involvement necessitating aortic surgery at twenty years of age (Grossfield et al., 1995).

There are only two other mutations involving exon 63, namely G2627R and R2680C. It is interesting to note the proximity of the two mutations G2627R and H2629P. The G2627R mutation, due to a G to A transition in FBN1 cDNA, was documented in the mother of a compound heterozygous Marfan patient (Karttunen et al., 1994). The mother had tall stature, myopia, arachnodactyly and joint laxity. She had no signs of cardiac disease on echocardiography as a teenager and refused examination thereafter. The substitution of an arginine residue for a glycine residue in the cbEGF-like motif at exon 63 of the FBN1 gene is significant, since the glycine residue at this position is conserved

through all the cbEGF-like motifs. Nuclear magnetic resonance studies have revealed that this particular glycine contains one of the two backbone carbonyls that serve as ligands in the binding of calcium ions (Selander-Sunnerhagen et al., 1992). The crucial role of this specific glycine strongly suggests that this mutation would disturb the secondary structure of the cbEGF-like motif. The R2680C mutation occurred in a family whose affected members have no cardiovascular manifestations of Marfan syndrome. The W2756X nonsense mutation in exon 65 is associated with marked skeletal and ocular manifestations of Marfan syndrome. The affected individual did not undergo aortic root repair until the age of 55, twenty years later than patients with Marfan syndrome typically have this surgery. One patient with a genomic deletion of exons 60-62 had classic Marfan syndrome (Kainulainen et al., 1992), while another individual with only skeletal features of Marfan syndrome had an R2726W mutation in exon 64.

Aboriginal Kindred with Marfan Syndrome

Collaborators in Houston originally reported a heterozygous K1317E mutation in P13 on direct sequencing of exon 31-specific amplified double-stranded PCR product. However, failure to identify this sequence alteration in four clones led the the researchers to conclude that their original findings might be spurious. Another possible explanation is that by chance, each sequenced clone happened to represent only the normal FBN1 allele, generating entirely normal sequence. In theory, one would need to sequence between 10-14 individual clones to be 97-99% certain of each allele being represented at least once (A Staples, personal communication). Thus, of itself, the failure to identify the expected K1317E sequence alteration from the four clones, does not necessarily imply that the mutation does not exist in P13. Once genomic amplicons were available for my use, SSCP analysis of exon 31-specific amplified PCR product was undertaken. However, no aberrantly migrating fragments were seen on 4.5 or 10% SSCP PAGE/glycerol gels in either P13 or her affected sister P14. Thus, it was not possible to resolve this apparent discrepancy further.

Neonatal and Infantile Marfan Syndrome

Two novel mutations were identified in two patients, one of whom had neonatal Marfan syndrome (P47), while the other (P44) had significant symptoms from early infancy although a diagnosis of Marfan syndrome was not made for several years. The mutations occurred in exon 25 and exon 27 of the FBN1 gene, respectively. The site of these

mutations is in keeping with the literature reports of clustering of FBN1 mutations between exons 24 and 32 in most individuals with severe disease phenotype (Kainulainen et al., 1994). However, many mutations causing classic or even mild Marfan syndrome also fall into this region. Therefore, the location of the mutation cannot be the sole determinant of phenotypic severity, and other factors including the character of the mutation and modifiers of phenotypic expression are also relevant. As predicted from the dominant negative model, mutations in this region that produce premature termination codons are not associated with neonatal Marfan syndrome. In addition, mutations that substitute cysteine residues are less likely to cause severe disease than those that alter other residues (Nijbroek et al., 1995). One hypothesis maintains that cysteine substitutions may be more likely to transmit structural perturbations to the amino- and carboxy-termini of the monomer, which are the proposed sites of nucleation for microfibrillar assembly (Dietz and Pyeritz, 1995).

There are thirteen published mutations that cause neonatal Marfan syndrome (Milewicz and Duvic, 1994; Kainulainen et al., 1994; Wang et al., 1995b; Putnam et al., 1995a, 1995b). They have the following distribution within the FBN1 gene: one in exon 4 (involving a non-calcium binding EGF-like motif); two in exon 24 (both of which affect a TGF-β1 BP-like motif); three each in exons 25 (one mis-splicing mutation) and 26, one each in exons 27 and 31, and two in exon 32 (mis-splicing mutations). The mutations comprise eight missense mutations, four deletions and one insertion. Excluding the mutations of exon 4 and 24, all the other mutations in this region affect calcium binding EGF-like motifs. Mutations resulting in severe neonatal Marfan syndrome cluster in exons 24 to 32 which code for the longest uninterrupted stretch of 12 cbEGF domains in the fibrillin polypeptide, and is flanked on either side by the third and fourth TGF-β1 BP-like domains (numbered from the amino terminus). The cbEGF domains in this region may be structurally interdependent due to the formation of a common β sheet between adjacent domains (Mosher et al., 1992; Pereira et al., 1993). It is conceivable that selected mutations in this region may structurally perturb the monomer over a wide distance.

Kainulainen et al. (1994) described three patients with a severe neonatal Marfan phenotype, each of whom had a mutation within the same region of FBN1. Two of the neonates died within the first day of life, and the third at nine weeks, all of congestive

heart failure. The three different mutations occurred in three consecutive motifs: a K1023N substitution in exon 24 (in the third TGF-β1 BP motif), a C1074R mutation in exon 25, and a deletion of exon 25 (both of these mutations involved calcium binding EGF-like motifs). Detailed structural analyses by rotary shadowing EM was performed on dermal fibroblasts from the patient with the deletion. The absence of any morphologically recognisable microfibrillar assemblies indicated that microfibril assembly was comprehensively disrupted. (Kielty et al., 1994). The authors suggested that mutations located in this central region of the fibrillin polypeptide may predispose patients to this lethal phenotype.

In addition to these cases, a rare compound heterozygote has been described (Karttunen et al., 1994). The nonconsanguineous parents both had classic Marfan syndrome. The father's heterozygous point mutation occurred in the hybrid motif at exon 6 of the FBN1 gene, and the mother's mutation substituted an arginine residue for a glycine residue in the cbEGF-like motif at exon 63 of the FBN1 gene. Their affected infant had a very severe form of Marfan syndrome and died from cardiac failure at four months of age. The mutant fibrillin alleles were shown to be transcribed with equal efficiency compared with the normal alleles, but metabolic labelling of fibroblast cultures from the child and both parents showed reduced biosynthesis and secretion of profibrillin. The amounts of fibrillin in cell-culture media and extracellular-matrix extracts were markedly diminished. Immunofluorescence analysis of the cell cultures of all three family members demonstrated a dramatic reduction in the amount of microfibrils, with no visible fibrils in the compound heterozygote. The biochemical studies of fibroblast cultures pointed to a severely disturbed assembly of microfibrils. This report of a compound-heterozygote Marfan patient with two characterised FBN1 mutations demonstrates incomplete dominance of FBN1 mutations and emphasises the fact that the normal allele plays a significant role in ameliorating the disease process in Marfan syndrome.

Of the neonatal mutations described so far, there has been only one insertion (Milewicz and Duvic, 1994). Interestingly, this mutation also occurred in exon 25 of the FBN1 gene, involved a 3-bp insertion that maintained the reading frame for fibrillin, and inserted a cysteine into one of the identified cysteine-rich EGF domains (Corson et al., 1993; Milewicz and Duvic, 1994). The insertion occurred in a region eleven bases (four

amino acids) downstream from the substitution determined in P47. The 3-bp insertion of a new cysteine into an EGF-like domain was predicted to disrupt disulphide bonding, either by preventing the proper pairing of cysteine within the immediate EGF-like domain, or by interacting with other cysteines in adjacent domains. Intermolecular disulphide bonds are important for fibrillin aggregation into microfibrils (Maddox et al., 1989), and it was postulated that the additional cysteine might disrupt these cross-links (Milewicz and Duvic, 1994). Pulse-chase studies of fibrillin processing, performed on the proband's dermal fibroblasts showed a delayed secretion of fibrillin and a diminished incorporation of fibrillin into the extracellular matrix.

Previous studies using electron microscope rotary shadowing of microfibrils and pulse-chase studies assessing fibrillin processing, have shown that the cells from all patients studied with neonatal Marfan syndrome produce only disorganised fibrillin aggregates, and no clearly defined microfibrils, suggesting a major role of this polypeptide region, encoded by exons 24 to 32, in the stabilisation and organisation of microfibrils (Peltonen et al., 1994). The C1055G mutation characterised in P47, is due to a single-bp T to G substitution into the cysteine-rich EGF-like domain of exon 25 in the FBN1 gene, with maintenance of the reading frame. The site of this mutation lends further support to the growing evidence for the clustering of neonatal Marfan syndrome mutations in the central portion of the FBN1 gene between exons 24 and 32. The presumed functional significance of this region in terms of microfibrillar organisation, structure and assembly is further supported by the disruption of a cysteine residue in a cbEGF-like domain, in association with a perinatal lethal phenotype.

The two exon 27 sequence alterations (E1139V and C1152Y) discovered in P44's plasmid DNA required careful and critical interpretation. This adopted patient has a relatively severe phenotype, and has survived beyond the neonatal period. Although parental DNA was not available for study, neither natural parent is known to have Marfan syndrome (E Haan, personal communication). There has been only one report of a compound heterozygote (lethal phenotype), born to two parents with classic Marfan syndrome (Karttunen et al., 1994). The possibility that P44 might represent a compound heterozygote was considered but dismissed, firstly because the parents are presumed unaffected, and secondly because *Bsm* I digest results on chromosomal DNA from P44 was in direct conflict with results obtained on *Bsm* I digests from P44's cloned plasmid

DNAs. The confirmation that the *Bsm* I site in exon 27 of both FBN1 alleles is intact in the normal population and in P44 led to the conclusion that the E1139V sequence alteration is unlikely to represent a silent polymorphism. Finally, it is presumed that this change is artefactual and does not accurately reflect P44's true genomic sequence at this site. Although considered unlikely, the possibility of mosaicism cannot be excluded.

Both the C1055G and C1152Y mutations in exon 25 and 27, respectively, involve the alteration of a wild type cysteine, and each is associated with a severe clinical phenotype. At first glance, these findings appear to be contrary to reports in the existing literature which suggest that in general, cysteine substitutions only translate clinically into a severe phenotype when they occur at the amino or carboxy terminus of the FBN1 gene (Sakai et al., 1991; Eldadah et al., 1995; Nijbroek et al., 1995). However, it has also been suggested that the location rather than the character of the mutation, might be the more influential factor in determining phenotypic severity (Kainulainen et al., 1994; Nijbroek et al., 1995). It is probable that selected mutations in the region between exons 24 and 32 can structurally perturb the fibrillin monomer over a wide distance. The C1055G and C1152Y mutation data lend further support to this correlation of genotype and phenotype.

4.8.6.1 FBN1 Mutations: An Overview

Fifty-five mutations in FBN1 have been reported to date (Dietz and Pyeritz, 1995). Of these, 69% are missense mutations, small in-frame deletions or insertions, 20% are frameshift or nonsense mutations that lead to the creation of a premature termination codon, and the remaining 11% comprise mutations that create a centrally deleted monomer either due to aberrant splicing events or genomic deletions that preserve the reading frame. With the exception of two recurrent FBN1 mutations identified in unrelated probands, all other mutations have been unique to individual families (Dietz et al., 1991b; Nijbroek et al., 1995; Dietz and Pyeritz, 1995). Eighty-four percent of missense mutations, small in-frame deletions or insertions in FBN1 occur in exons that encode EGF-like domains (Figure 4.13), and most occur in domains that satisfy the calcium binding consensus. Of the four mutations that occur in non-calcium-binding EGF-like domains, three substitute one of the six highly conserved cysteine residues and one creates a new cysteine. Exactly half of the mutations that occur in calcium-binding EGF-like domains substitute cysteine residues, while one third specifically alter residues

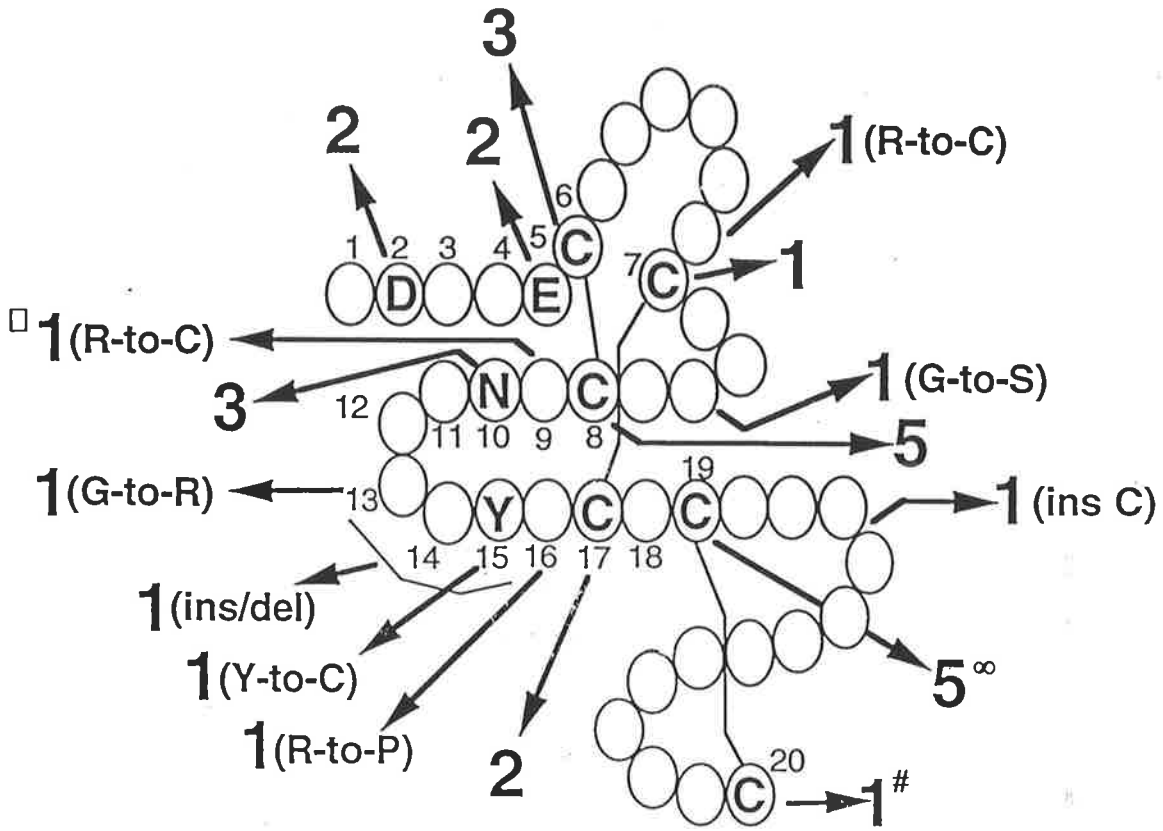
with isolated significance in dictating calcium binding (the D at position 2, the E at position 5, the N at position 10 and the Y at position 15); the remainder create a new cysteine (Figure 4.14). Based on the study of EGF-like domains in other proteins, only 9% of FBN1 mutations in EGF-like domains fail to suggest an obvious mechanism for disturbance of domain structure and function. Within this group, the R1137P and G2627R mutations substitute residues that are relatively conserved in EGF-like domains of fibrillin (the G at domain position 13 and the R at domain position 16), both of which occur within the sequence that promotes β-hydroxylation of the N at position 10 (Dietz et al., 1991b; Karttunen et al., 1994). The G1127S mutation substitutes a residue that is highly conserved in EGF-like domains in fibrillin and in other proteins, but its location provides no clue as to its functional significance (Francke et al., 1995).

Twenty percent of FBN1 mutations create a premature signal for the termination of translation of mRNA, and most of these occur prior to the penultimate exon of the gene. These mutations result in low mutant transcript levels (ranging between 6 and 25% of that from the wild-type allele). In contrast, alleles containing premature termination codons in the last exon are associated with transcript levels equal to that from the wild-type allele (Nijbroek et al., 1995). Considering that the predominant mutation-screening technique has involved the assay of amplicons generated by RT-PCR, and that most of the premature termination codon mutants dramatically decrease the level of mutant transcript, then it seems likely that the prevalence of these particular mutations has been underestimated. Two of four unrelated Marfan patients who were heterozygous for a dimorphism in the 3' untranslated region of FBN1 showed apparent loss of heterozygosity when RT-PCR products were typed by restriction analysis (Hewett et al., 1994b). More recently, the screening for FBN1 mutations from genomic amplicons spanning each of the sixty-five exons showed that 28% of identified mutations created a premature termination codon (Nijbroek et al., 1995). None of the mutations identified in this study resulted in creation of a premature termination codon.

4.8.7 Genotype-Phenotype Correlation in Patients Without Marfan Syndrome

Heterozygous FBN1 gene mutations may be found in individuals who do not have Marfan syndrome, as has been demonstrated by the recent genotype-phenotype correlation in familial ectopia lentis and in individuals in a family manifesting either skeletal features of Marfan syndrome or tall stature alone.

Figure 4.14 Mutations in Fibrillin EGF-like Domains



The fibrillin EGF-like domain consensus, modelled after the solution structure of native EGF (Cooke et al., 1987). Residues with putative significance for calcium binding are numbered sequentially. Highly conserved residues are designated by their single-letter amino acid code. Lines indicate disulphide linkages between cysteine (C) residues. The number of mutations that substitute each residue is indicated (arrows). The nature of the amino acid change is indicated for residues that are not absolutely conserved in calcium binding EGF-like domains. The location of the three mutations in EGF-like domains identified in my research are indicated as follows: □ H2629P, ∞ C1055G, and # C1152Y

(after Dietz and Pyeritz, 1995)

Familial Ectopia Lentis

To date, the only published mutation causing ectopia lentis is located in exon 59 (Lönnqvist et al., 1994; Kainulainen et al., 1994). The E1549K mutation was identified in a four-generation family with three living subjects suffering from dominantly inherited ectopia lentis. The phenotype included dislocated lenses, some skeletal mainfestations but no cardiovascular abnormalities. The mutation was detected in the DNA of all the subjects with dislocated lenses as well as in the DNA of three other individuals with only skeletal manifestations of the disorder.

SSCP screening analysis of a large autosomal dominant ectopia lentis kindred was performed and exon 59 was specifically targetted. The clinical details of this kindred have been published previously (Edwards et al., 1994). The DNA samples of seven affected individuals, four unaffected individuals and one normal (control) were analysed. Two standard gel conditions were used (see Section 2.7.4-2.7.6) and in addition, the patient DNA samples were analysed using a gel system identical to that in which the band shift was originally detected (Lönnqvist et al., 1994). The size of the amplified PCR fragment was 222 bp. Despite the use of three different gel conditions, there were no detectable band shifts in exon 59 of this kindred. In addition, SSCP screening of exons 15-18 and 23-32 under 4.5% and 10% gel conditions, was performed on three DNA samples (one normal individual, one unaffected family member, and one affected family member). No band shifts were detected. Given that the size of the PCR fragment for exon 59 is smaller than that of exon 25 and comparable to that of exon 28 (for which band shifts have been identified in this research), this lessens but does not exclude the likelihood of a mutation in exon 59 in this kindred. However, further analysis using restriction enzyme digestion may clarify this issue. Until a mutation in exon 59 has been excluded and/or a mutation is found in some other region of the FBN1 gene, the question of whether there is allelic heterogeneity for ectopia lentis remains unresolved.

Skeletal Manifestations of Marfan Syndrome and Tall Stature in a Family

A family was recently identified that segregates a mutation (R2726W) that apparently disrupts the processing of pro-fibrillin to fibrillin (Milewicz et al., 1995). Half of the secreted monomers were abnormally large, presumably due to failure of cleavage of the C-terminal propeptide. Fibroblasts from patients deposited a half-normal level of fibrillin in the matrix, only the processed form of fibrillin, and ultrastructurally normal

microfibrils. The proband's clinical manifestations were limited to the skeletal system. Other family members who were heterozygous for the mutant genotype had isolated tall stature. Towards the end of the thesis, several families with clinical features strikingly similar to this kindred were referred for FBN1 gene analysis. These families provide the opportunity for further examination of genotype-phenotype correlation in individuals without Marfan syndrome.

4.8.8 Special Studies

The results obtained in two patients in whom special studies were undertaken, were uninterpretable. This is illustrated by findings in P26 (abnormal fibrillin studies) and P37 (abnormal collagen studies). The clinical findings, together with a normal adult level of testosterone in P26, were considered unusual. The elevated FSH is most likely attributable to delayed testicular descent. The normal androgen receptor assay excluded the possibility of androgen insensitivity and 5-alpha reductase deficiency. Genital hypoplasia and ectopic or undescended testes have been reported in a few instances of Marfan syndrome (Sinclair et al., 1960). Sipione et al. (1986) described a 7-year-old child with Marfan syndrome and bilateral cryptorchidism. Testicular biopsy showed the histopathological changes of Sertoli-cell-only syndrome, a syndrome characterised by azoospermia and infertility. Gershoni et al. (1990) reported a patient with Marfan syndrome associated with bicuspid aortic valve, premature ageing and primary hypogonadism. The authors concluded that the occurrence of premature ageing and hypogonadism with Marfan syndrome or marfanoid characteristics may represent a distinct clinical entity. Fibrillin pulse chase studies in P26 demonstrated abnormal intracellular retention of fibrillin and delayed secretion into the extracellular matrix. The interpretation of this result in the light of the patient's clinical features is difficult and may only be resolved if a FBN1 gene mutation is found in future.

The screening of types I and III collagen allowed for the exclusion of the diagnosis of EDS IV in P28 and P35, and identified a collagen abnormality in P37, whose results although interesting are uninterpretable at present. In all cases of EDS IV studied at the molecular level, structural defects in type III collagen lead to impaired secretion, intracellular storage and degradation, to reduced stability of the secreted molecules, or to both (Steinmann et al., 1993). The overall result is a reduced proportion of type III to type I collagen production relative to control fibroblast values. The presence of only a

small amount of type III collagen in the cultured dermal fibroblasts of P37 caused interpretative difficulties, since there was not enough type III collagen present to determine if a subtle structural abnormality was present. A small number of families with EDS IV have been described, in whom the synthesis of procollagen is reduced to about half the normal level, and there is no evidence of intracellular storage (Steinmann et al., 1993). Such a biochemical phenotype would be expected in the presence of a nonfunctional COL3A1 allele, and the clinical phenotype may be milder than the classical EDS IV phenotype. Confirmation of preliminary biochemical studies with this type of defect (Deak et al., 1992) would establish a distinct subclass (mild variant) of EDS IV analagous to mild (type I) osteogenesis imperfecta. It is possible that P37 has such a heterozygous type COL3A1 gene mutation. Thus, P37's disease phenotype could result from reduction of the gene product to 50% of normal level, generated by the constitutively expressed wild type allele, as has been seen in osteogenesis imperfecta type I (Tynan et al., 1993). The inability to confirm a diagnosis of autosomal dominant EDS IV in P44 creates difficulty in genetic counselling of the patient and his family.

In the two patients with congenital aneurysms of the great vessels (P28 and P35), no fibrillin abnormality was detected at either the protein processing level (pulse-chase study), or at the molecular level (partial screening of the FBN1 gene). In addition, collagen screening failed to detect an underlying collagen defect. This led to the hypothesis that the aneurysms in these children might be attributable to a defect in elastin, another important component of elastic tissue. This hypothesis was supported by recent findings related to familial supravalvular aortic stenosis (SVAS), a congenital narrowing of the ascending aorta originating at the sinotubular junction, just distal to the coronary ostia (Peterson et al., 1965). This disorder may occur sporadically or as an autosomal dominant defect (Chiarella et al., 1989; Schmidt et al., 1989). It may also occur in association with Williams syndrome (Williams et al., 1961; Beuren et al., 1962), and it has been suggested that Williams syndrome may be a contiguous gene syndrome that involves the genetic locus for SVAS (Schmidt et al., 1989). Familial cases of SVAS suggest a single gene defect, but the underlying molecular basis of SVAS is unknown. Familial SVAS has been localised to chromosome 7q (Olson et al., 1993).

On the basis of the proposed hypothesis and existing knowledge regarding an elastin defect in the causation of familial SVAS, FISH of the elastin gene was performed in P35

to specifically analyse this chromosomal region. No elastin gene defect or rearrangement was found, and the cause of the aneurysms in these children remains unknown.

Collagen cross-linking was studied in P35's cultured dermal fibroblasts under conditions that stimulate collagen matrix formation and maturation. This analysis was undertaken because of the striking similarity between the arterial abnormalities in P35 and those seen in Menke's disease, a disorder of copper metabolism. While the proportion of the total matrix collagen extracted by pepsin varied in control cells from 32% to 63% of the total collagen, the amount of collagen in P35 requiring pepsin extraction fell within this range. Since this collagen fraction requiring pepsin proteolytic extraction represents the insoluble mature crosslinked collagen fraction, these data demonstrate that the proportion of collagen forming mature crosslinks in P35 fell within the normal range in this *in vitro* system. The proportion of collagen β-components (crosslinked α-chain dimers) was also the same in fibroblasts from controls and P35. To determine if there was a difference in the kinetics of crosslink formation, a pulse chase experiment was performed. Again, there were no detectable differences between controls and P35, with 79% of the pulse labelled collagen forming an insoluble crosslinked matrix requiring pepsin extraction within 24 hours (Table 4.3).

Finally, preliminary work suggests the possibility of a mutation in the FGFR2 gene in one patient (P27) with Shprintzen-Goldberg syndrome. This research will be continued, and if the results can be confirmed, then molecular analysis of the FGFR2 gene should also be extended to include patients with Furlong syndrome.

CHAPTER FIVE

SUMMARY AND CONCLUDING REMARKS

5.1 Summary

5.1.1 The Research

This research was undertaken in order to study two patient populations, the first with unequivocal Marfan syndrome, and the second with clinical evidence of a connective tissue disorder with some features in common with Marfan syndrome, but in whom the diagnosis was unknown. The clinical features of forty-eight patients were documented in detail, diagnoses were sought in the patients not considered to have Marfan syndrome on clinical grounds, and a search for putative FBN1 gene mutations was embarked upon in all patients. If one includes both the detection of FBN1 gene mutations (a total of five mutations in six Group 1 patients), and the diagnoses made and excluded in Group 2 patients, the research generated new information in 11/48 patients (22.9%). Further information was available for two patients (P26 and P35), but interpretation of these findings remain unclear at the present time.

At the commencement of the project, only the partial cDNA sequence of FBN1 was known and mutation analysis was based wholly on reverse transcription of mRNA to cDNA from cultured dermal fibroblasts. This was a time-consuming process and progress was slow. Two false positive results but no true FBN1 gene mutations were found using this approach. Part-way through the research, the entire genomic DNA sequence was determined. The availability of primer sequences for genomic-based amplicons dramatically altered the subsequent course of the research, with identification of two polymorphisms and five novel FBN1 gene mutations in a relatively short time. Numerous methods and approaches were adopted in the SSCP analyses of 44% of the FBN1 coding sequence in all patients. The advantages, disadvantages and potential refinements of these systems have been discussed. At the time of completion of the research, there were fifty-five FBN1 gene mutations reported in the world literature. The definition of a further five novel FBN1 mutations represents a substantial contribution.

5.1.2 Observations Based on the Clinical Studies of Group 1 Patients

Among the twenty-six Group 1 patients with classic Marfan syndrome, there were ten sporadic cases, and sixteen familial cases, including a three generation Aboriginal kindred. Observations and recommendations based on clinical studies of these patients are outlined below.

1. Some individuals with Marfan syndrome are of normal height and can be obese. Absence of a typical Marfan body habitus should not deter the clinician from considering the diagnosis if other salient features are present.

2. Physicians caring for groups of people selected on the basis of tall stature eg basketballers, some other classes of athletes, soldiers and policemen, should be aware of Marfan syndrome and its features so that affected individuals are diagnosed.

3. Marfan syndrome has not been documented in the Aboriginal population until now. Aborigines may be underdiagnosed because they have a naturally lean build and relatively long legs. In addition, many live in isolated areas without ready access to medical care. The latter problem places affected Aborigines at increased risk of cardiovascular complications because of the unavailability of facilities for surveillance.

4. Aortic root dilatation can occur late in life in Marfan syndrome. Though this is not common, it underscores the need for regular echocardiographic surveillance in those patients in whom the clinical suspicion of Marfan syndrome is high, regardless of whether or not the diagnostic criteria are met at the time of initial contact.

5. Neurological symptoms in Marfan syndrome patients can be significant. Paraesthesia was present in four patients, all of whom subsequently required aortic surgery. Nerve root compression may mimic an intra-abdominal emergency, and if this is not considered in the differential diagnosis of abdominal pain in Marfan syndrome patients, some may be subjected to unnecessary surgery.

6. Macrocephaly (relative or absolute) was common and two patients had communicating hydrocephalus. Two patients had pyloric stenosis, and uterine prolapse occurred at an early age in one patient. These findings, though based on a small number of patients, raise questions about a possible functional role of fibrillin in the pyloric sphincter muscle, the uterine ligaments, in cranial skull bone formation and cranial sutural closure. Investigative studies of more patients are needed to address these questions.

7. Genotype-phenotype correlations in Marfan syndrome remain of great interest, but in most cases, do not appear useful in terms of predicting disease severity or prognosis in a

given individual.

5.1.3 Clinical Observations Based on the Studies of Group 2 Patients

The twenty-two Group 2 patients were distinctly heterogeneous. Although no FBN1 gene mutations were identified in any of them, a diagnosis was made in three (Furlong syndrome [2 cases], Shprintzen-Goldberg syndrome [1 case]). The ascertainment of two children with congenital aneurysms of the great vessels resulted in an international collaboration describing the clinicopathologic features of four children with these rare abnormalities.

During the research period, there were several referrals by colleagues of interesting cases or families in Australia and New Zealand. These included a large kindred with autosomal dominant ectopia lentis (referred by Dr M Edwards, Newcastle), the only known kindred in the world with Shprintzen-Goldberg syndrome in siblings (referred by Professor D Sillence, Sydney), an interesting family of two sisters with aortic root dilatation and skeletal features of Marfan syndrome, in whom the third sister and both parents are completely normal (referred by Dr J Morreau, Rotorua), a baby with neonatal Marfan syndrome (referred by Dr A Colley, Newcastle), and a child with a neonatal Marfan phenotype and proven mitochondrial complex I deficiency (referred by Dr J Christodoulou, Sydney) (Christodoulou et al., 1993). There were also numerous enquiries from colleagues, including a thoracic physician who requested molecular study of the FBN1 gene in a 25-year cohort of patients with isolated pneumothoraces. Due to time constraints, only some of these patients were studied but the remaining patients will form the basis of future research.

5.2 Concluding Remarks

Many unanswered questions have sprung from the research undertaken over the last two years. Future research will involve analysis of the remaining portion of the FBN1 gene in the patients in whom a FBN1 mutation has not been found as yet. Localisation of an FBN1 gene mutation in the ectopia lentis kindred, in the child with mitochondrial complex I deficiency, and in individuals from other families referred by colleagues, await further investigation.

The recent report of a sequence alteration in exon 28 (P1148A) of FBN1 in Shprintzen-Goldberg syndrome is of great interest and further studies will need to be done to determine whether the polymorphism identified in exon 28 in some of our Marfan syndrome patients is the result of the same or a different sequence change. In addition, the report of a FBN1 mutation (C1223Y) in exon 29 in a girl with Shprintzen-Goldberg syndrome (Dietz et al., 1995b) highlights the need to re-examine this and surrounding regions of the gene in our Shprintzen-Goldberg patients.

The question of possible involvement of the FGFR2 gene in the pathogenesis of marfanoid-craniosynostosis syndromes such as Shprintzen-Goldberg syndrome and Furlong syndrome is presently unresolved. So too, the question of a possibile interaction between the FGFR2 and FBN1 genes in determining the phenotype of these disorders. Both these issues demand further investigation.

There is potential within Australia to collaborate with a research group that is currently developing the pulse-chase analysis technique, so that results of the clinical and molecular work can be combined with biochemical analyses in future. Preliminary discussions have already commenced with this in mind.

In the longer term, given the prevalence of Marfan syndrome, a case could easily be made for establishment of a National Marfan Consortium group in Australia, aimed at compiling clinical, biochemical and molecular data on Marfan syndrome patients in an integrated and comprehensive manner. This could be extended to allow for the provision and sharing of resources between interested research groups, since the cost of many of the techniques required for biochemical and molecular analyses prohibit any one research group from undertaking all aspects of the necessary research.

5.3 Current Knowledge and Future Directions

Marfan syndrome is an autosomal dominant condition that involves multiple organ systems and, if untreated, shortens life expectancy because of cardiovascular complications. The incidence is at least 1/5000 individuals, without regard to ethnicity and geography, although it is difficult to specify precisely because the phenotype represents a continuum, one end of which merges with the range of normal in all systems

affected.

The majority of FBN1 mutations substitute single amino acids and are associated with classic Marfan syndrome. There is some bias in this assessment because until recently, mutation screening has focussed exclusively on patients with Marfan syndrome. Although mutations causing Marfan syndrome appear to be evenly distributed throughout the length of the fibrillin gene and protein, the domain organisation of fibrillin and the putative functional significance of the mutations are pertinent. An understanding of how mutations in FBN1 result in the expression of a clinical phenotype must be founded on the biology of fibrillin monomers and the assembly of microfibrils. The variable clinical phenotypes that result from specific mutations is also pertinent. Mutations associated with very low levels of mutant transcript and protein can result in mild disease phenotypes. Other premature termination codon-mutants associated with a higher level of steady-state mutant transcript, have the capacity to produce the classic and severe Marfan syndrome phenotype.

Genotype has a major influence on phenotype in Marfan syndrome. This is supported by the facts that interfamilial variability tends to be greater than that seen within families, and the biochemical phenotype (as defined by quantitative pulse-chase analysis) in a given family member is generally concordant with that observed in other family members (Milewicz et al., 1992). The most typical biochemical profile seen in patients with Marfan syndrome is characterised by a deficiency of fibrillin incorporated into the extracellular matrix, far below the amount that is predicted to be derived from the wild-type allele (Milewicz et al., 1992; Aoyama et al., 1993; Aoyama et al., 1994). Such a profile can be associated with many different classes of mutations, including missense mutations that perturb monomeric structure and/or calcium binding, mutations leading to central deletions of the monomer, and premature termination codon-mutations causing carboxy-terminal truncations. Quantitative assessment of fibrillin deposition must be complemented by a consideration of the functional integrity of the protein and the genetic background upon which the protein is expressed.

While allelic heterogeneity and genotype-specific differences in the pathogenicity of fibrillin mutant gene products may account for interfamilial variability, intrafamilial differences in phenotypic expression are less easily reconciled. Assessment of one

particular kindred (Dietz et al., 1992a) has led to speculation that epistatic loci, environmental or stochastic factors may have the capacity to modify clinical expression of a given mutant FBN1 allele. Another explanation for apparent intrafamilial clinical variability stems from the inadequacy of current clinical diagnostic criteria for Marfan syndrome. In selected families, both linkage and direct mutation analyses have shown that the copy of the fibrillin gene cosegregating with classic Marfan syndrome was not inherited by family members with milder phenotypes, previously diagnosed as Marfan syndrome (Nijbroek et al., 1995; Pereira et al., 1994). Theoretically, a gene conversion event might explain these findings, but segregation of haplotypes in these families is not consistent with such a postulate. Direct mutation analyses of all affected individuals (whether of mild or classic phenotype) have yet to be reported by the authors. The occurrence of phenotypically similar but aetiologically distinct disorders in the same family emphasises the need for modification of the current diagnostic criteria (Berlin criteria). In recognition of these deficiencies, revised criteria (Ghent criteria) have been formulated, are being scrutinised by international experts and should be published in the near future (R Pyeritz, personal communication).

The new knowledge being generated about the aetiology and pathogenesis of Marfan syndrome is only just beginning to reach the clinic. Some patients are already benefitting from the availability of methods for presymptomatic and prenatal molecular diagnosis. Once a disease-producing mutation is identified in a proband, methods such as restriction analysis or allele-specific oligonucleotide hybridisation analysis can be used to genotype relatives. Application of this highly sensitive and specific approach is limited by the difficulties and time constraints inherent to the mutation screening process. Alternatively, molecular diagnosis in families with Marfan syndrome can be provided by conventional linkage analysis. Four highly informative microsatellite polymorphisms can be used to distinguish between the many copies of the fibrillin gene that segregate in any given family (Pereira et al., 1994). Determination of which allele cosegregates with Marfan syndrome will allow molecular diagnosis for asymptomatic or equivocally affected individuals. Early diagnosis will allow for refined natural history studies and prophylactic management. Verification of the dominant negative model will offer mutant allele knockout as a potential strategy for gene therapy. Likely obstacles will include design of effective targetting vectors, improvement of delivery systems, and attainment of a better understanding of pathobiology of early development.

Techniques that assay the deposition of fibrillin into the extracellular matrix of skin or cultured cells have the potential to allow diagnosis without knowledge of FBN1 genotype. However, the fact that both immunohistochemical and pulse-chase analyses produce a low but important rate of false-positive and false-negative results has lessened enthusiasm for their widespread clinical application (Hollister et al., 1990; Milewicz et al., 1992; Aoyama et al., 1994). While both of these tests give an indication of the amount of protein that is deposited, neither assesses the functional integrity of resultant microfibrils.

There is no currently available biochemical or genetic test that, in isolation, allows the assignment of affected status for Marfan syndrome. Both protein abnormalities and FBN1 mutations have been identified in individuals who do not satisfy even the revised diagnostic criteria for the disorder. Thus, Marfan syndrome remains a clinical diagnosis. Molecular diagnosis may be more readily available in the future but prognosis should not be based on FBN1 mutation anlysis alone, since there are likely to be other genes and non-genetic factors which influence phenotype.

Research over the past five years has identified mutations in the fibrillin gene as the cause of Marfan syndrome. Characterisation of mutations in individuals with the disorder has provided some preliminary information about the functional significance of some regions of the fibrillin protein, but full clinical application awaits a more comprehensive appreciation of pathogenesis, a task perhaps best approached using experimental strategies that mimic the physiologic complexity of the human system. The development of murine and nonmurine models for Marfan syndrome should provide a means to examine conventional medical therapies. Animal research will also be necessary to test novel gene therapy strategies. Further work is in progress to determine the full range of clinical disease associated with FBN1 mutations. Ultimately, the answers provided by this combined research approach may pave the way for the development of laboratory-based prognostic and diagnostic tests for patients with severe disease, cardiovascular risk stratification in Marfan families, and exclusion of the disease in those individuals with a Marfan-related phenotype, or in those who do not meet the diagnostic criteria for Marfan syndrome yet have some features that overlap the disorder.

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APPENDIX 1A

Amino Acid Abbreviation Table

Amino Acid	Three Letter Code	One Letter Code
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	Q
glutamine	Gln	E
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

APPENDIX 2A

Medical Record: Connective Tissue Disorder

Date / /

Name:
Pedigree no:
Hospital medical record no:
Date of birth:.

Reason for visit: first evaluation follow-up research other

Referral source: self general practitioner other

Review of patient's general situation:

current age:
cardiac surgery: composite graft date / /
other
pregnant: time since last menstrual period:
current level of exercise:

Review of patient's current complaints and connective tissue disorder:

General:

Skin:
elasticity
fragility/bruisability
striae

Skeletal:
joints
lower back
upper back and neck
shoe size

Ocular:
myopia
lens dislocation

Cardiopulmonary:
chest pain
palpitations
dyspnoea
oedema
syncope other

Gastrointestinal:

Neurologic:

Other systems:

Past medical history:

Social history:

Family pedigree and family history:

Allergies:

Current medications:

none

-blocker: none atenolol propanolol other

age any -blocker begun:
current daily dose:
optimal dose:
misses doses: never occasional frequent
side effects: yes no
if yes, physical fatigue yes no
decreased libido yes no
vivid dreams yes no
mental fatigue yes no
sleeplessness yes no
wheezing yes no
other yes no

anticoagulant: yes no
dose:
most recent coagulation profile:

antiarrhythmics: yes no
name:
dose:

calcium antagonist: yes no
name:
dose:

digoxin: yes no
dose:

sex hormone: current past never

current drugs and doses:

if ever used, indication:
birth control
weight gain
growth modulation

other

other medication:

Examination name:

Date of examination: / /
Dates of previous examinations:

Habitus:

height	cm	percentile
weight	kg	percentile
head circumference	cm	percentile
arm span	cm	
lower segment	cm	
upper:lower segment ratio		

adiposity:
average minimal increased truncal increased general

musculature: average development decreased well developed

Skin:

striae:	none	trace	marked
lumbar			
shoulders/axillae			
breast/chest			
abdomen			
hips/thighs			

elasticity: normal mild increase marked increase
scars: none normal wide and thin keloid

Skeletal:
(- = decreased; 0 = nil; + = mild increase; ++ = marked increase)

joint mobility:

fingers	toes	ankles
elbows	shoulders	hips
carrying angle	knees	

vertebral column: kyphosis lordosis scoliosis
cervical
thoracic

lumbar

anterior chest deformity:
none excavatum carinatum combination

arachnodactyly: (subjective) none mild marked
mid finger length cm percentile
hand length cm percentile
thumb sign negative equivocal positive
wrist sign negative equivocal positive
foot length cm percentile
arch of foot: normal pes planus pes cavus

Head and facies:

mandible normal prognathic retrognathic
palate normal high and narrow high narrow
teeth: normal overbite underbite crowding none
malar region normal hypoplastic prominent
ears crumpled helix simple helix soft helix other
eyes deeply set
inner canthal distance cm percentile
inter-pupillary distance cm percentile
outer canthal distance cm percentile
epicanthus
ptosis
iridodenesis

Pulmonary:

breath sounds clear wheezes other

Cardiovascular:

blood pressure left arm supine mmHg
sitting mmHg
cardiomegaly
cardiac thrill
cardiac heave

rhythm:
regular sinus arrhythmia irregularly irregular
premature beats none occasional frequent

first heart sound:
normal increased decreased
second heart sound:

normal	increased	decreased	prosthetic	
split	none	physiologic	paradoxic	
gallop	none	third sound	fourth sound	both
systolic click	none	apical	parasternal	ejection

murmur	none			
	systolic:			
	apex mid-late	holosystolic	grade	/VI
	base aortic	pulmonary	grade	/VI
	diastolic:	aortic	grade	/VI
		pulmonary	grade	/VI
other	pulses	normal	abnormal	
	oedema	none pedal	pretibial	

Abdomen:

organomegaly/tenderness/masses			
abdominal aorta	normal	wide	not felt
abdominal bruit	yes	no	
hernia:			
umbilical	yes	no	repaired
inguinal	none unilateral	bilateral	repaired

Neurologic:

cranial nerves
muscle power
muscle tone
deep tendon reflexes
sensation

Other:

Echocardiogram:

LVES			
LVED			
RV			
LA			
aorta diastole	mm		
aorta systole	mm		
pulmonary artery	mm		
mitral valve prolapse:			
no yes	mid-late sytolic	holosystolic	
pericardial effusion	none small	large	
other findings			

ECG:

within normal limits abnormal (specify)

Assessment:
Diagnosis

Group 1: Marfan syndrome

Group 2: Not Marfan syndrome

Mitral valve prolapse syndrome
Annuloaortic ectasia
Ehlers-Danlos syndrome type I II IV type other
Homocystinuria
Pseudoxanthoma elasticum
Osteogenesis imperfecta
Other connective tissue disorder
No connective tissue disorder
Other diagnosis

Diagnostic justification:

Clinical problems:

Issues:

Genetic counselling done this visit planned for return visit
Psychosocial counselling
Summary of evaluation discussed with patient
letter of summary to referring doctor/s
letter of summary to specialist/s
letter of summary to patient/family
Next medical genetics clinic visit:

Studies: date collected

blood for DNA
blood for karyotype
blood for other studies(specify)

urine for homocystinuria

slit lamp examination of lenses

echocardiogram
ECG
Holtor monitor

clinical photography

skin biopsy

radiology bone age
chest radiograph
scoliosis series
CAT scan

MRI scan

Consultations:

cardiac surgery
dental
general surgery
neurology
dietitian
obstetric/gynaecology
ophthalmology
orthopaedics
vascular surgery
social work

APPENDIX 2B

Invitation to Participate in a Research Study: Definition of Fibrillin Gene Mutations in Marfan Syndrome

We are writing to you in the hope that you will be willing to participate in a research study into the genetic changes which cause Marfan syndrome. We have seen your family because..... has Marfan syndrome/ has a disorder which shares features with Marfan syndrome.

Marfan syndrome has been shown recently to result from mutations (changes in the genetic code) in the gene for fibrillin (a protein associated with elastic fibres of connective tissue) on chromosome 15. Abnormalities in the metabolism of fibrillin have been demonstrated by examination of the skin from patients with Marfan syndrome.

We wish to find the mutations responsible for Marfan syndrome in a group of South Australians with the disorder. We also wish to study the fibrillin gene and other important connective tissue proteins in people having disorders which share features with Marfan syndrome.

Discovery of a fibrillin mutation in someone will mean that the diagnosis of Marfan syndrome is confirmed and will allow us to check other family members to see if they also have the disorder. As more mutations are described world-wide, it may be possible to predict the progress of the disorder in an individual based on their particular mutation. In addition, information about specific mutations may help in designing treatment for individuals with Marfan syndrome. The research is difficult and we may not find a fibrillin mutation in some people, even though they definitely have Marfan syndrome.

We would like to obtain a blood sample (10 mls from young children and 20 mls from older individuals) from each affected person, both of his/her parents, and from any children of an affected person. Blood would be collected from a vein using a needle and syringe. The genetic material, DNA, will be extracted from the blood sample and stored frozen, awaiting use for the research.

We would also like to obtain a small piece of skin (a skin biopsy) to look for fibrillin abnormalities in the skin. The skin would be taken from the upper arm (punch biopsy) under local anaesthetic. A single stitch will be needed and the wound heals rapidly. The stitch will need to be removed by your local doctor in one week.

To interpret the results of the blood and skin tests, we need to be sure which family members have Marfan syndrome and which do not. For this reason, we would like to examine each affected person, their parents and all their children, and to take photographs to provide a record of their appearance. For some people, it may also be necessary to carry out additional assessments including echocardiography (ultrasound examination of the heart and aorta), slit-lamp examination of the eye, and orthopaedic consultation. If required, these appointments will be made for you at a convenient time and another time will be arranged so that the results of these assessments can be discussed with you.

The research we propose may prove to be difficult and may take a long time, and may

not be successful in defining a fibrillin mutation in all families. We will let you know the results of the research project once it has been concluded. The results of the research study may be published in the medical literature and if this happens, you will not be identified, and your confidentiality and privacy will be preserved. No photograph will be used without written permission.

Even though you agree to assist in this research project, you can withdraw at any time. A decision not to participate in the research will not affect the way in which your doctors care for you or other members of your family. We will ask you to sign a consent form before taking blood or skin specimens.

All enquiries concerning this project should be directed to Dr L. Adès or Sister S. White of the Department of Medical Genetics, telephone 2047375.

APPENDIX 2C

Title of Project: Definition of Fibrillin Gene Mutations Responsible for Marfan Syndrome

Consent Form 1: Participation in Study

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree to (my child) taking part.

2. I understand that I/my child may not be directly benefited by taking part in the research.

3. I understand that while information gained in the study may be published, I/my child will not be identified and information will be confidential.

4. I understand that refusal to participate in the research will not affect my/my child's medical care.

5. I understand that there will be no payment to me/my child for taking part in this study.

6. I have had the opportunity to discuss taking part in this investigation with a family member.

7. I am aware that I should retain a copy of the Consent Form when completed and the Information Sheet.

Signed .....

Relationship to patient: .....

Full name of patient: .....

Dated: / /

I certify that I have explained the study to the patient/parent and consider that he/she understands what is involved.

Signed: .....

Title: .....

Consent Form 2: Blood Collection

I..... give consent to the collection of a blood sample from myself/my child for DNA extraction and indefinite storage, having read the Information Sheet concerning the research project and having had the research project explained to my satisfaction.

Signature: ..... Date: .....

I agree to the use of my DNA for the following purposes:

1. To search for a mutation in my fibrillin gene. (The current research project).

   Signature: ..... Date: .....

2. For use in any additional projects into the genetic basis of Marfan syndrome which the researchers may conduct in the future, or which other researchers may conduct elsewhere in Australia and overseas for the same purpose.

   Signature: ..... Date: .....

3. For use in tests which may be able to determine whether or not others in my family have the Marfan syndrome gene.

   Signature: ..... Date: .....

Witness: Signature: ..... Date: .....

Consent Form 3: Skin Biopsy

I.....give consent to the collection of a skin biopsy from me/my child..... to aid in diagnosis. I also give consent for the tissue to be used for research purposes. I understand that the genetic material (RNA/DNA) will be extracted from the skin cells for study, and that the proteins made by the skin cells (fibrillin and some others) will also be studied.

I have read the Information Sheet concerning the research project and have had the project explained to my satisfaction.

Signature: ..... Date: .....

Consent Form 4: Permission to use Clinical Photographs for Educational Purposes and Scientific Articles

1. For adults- 18 years and over

I, ...of ..... hereby give permission for clinical photographs of myself to be used for the purposes stated above.

Signature: .....

Witness: .....

Date: .....

2. For minors- younger than 18 years

I, ...of ..... being the parent/legal guardian of ..... hereby give permission for clinical photography of my child to be used for the purposes stated above.

Signature: .....

Witness: .....

Date: .....

APPENDIX 2D

South Australian Marfan Syndrome Research Project Update, December 1993

It is already nine months since the commencement of the Marfan Syndrome Research Project. One of the patients I reviewed during the course of the year suggested that it would be valuable for all the participants in the research to receive some kind of update on a regular basis. I decided to follow his advice and so here is the first update.

Most of you will know that originally the funding for this project (which is funded through a clinical research fellowship granted by the Women's and Children's Hospital) was initially for a 12 month period only. This was reviewed recently and the Women's and Children's Hospital was gracious enough to extend this funding for 1994. There has been a very good response to the Marfan Syndrome Research and the number of patients now enrolled in the study is certainly more than I had anticipated at the start of the project. More hospital centres have started to hear about the research and we still seem to be receiving a few new referrals every few months. So far forty patients with either Marfan syndrome, a Marfan-like syndrome or an undiagnosed connective tissue disorder have enrolled in this study.

The clinical component of the research study was completed around the middle of this year. Patient information is regularly being entered and updated into the Marfan Syndrome International Consortium Database. This information is forwarded on to Dr Petros Tsipouras who is the coordiantor of this entire data collection system. The patient information is sent on disc, is completely confidential and does not include details such as the patient's name or address. The idea behind data collection of this sort is to try and gather enough information world-wide so that information about the natural history of Marfan syndrome can be generated as quickly as possible. Once this information has been analysed, then it can be passed directly back to you. All that I ask is that you notify me in the event that you change address so that we always have a current address and phone number for contacting you.

The laboratory component of the research was commenced in July of this year. There were some initial hurdles but these have slowly been overcome. At short notice, I was invited by one of the leading research groups in Houston to attend the American Society of Human Genetics Annual Meeting in New Orleans in early October. The Houston research group was interested in undertaking some collaborative research and this enabled me not only to attend the conference but also to spend another five weeks working in Houston in Dr Dianna Milewicz/s laboratory. Dr Milewicz is one of the leaders in Marfan syndrome research in the United States. Prior to the commencement of the annual meeting, there was a half-day Marfan Syndrome Clinical Database Consortium Meeting. This was attended by experts in the field from the United States, the United Kingdon, and Finland. Topics presented included work on fibrillin gene mutations in Marfan syndrome and an update on the life expectancy in Marfan syndrome.

There was some interesting information presented with regard to the long-term follow-up of patients after aortic aneurysm repair. When survival of a study population was compared with 45 affected relatives who did not receive surgical intervention for aortic aneurysms, the operative group was found to have significantly better survival (expected survival of 61 years) than that of affected relatives (30 years). Whilst aortic aneurysm repair prolongs the lives of patients with Marfan syndrome, evidence was presented

which showed that as these individuals live longer, they may be at risk of developing aortic aneurysms at other sites.

Several research groups presented work regarding the creation of animal models for Marfan syndrome and the possibility for the development of gene therapy. I must stress that this research is in its infancy and it will be some years yet before this may have any direct application to humans. None-the-less it is important for you to know about the many directions in which Marfan syndrome research is proceeding.

I was fortunate enough to receive funding from the Australian College of Paediatrics, the National Heart Foundation, and Professor Grant Sutherland's laboratory to help fund my time in the United States.

I spent the following five weeks working in Dr Dianna Milewicz's laboratory at the University of Texas Health Science Center at Houston. I took samples of DNA from all the patients that have enrolled in the study, and skin cells from some selected patients so that we could undertake collaborative research in Houston. I was taught many different technologies, all of which worked well and I am hopeful that I will be able to translate this work back into the Adelaide laboratory now.

Although many of you may not have heard from me individually during the course of the year, you will see that it has been a busy time, and progress is being made. As yet, I have not found any fibrillin gene mutations, but this area of the research is really only just beginning, and of all the areas, this is the most time-consuming.

Research aside, I wanted to mention one very important aspect that many of you have raised individually with me over the course of the year. This is to do with the formation of a South Australian Marfan Syndrome Support Group. Most of you will know that there is a support group in Victoria and also one in New South Wales, but we do not have a South Australian support group. I have explained to some of you that the initiative to consider formation of a Support Group must come from individuals within the community rather than being seen as a hospital-based undertaking. However, it is important for you to know that I would be available as a resource person if you seriously wish to think about forming such a group.

At the end of this update, you will find a section with space available to enter your name and telephone number and a "yes" or "no" box. I would be grateful if you could tick whether or not you are interested in the formation of a South Australian Marfan Syndrome Support Group. Depending on the response, we may then be able to formulate a list of individuals who are interested to embark on informal discussions as to how to go about setting up a Support Group. I should warn you that this will probably require a considerable amount of lobbying and sheer hard work especially with regards to raising public awareness, and potential fund-raising activities. However, I would be fully supportive of the formation of such a group. As individuals with the disorder, you know better than anyone else which services you would like that are currently not being provided, and the areas in which your needs are not being met. It is important to think about these issues since part of the reason for forming a Support Group apart from providing moral support, is to try and fill these gaps. Education is usually another significant issue and depending on your needs, it would be possible to explore available avenues (eg invitation to cardiologists to talk to the group, discussions or information nights for those patients facing surgery and for those recovering from surgery,

rehabilitation issues etc).

I have had several enquiries over the course of the year about the possibility of specialised training programs for individuals with Marfan syndrome. Some of you have had aortic surgery +/- valve replacement and are wanting to continue on with a safe and regular fitness program. Fortunately, there is a very reputable organisation dedicated to individuals with specific needs such as yours. This is the Institute for Fitness Research and Training Incorporated and it is located at 64 MacKinnon Parade, North Adelaide. The contact phone number is 267 1887. If you are interested you can ask to speak to Ann Lang. The program is run by Tony Sedgewick. Fitness programs are designed, taking into account an individual's medical history and past cardiac surgery, and these programs are overseen by a cardiologist. Fitness classes for cardiac rehabilitation patients are held on a twice weekly basis, Wednesday evenings and Saturday mornings at the University gym located at 127 MacKinnon Parade. Saturday morning classes are taken by Dr Gavin Beaumont. A cardiac-trained sister attends these sessions, all of which are conducted and supervised by a doctor. There is a physiotherapist who is due to start in mid-January who is also interested in developing specialised exercise programs for individuals. The Institute is closed between the 17th December and the 17th January, but otherwise is operational all year. Cost of the classes for those wishing to attend twice weekly over a 12 week period is roughly $120. There is a tie-in with Mutual Community. The classes are of one hour duration. If you are interested in this for yourself, you can ring the Institute and make further enquiries. All that the Institute requires is a letter either from your doctor or the cardiologist who knows you well, outlining details of your medical and surgical history. I am happy to help with the provision of such a letter if you wish.

I am hopeful that the next 12 months will bring us new information about Marfan syndrome. I will be contacting you individually when the results of your fibrillin mutation work is available. Please do not hesitate to contact me at any time if you have any further questions or concerns. I want to thank you for agreeing to participate in this research, which would not have been possible without your cooperation.

Dr Lesley C Adès
Marfan Syndrome Research Fellow

Name: .....

Phone Number: .....

Are you in favour of a SA Marfan syndrome support group?

Yes: .....

No: .....

Are you happy for other Marfan patients to contact you by phone?

Yes: .....

No: .....

Which areas would you like assistance with?

Moral support: .....

Pre-operative information: .....

Post-operative information: .....

Post-operative rehabilitation: .....

Fitness program: .....

Counselling: .....

Other(please specify below):

.....

.....

.....

.....

South Autralian Marfan Syndrome Research Project Update, January 1995

Most of you participating in this study will be aware that I have been overseas working in a genetics department in Manchester for the last 7 months or so. This work was not related to Marfan syndrome, or to my research, but it did give me the opportunity to attend four separate conferences. One of these, the Third International Symposium on the Marafn syndrome, held in Berlin in Sepetember of last year, was of particular relevance to my research. I was fortunate enough to be able to attend this meeting, to present some of my work there, and also to hear all the latest developments coming from international researchers working in this field. En route back to Australia, I spent several days working with my collaborators in Houston. This group is continuing to provide support relating to the laboratory component of my research, and the sharing of information about their population of Marfan patients. They are also performing special analyses on some samples from some selected patients from my research project, the results of which are still awaited.

Although most of you will not have heard from me directly over the last 12 months, the research has been continuing on and now that I am back, I have a further 6 months of funding to complete the project. The scope of the project has proved to be much greater than the time or funding allocated to it. This has meant that I have had to be somewhat more selective in looking at particular regions of the fibrillin gene for mutations, rather than attempting to look at the entire fibrillin gene in each of you.

Once the research is complete, I will be writing to you all individually, to let you know the outcome of my findings. If further follow-up is warranted, this will, of course, be available to you through the Medical Genetics department.

I would like to summarise some of the main new pieces of information that arose from the Berlin meeting.

Over thirty mutations in the fibrillin gene have been reported so far. Each of these has been unique to individual families, with the exception of one particular mutation which has been reported twice in individuals from two unrelated families. Originally it was thought that there may be a "hot spot" for mutations within the fibrillin gene; ie one section of the gene in which most mutations occurred. Unfortunately, this has not proved to be the case and the mutations seem to be scattered fairly randomly throughout the entire length of the fibrillin gene.

One of the aspects of the research has been to try and determine if there is a correlation between particular mutations within the fibrillin gene and a patient's clinical status. For example, is a patient more likely to have aortic root dilatation if the mutation involves one region of the gene, and more likely to have problems with lens dislocation etc if a different region of the gene contains the mutation. We call this an attempt to look for a correlation between the genotype (ie where the mutation is in the fibrillin gene) and the phenotype (ie the particular clinical features of a Marfan syndrome patient). Whilst earlier work suggested that there might in fact be a definite correlation between genotype and phenotype, this has not been borne out by more recent studies.

The whole unfolding story about the fibrillin gene has become more interesting and decidedly more complex. At the beginning of my research, it was known that mutations in the fibrillin gene on chromosome 15 had been found in some 10-15% of all patients

with Marfan syndrome. This figure has not changed. One group in Baltimore claims that they can find mutations in over 70% of their patients, but these results were based on a very small number of patients and additionally, the findings from this group have not been reproduced by other researchers.

We now know that apart from fibrillin mutations leading to Marfan syndrome, they may also be responsible for some families who have aortic aneurysms only without any other manifestations of Marfan syndrome. Similarly, some families with dominantly inherited dislocation of the lenses of the eyes, have also been shown to have mutations in the same fibrillin gene that causes Marfan syndrome. There has also been a recent report of a family with just the skeletal features of Marfan syndrome (without having heart or eye problems), in which a fibrillin gene mutation has been identified.

In addition to this, there has been one study suggesting that a fibrillin gene on another chromosome (chromsome 3) may be responsible for the features in a family with a dominantly inherited pattern of features closely resembling the Marfan syndrome.

You will see from all this new information that perhaps the Marfan syndrome is a continuum or spectrum, wherein fibrillin gene mutations may be found in some individuals and where the clinical course may be extremely variable, and range from mild through to severe involvement. We still do not have a "handle" on how to determine disease severity. The original diagnostic criteria that were laid down to help doctors make the diagnosis of Marfan syndrome, may, as we suspected, be very inadequate. Unfortunately, at present the diagnosis is still clinical, and there is, as yet, no definitive diagnostic blood test available.

Clearly, there is much more to the Marfan syndrome story than meets the eye, and it seems that determining the mutation in the fibrillin gene on its own, will only provide us with one piece of the jigsaw puzzle. Some groups are now concentrating their efforts more on looking at the effect that a particular fibrillin gene mutation has on the way in which the fibrillin protein is processed. A combination of these various approaches will hopefully help to answer some of our many questions about Marfan syndrome.

I would like to take this opportunity to extend my best wishes to you all for 1995, and to thank you for your patience whilst this research continues. If you have had a change of address or telephone number since we last spoke, I would be grateful if you could let me know. Please do not hesitate to contact me if you have some questions that I may be able to answer for you. I can be reached in the Department of medical genetics on 204 7375 and will do my very best to help.

Dr Lesley C Adès
Marfan Syndrome Research Fellow

APPENDIX 3

Publications Arising From Thesis

(see overleaf)

APPENDIX 3A

Adès LC, Morris LL, Power RG, Wilson M, Haan EA, Bateman JF, Milewicz DM, Sillence DO. Distinct skeletal features in four females with Shprintzen-Goldberg syndrome. *Am J Med Genet* 1995;57:565-572

(see overleaf)

Adés, L. C., Morris, L. L., Power, R. G., Wilson, M., Haan, E. A., Bateman, J. F. et al. (1995). Distinct skeletal abnormalities in four girls with Shprintzen-Goldberg syndrome. *American Journal of Medical Genetics, 57*(4), 565-572.

APPENDIX 3B

Grossfield J, Kielty C, Adès L, Shapira SK, Towbin JA, Coselli J, Cao S, Milewicz DM. Mutations in exons 60 through 65 of the FBN1 gene in individuals with the Marfan syndrome: effect on fibrillin processing and microfibril formation. *Hum Mol Genet* (in press).

Adès LC. Marfan Syndrome in Aborigines. *Med J Australia* (in press).

Adès LC, Knight BK, Byard RB, Bateman JF, Desouza Esquivel JA, Mee RBB, Haan EA, Milewicz DM. Clinicopathological Features of Congenital Aneurysms of the Great Vessels. *Am J Med Genet* (submitted).

Adès LC, Haan EA, Colley A, Richards RI. Characterisation of four novel fibrillin-1 (FBN1) mutations in the Marfan syndrome. *J Med Genet* (submitted).